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Developmental biology and endocrine research for a successful pregnancy

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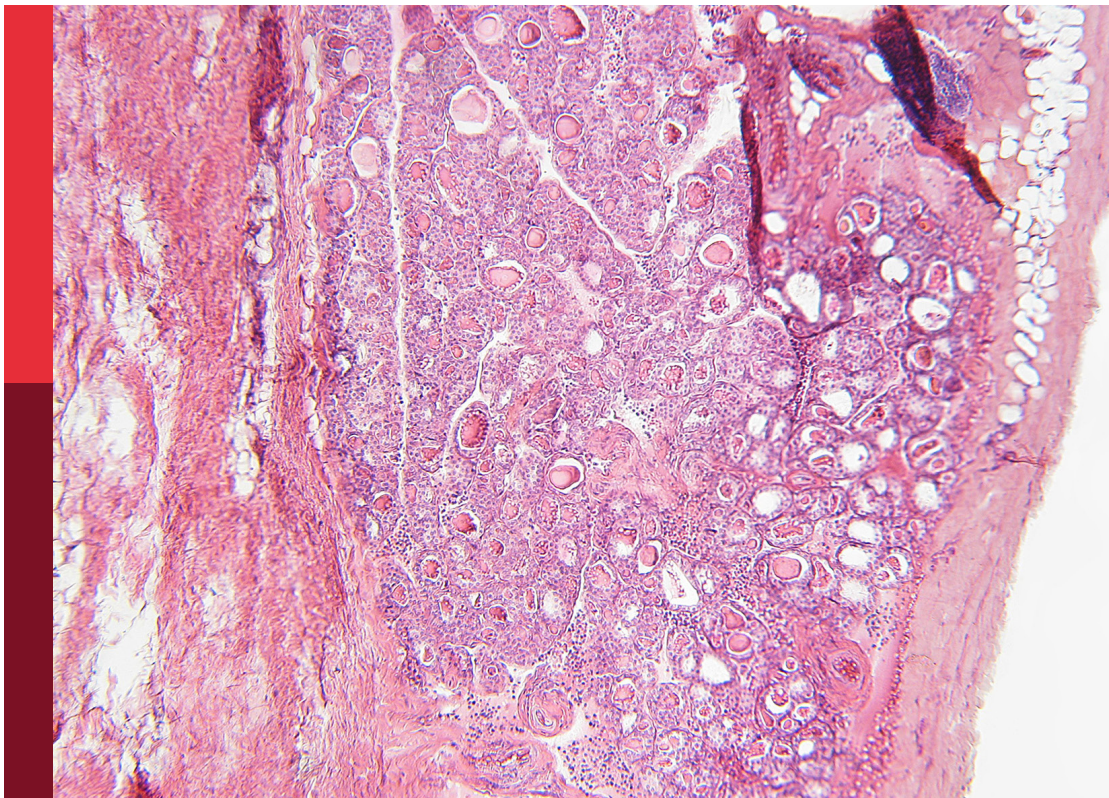

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Developmental biology and endocrine research for a successful pregnancy

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Editorial: Developmental biology and endocrine research for a successful pregnancy

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Editorial on the Research Topic
Developmental biology and endocrine research for a successful pregnancy

Pregnancy is central to mammals' lives, growth, and development. A healthy pregnancy is not only a prerequisite for a species' survival and existence but also crucial for the growth and development of livestock and agriculture sectors, such as dairy and beef animals. Pregnancy failure and fertility have long been studied in developmental biology and endocrinology, and new inventions and technologies are welcome in this field.

Many factors, including endocrine disruptors and sedentary lifestyles, are becoming more prevalent in modern culture, limiting and impeding pregnancy success. Thus, reproductive development, endocrinology, and infectious mechanisms in pregnancy must be understood. Proper knowledge of reproductive development and endocrinological techniques will enable us to develop strategies to mitigate the pathologies and factors affecting a successful pregnancy.

Multiple reproductive organs regulate developmental biology. Starting from external and accessory reproductive organs, the uterus, oviduct, ovaries, and their health and optimum function are prerequisites for a successful pregnancy. Along with the reproductive organs, many other factors also regulate pregnancy success. Lifestyle factors, such as physical activity, good nutrition, and exposure to endocrine disruptors, influence reproductive and developmental outcomes. Knowing about all disorders and conditions affecting reproductive development and their management is crucial.

The objective of the Research Topic *"Developmental Biology and Endocrine Research for a Successful Pregnancy"* was to bring together and highlight the original research and reviews on the recent advancements in the work for successful pregnancy. This Research Topic includes a diverse range of studies, including seven original research articles, six reviews, and two retrospective cohort studies.

A successful pregnancy concerns us all. Knowing the phases and disorders of female reproductive stages is crucial. Liu et al reviewed the role of oxytocin (OT) in the health and

disease of women. They explained how OT controls the reproductive cycle and pregnancy's physiological phases. OT helps GnRH release, ovulation, lactation, fetus expulsion, milk ejection, and maternal behavior. This review also discusses how OT affects maternal depression and hypogalactia. Endocrine-Disrupting Chemicals (EDCs) and physiological endocrine or hormonal systems are of concern today. EDCs originate from both natural sources, such as plants, and industrial/artificial sources. Pool et al. examined how phytoestrogens affect sheep reproduction. They discussed the way estrogenic pasture impairs sheep's reproduction. The relevance of estrogenic herbs on reproduction and pregnancy problems, including dystocia, stillborn lambs, and uterine prolapse after parturition (1–4), was stressed in their review. Endocrine disruptive substances' effects on specific *in-utero* development periods are poorly understood. If phytoestrogens breach the blood-placenta barrier and enter fetal tissue (5, 6), Pool et al. warn that estrogenic substances may affect gestational outcomes *in utero*. Although the long-term effect of *in-utero* exposure to phytoestrogens have not yet been established, neonatal exposure has been documented to cause precocious vaginal opening (7), ovarian follicle atresia (8), increased uterine fluid content (9), and hyperplasia of the endometrium (9–11) in rodents. Basak et al. also discussed that maternal endocrine balance is crucial for pregnancy. EDCs in daily life can impact pregnancy and outcomes if they exceed permissible concentrations, affecting implantation, placental development, fetal organ development, and epigenetics (12–16). Therefore, EDC awareness is crucial for reproduction, fertility, and pregnancy success. Basak et al review the effects of EDCs, specifically bisphenols and phthalates, on fetoplacental growth and pregnancy outcomes.

Maternal obesity, gestational diabetes, preeclampsia, and cardiomyopathy are prominent pregnancy-related disorders. Exercise is safe and useful during pregnancy. Regular aerobic, anaerobic, and circular activities help pregnant humans and animals (17–19). Pahlavani reviewed the role of exercise in combating pregnancy complications, focusing on apelin in exercise-induced pregnancy problem protection. Pahlavani's review sheds light on the molecular mechanisms of exercise, including the APLN/APJ system, which has been extensively studied across various body systems and species (20–25). Shokrollahi et al. found that buffalo ovarian follicles regulate the ALPN/APJ system, affecting buffalo fertility. They also highlighted the role of adipokines in endocrine control and steroidogenesis, suggesting that IGF1 or FSH-based involvement could aid in therapeutic ALPN/APJ system use.

The placenta and trophoblast are crucial for pregnancy establishment and maintenance, but abnormalities in the trophoblast and its protein and gene functions can lead to pregnancy termination. S100P is one of them, which was first purified and characterized from the placenta (26–28). Zhou et al. found that S100P positively regulates trophoblast syncytialization during the earlier stages of pregnancy establishment through the regulation of YAP1 protein and is present in many other tissues as well (29, 30). The results from Zhou et al. showed lower S100P as a poor pregnancy outcome marker for humans. Another peptide and its receptor, kisspeptin, have an established role in reproduction as a whole (31–33). It is expressed in a wider range of tissue along with the placenta. Researchers depicted its role in pregnancy (34, 35). Tsoutsouki et al have explored kisspeptin levels as potential pregnancy complication markers. They suggest kisspeptin levels could be useful in early pregnancy losses alongside beta-HCG measurement. Kisspeptin levels have been found to be variable during preeclampsia, suggesting a need for further study to ensure pregnancy health and success. In this Research Topic, Zhou et al. studied the role of VTCN1 at the human maternal-fetal interface. VTCN1 (B7-H4)'s expression in the first week's villous trophoblast was recently described (36). As VTCN1 has several functions and roles in immune homeostasis, its immunological involvement at the maternal-fetal interface could also be an important factor. Zhou et al. explained VTCN1 as an important regulator of trophoblast syncytialization and invasiveness during early placentation. Hence, VTCN1 could also be involved with abnormal placentation and diseases associated with abnormal placentation during pregnancy. The normal function of the placenta is also dependent on the ubiquitously expressed renin-angiotensin system (RAS) (37). The components of RAS, Angiotensin II (Ang II), and Angiotensin (1-7) (Ang-(1-7)) contributed to the normal physiological function of the reproductive processes such as follicular growth and development and the function of the placenta. Liu et al. summarize the localization and role of Ang II and Ang-(1-7) in the female reproductive system. A concreted understanding of the RAS and involvement of Ang II and Ang-(1-7) may aid in the understanding and maintenance of a healthy pregnancy. Whenever we consider the placental abnormalities, Kong et al., with a retrospective cohort study, found that IVF and maternal age are related to placental abnormalities. Extensive research is needed to determine if there is a connection between biological and molecular mechanisms causing placental pathological conditions in IVF and aged human pregnancies. Along with human studies, Bai et al. studied the insight into bovine pregnancy establishment and the role of the stromal protein PGE2 in bovines. Along with IFN tau, PGE2 from endometrial stromal cells helps maintain pregnancy with its luteoprotective function. Bai et al. demonstrated the molecular mechanism of how PGE2 helps in pregnancy maintenance. Bai et al. identified the PGE2-mediated factors viz. NFIL3 and CEBPA expression, might help in early-stage pregnancy establishment.

Polycystic ovary syndrome (PCOS) affects 6 to 15% of women of reproductive age (38). PCOS is also associated with miscarriage, gestational diabetes mellitus, hypertensive disorders of pregnancy, preterm delivery, and the birth of small-for-gestational-age (SGA) infants. Considering mRNA levels, Ren et al. stated that the PNA mouse is the best animal model for studying PCOS. Although a high percentage of women are affected by PCOS, its screening is not yet straightforward. Therefore, biomarkers could be of paramount importance here. Earlier studies highlighted that follicular microenvironments are related to PCOS (39). The pilot study by Ding et al., with the aim of searching for biomarkers of PCOS from follicular fluid, has successfully identified twenty-three lipid subclasses as potential biomarkers of PCOS in women. This study

could help in developing diagnostic markers and an accurate and early screen of PCOS in women.

SGA and large-for-gestational-age (LGA) infants are always at greater risk of obstetrical and gynecological complications (40, 41). Much research has been conducted with regard to the transportation functions of the placenta. Maternal lipid viz, total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol (HDL-c) are taken up by the placenta and actively participate in maternal-fetal metabolism and development (42, 43). In their retrospective study, Zhu et al. found that a higher lipid profile is associated with higher birth weight in the first trimester. They also found an increased risk of (LGA) and macrosomia. Although it is not routinely practiced, Zhu et al. show that measuring pregnant women's first trimester's lipid profile is advisable.

However, comparatively less research has been conducted on the placenta, an endocrine organ. Placental-secreted hormones and growth factors directly contribute to fetal development and pregnancy maintenance (44–49). Lopez-Tello; Sferruzzi-Perri explored the placental endocrine function and identified placental hormones as key fetal growth and pregnancy maintenance regulators. In conclusion, it could be stated that this Research Topic combined research articles that could help researchers and scientists generate ideas for a sound and healthy pregnancy outcome.

Author contributions

JB: Conceptualization, Writing – original draft, Writing – review & editing. AR: Writing – review & editing. BB: Writing – review & editing. KI: Writing – review & editing. LE: Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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Oxytocin in Women's Health and Disease

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Oxytocin (OT) is a nonapeptide mainly produced in the supraoptic and paraventricular nuclei. OT in the brain and blood has extensive functions in both mental and physical activities. These functions are mediated by OT receptors (OTRs) that are distributed in a broad spectrum of tissues with dramatic sexual dimorphism. In both sexes, OT generally facilitates social cognition and behaviors, facilitates parental behavior and sexual activity and inhibits feeding and pain perception. However, there are significant differences in OT levels and distribution of OTRs in men from women. Thus, many OT functions in men are different from women, particularly in the reproduction. In men, the reproductive functions are relatively simple. In women, the reproductive functions involve menstrual cycle, pregnancy, parturition, lactation, and menopause. These functions make OT regulation of women's health and disease a unique topic of physiological and pathological studies. In menstruation, pre-ovulatory increase in OT secretion in the hypothalamus and the ovary can promote the secretion of gonadotropin-releasing hormone and facilitate ovulation. During pregnancy, increased OT synthesis and preterm release endow OT system the ability to promote maternal behavior and lactation. In parturition, cervix expansion-elicited pulse OT secretion and uterine OT release accelerate the expelling of fetus and reduce postpartum hemorrhage. During lactation, intermittent pulsatile OT secretion is necessary for the milk-ejection reflex and maternal behavior. Disorders in OT secretion can account for maternal depression and hypogalactia. In menopause, the reduction of OT secretion accounts for many menopausal symptoms and diseases. These issues are reviewed in this work.

Keywords: lactation, menstruation, parturition, pregnancy, menopause

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1 INTRODUCTION

Why should we concern women's health and diseases? Women exhibit menstrual cycle, pregnancy, parturition, lactation, menopause and other unique physiological activities, such as maternal behaviors. Correspondingly, women have some unique reproduction-associated diseases, such as postpartum depression and menopausal syndromes. Women's biological activities are regulated not only by the hypothalamic-pituitary-gonad (HPG) axis, but also by oxytocin (OT). While OT commonly influences sexual behaviors, production of sex steroids and the maturation of gemmates

of both sexes (1), it differently influences women's health and disease at different reproductive stages. In this work, we review the roles of OT in women's health and disease.

2 GENERAL VIEW OF THE OT SYSTEM

OT, a classical neuropeptide, is mainly produced in hypothalamic OT neurons. Changes in OT neuronal activity can modulate cognitive, endocrine and physical activities as well as autonomic and visceral neural activities. In addition, scattered OT cells are also present in extrahypothalamic brain regions and peripheral sites, exerting autocrine and paracrine functions at local levels.

2.1 Histological Features of the OT System

OT neurons in the brain are largely aggregated in several neuroendocrine nuclei, typically the supraoptic nucleus (SON) and paraventricular nucleus (PVN). In the SON and PVN, most OT neurons send axons to the posterior pituitary and are magnocellular neuroendocrine cells. These OT neurons can release OT into the blood from OTergic terminals in the posterior pituitary and into the forebrain from OTergic axon collaterals (2). Another type of OT neurons in the CNS is parvocellular OT neurons. They are mainly present in the parvocellular division of the PVN and project to the brainstem and spinal cord but not the posterior pituitary (3). Alongside the hypothalamic OT neurons, some OT cells are also present in extrahypothalamic regions in the CNS and peripheral sites, such as amygdala, the median preoptic nucleus, uterus, placenta, amnion, corpus luteum, testis, heart and colon (4–6). Notably, OT gene expression in chorio-decidual tissues can increase three- to fourfold around the time of labor onset (7–10), supporting OT functional role in parturition. These histological features allow OT to modulate body functions at multiple levels and patterns including neuromodulation, neurosecretion, endocrine, autocrine and paracrine effects.

2.2 Features of OT Neuronal Secretion and Its Regulation

OT release from OT neurons is microdomain-specific (11). In response to changes in the neurochemical environment around OT neurons, changes in cytosolic Ca^{2+} level and/or firing activity occur. Increased firing rate causes OT release at the axonal terminals *via* excitation-secretion coupling. Increased cytosolic Ca^{2+} level triggers OT release from somata and dendrites. The somatodendritic OT release can be in synchrony with the firing activity under physiological conditions, such as suckling stimulation (12). It can be independent of the firing activity under some pathological conditions, such as maternal depression following offspring deprivation (13) and cesarean delivery (14).

The electrical activity, cytosolic Ca^{2+} level and the resultant OT secretion are under the regulation of extracellular and intracellular factors (**Figure 1**). These factors include changes in neurochemical environment (15), tonic and clustered synaptic inputs (16, 17), intercellular junctional coupling such as connexin 36 (18), and astrocytic plasticity (19, 20). Moreover, autoregulation of OT neuronal activity is a key regulator of OT neuronal activity (21). By activation of OT receptors (OTRs), OT causes activation of a series of intracellular signaling events, such as increased cyclooxygenase-2 (21), extracellular signal-regulated protein kinases 1 and 2 (22), and protein kinase A (13). These signals can activate hyperpolarization-activated cyclic nucleotide-gated channel 3 (13, 23), which can promote OT secretion (24, 25). Under the regulation of these extracellular and intracellular factors, OT neuronal activity and OT secretion can meet the demands of body activity in response to environmental changes.

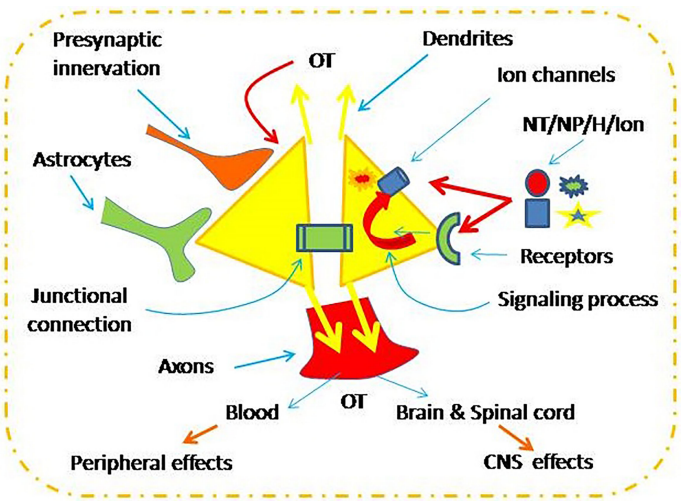

FIGURE 1 | Neurochemical regulation of oxytocin (OT) neuronal activity. CNS, the central nervous system; NT/NP/H/Ion, neurotransmitters, neuropeptide, hormone and ions.

2.3 Expression of OTRs and Its Regulation

OTRs are the protein product encoded by OTR gene that is localized at 3p25-3p26.2 in the human chromosome (4). OTR belongs to the G-protein-coupled receptor superfamily and its activation is regulated by cholesterol as an allosteric modulator (26). The expression profile of OTRs is a tissue- and stage-specific, such as upregulation of the nuclear fractions in term myometrium and down-regulation in non-pregnant myometrium. The OTR gene appears to be highly methylated. Methylation around intron 1 and in intron 3 might contribute to tissue-specific suppression of the gene. OTRs are also regulated by the mechanisms of desensitization, which causes the loss of ligand-binding activity of the protein as well as suppression of OTR mRNA transcription. OTR mRNAs are present in different sizes, for instance 3.6 kb in human breast and 4.4 kb in ovary, endometrium, and myometrium. In posttranslational modifications, OTRs are further palmitoylated and glycosylated (27, 28). In addition, species differences are present and may be due to the existence of different clones of OTR genes of the myometrium and the hypothalamus at different reproductive stages (29, 30). Thus, the phenotype of OTRs from different species and different tissues of the same species could appear in different sizes.

In mammals, OTRs have been identified in a broad spectrum of tissues, including the kidney, heart, thymus, pancreas, adipocytes and other sites in addition to the CNS (4). Expressions of OTRs in the hypothalamus, uterus, and mammary glands are stimulated by estrogen (31). In females, OTRs are specifically localized in the myoepithelial cells of the mammary glands, and in the myometrium and endometrium of the uterus. Peripheral actions of OT are commonly associated with smooth muscle contraction, particularly within the female and male reproductive tracts (32).

OT expression during pregnancy and parturition in females has some unique features unseen in males. Changes in brain OT binding sites during pregnancy may influence the sudden onset of maternal behavior in female rats at parturition. Transcriptional regulation of OTR gene expression mediates changes in receptor density in the brain in a region-specific manner during pregnancy, such as the uterus (33), the PVN, SON, the bed nucleus of stria terminalis (BNST) and the medial preoptic area (mPOA) (34). Moreover, peak OTR mRNA expression was observed at parturition in the SON, brainstem regions, mPOA, BNST, and olfactory bulbs. Postpartum OTR expression in all brain regions returned to levels observed in virgin rats (35). These features of OTR expression are in agreement with the demands of establishing maternal behaviors and parturition.

Correspondingly, the uterus transitions from a quiescent non-contractile state to an active contractile state at the end of pregnancy. This is in association with increased OT/OTR signals (10, 36). Uterine quiescence requires prevention of excessive Ca^{2+} influx through voltage-dependent Ca^{2+}channels at the human myometrial smooth muscle cells. Through most time of pregnancy, the K^+ leak current is dominant and maintains the cell at a sufficiently negative membrane potential to prevent premature uterine contraction. However, increased myometrial OT/OTR expression near the term (10, 36) significantly increases OTR-associated Gαq-protein activation of protein kinase C (33), which then inhibits the activity of sodium-activated, high-conductance, K^+ leak channel. This results in depolarization of the uterine smooth muscle cells and calcium entry that causes uterine contraction (37). This effect is associated with the induction of cyclooxygenase 2, production of prostaglandin F2α and connexin 43 of the uterus (38, 39).

3 OT FUNCTIONS AND SEXUAL DIMORPHISM

3.1 General Functions

The functions of OT depend on the distribution of OTRs and relative change in OT levels. In the brain and spinal cord, activation of OTRs is associated with a variety mental activities, such as social memory, pair-bonding, maternal behavior, and aggression and instinctive behaviors such as sexual activity, anxiety, feeding and pain perception (40–42). In the circulation, OT can facilitate parturition and the milk-ejection reflex and regulate activities of other organ systems (43, 44). Locally-produced OT can promote the differentiation of thymic cells, inhibit inflammation (45), protect the heart from ischemic injury (46), and suppress metastasis of colorectal cancer (5) among many other functions (1, 4).

In the CNS sites, OT can regulate social activity, instinctive behaviors, and visceral functions. For example, by activation of inhibitory neurotransmission in the medial frontal cortex, the amygdala and hippocampus, OT can promote social recognition and pro-social behaviors while reducing stress and fear (47–49). Released from parvocellular OT neurons in the PVN, OT can act on the ventral tegmental area (VTA) to activate rewarding process. OT can suppress nociception ad pain by acting on the periaqueductal gray and spinal cord. By acting on the dorsal vagal complex, OT can regulate visceral activities *via* vagus. By innervating median eminence and median preoptic area, OT can increase gonadotropin-releasing hormone (GnRH) release and the activity of HPG axis (1, 50). In addition, intrahypothalamic OT can inhibit corticotrophin-releasing hormone neurons and social stress *via* acting on the parvocellular division of the PVN (51).

By contrast, circulating and locally-produced OT can influence body functions at cellular, tissue, organ and system levels. For instance, OT can promote insulin secretion (52) and immunological homeostasis (53), protects cardiovascular system (46), suppresses colorectal cancer migration (5), and potentially antagonizes COVID-19 pathogenesis (54). In addition, OT is a natriuretic hormone that acts directly on the kidneys (55). On the other hand, its synthesis and secretion are regulated by changes in plasma osmolality and blood volume (56, 57). This process proceeds in an estrogen-dependent manner in females (58, 59). Thus, OT extensively modulates body functions.

3.2 Sexual Dimorphism of OT Functions

In studies on OT functions, significant difference between males and females emerges, which is particularly significant in psychological and reproductive functions.

3.2.1 OT/OTR Signaling

Sexual dimorphism of OT functions is based on expression levels of OT and OTRs. For instance, serum OT levels are significantly higher among women than men (60), which makes women more sensitive to OT level reduction and likely accounts for menstrual pain (61) and higher incidence of depression in women (62). By contrast, OT binding sites in the VMH and dorsal horns are significantly high in males relative to females (63), which may contribute to the central regulatory actions of OT on feeding, reproduction *via* VMH (64) and nociception *via* the spinal cord (65). Males also exhibit higher OTR levels in the medial amygdala irrelevant to the reproductive status (66), which likely makes men less fearful facing stressful challenges because OT acing on the medial amygdala inhibits fear. Higher OTR levels in the nucleus accumbens are present at breeding males but not breeding females (66), which makes paternal behaviors conditional (67) and more rewarding (68). The sex dimorphism in the distribution OT and OTRs sets a histological basis for gender-specific functions and behaviors.

3.2.2 Psychological Functions

The most relevant OT-associated sexual dimorphism is present in empathy, social skills and other higher brain functions. OT facilitates familiar-partner preference with females being more significant and it increases trust in others and reduces anxious emotion, especially for males (69). Autism spectrum disorder is relatively low in the female gender. This is related to the higher levels of OT and better pragmatic language in girls than boys (70). In nulliparous women, OT enhances attention to the baby schema and morphological characteristics of an infant's face (71). In animal studies, the sexual dimorphisms of OT psychological functions are also identified. For example, in prairie vole, compared to females, males with less OTR expression perform better than females in a spatial memory and spatial learning test (72). Intranasal administration of OT (IAO) before the acquisition or recall sessions enhances conditioned safety memory in female rats while OT has no effects in male rats (73). In male prairie voles exposure to IAO during the peri-adolescent period impairs adult pair bonding in a dose-dependent fashion while IAO appears to facilitate pair bonding in females. This is associated with that IAO in females but not in males increases OTR binding in the nucleus accumbens shell (74). Thus, differences in the OT/OTR signaling determine sex dimorphisms between males and females.

3.2.3 Peripheral OT Effects

In association with hypothalamic OT neuronal activity and OT secretion, OT can extensively modulate body activities at peripheral sites. In the rat with left ventricle infarction, OTR is down-regulated in females while up-regulated in males. Thus, OTR signaling and OT protection are suppressed stronger in ischemic myocardium in females than males. It also accounts for why females have higher risk of heart failure and death following myocardial infarction relative to males (75). Moreover, OT is involved in bone formation in both sexes during development; however, OT treatment has no effect on male osteoporosis because estrogen amplifies OT local autocrine and paracrine secretion (76). Decreased OT and increased OTR occur in male but not female alcohol- dependent rats and patients (77). Thus, differences in OT/OTR signaling also exist in the sex dimorphism of circulation and peripheral OT functions.

3.2.4 Male Reproductive Functions

Among all OT functions, the most dramatic sexual dimorphism is reproductive functions. Relative to the periodic changes in females' reproductive physiology, males do not have dramatic monthly periodic alteration in reproductive endocrine activity and reproductive functions. It is known that plasma OT levels increase during sexual arousal and are significantly higher during orgasm/ejaculation in both women and men (78). In the CNS levels, OT from the PVN in the VTA of rats induces penile erection by activating OT neurons-spinal cord pathway (79). This pathway involves activation of dopamine, glutamate and other neurons in the VTA that project to nucleus accumbens, prefrontal cortex, amygdala, and other forebrain regions (80). Moreover, this OT-PVN-VTA-forebrain pathways play a role in the motivational and rewarding aspects of sexual behavior (81). At peripheral sites, OT directly targets the erectile tissues including corpus spongiosum and corpus cavernosum, and promotes ejaculation by increasing sperm number and contracting ejaculatory tissues (82). Thus, OT is a pivotal regulator of male reproductive functions.

3.3 Maternal Behavior

Maternal behavior begins before breastfeeding near the term and is clearly associated with the actions of OT. For example, in OT knockout mice, maternal behavior is disrupted (83) and maternal behavior is reduced significantly when OTR signaling is blocked (84). By contrast, IAO rapidly increases maternal care in mice (85). It is believed that OT neurons in the PVN are sensitive for the smell of offspring in lactating and multiple-parturient rats (86). OT neurons in the PVN and SON project to the hippocampus, amygdala, ventral striatum, hypothalamus, nucleus accumbens and brainstem nuclei; they can extensively modulate maternal behaviors (2, 3). By modulation of the maternal behaviors, OT convincingly promotes human development as recently reviewed (87, 88).

Notably, women with peripartum exposure to synthetic OT have a higher relative risk of depressive or anxiety disorder diagnosis or antidepressant/anxiolytic prescription within the first year postpartum than women without synthetic OT exposure (89). This is likely due to the suppression of excessive OT on OT neuronal activity *via* a mechanism of post-excitation inhibition of OT neuronal activity (14, 23, 90).

3.4 OT and Diseases

Under pathological conditions, reduced OT release or disorders in OTR signaling can cause many diseases, such as social stress (51), schizophrenia and depression (91), obesity (92), lactation failure, postpartum depression (13, 13) and even mammary tumor (93). For instance, schizophrenia is a form of mental disorders and involves dysregulation of the OT system. From animal models to human studies, observations have revealed that OT can improve the psychopathology of patients with schizophrenia by regulating social cognition and behavior (94). Postpartum depression is associated with disorders on maternal

OT system. As reported in rats, lack of suckling stimulation and bolus OT release during lactation can decrease maternal blood OT levels and cause milk insufficiency and maternal depression in lactating mothers deprived of offspring (13, 95) or following cesarean delivery (14). However, disrupted maternal behavior and lactation performance can be largely improved by IAO during offspring deprivation (13) or following cesarean delivery (14). Obviously, disorder in OT system activity contributes to disorders in mental activity while IAO has the potential to correct abnormal social behaviors such as those in schizophrenia and postpartum depression.

Notably, in mediating OT actions, the efficiency of OTRs depends on their gene polymorphisms, expression levels and posttranslational modification (96–98). For example, the GG homozygotes of OTR rs2254298 are associated with childhood adversity (99); DNA methylation in the 1st intron of the OTR gene causes common learning and behavioral impairments (100). Thus, OT-associated diseases are not only associated with disorders in OT secretion but also with abnormality of OT/OTR signaling.

4 OT FUNCTIONS AT DIFFERENT REPRODUCTIVE STAGES OF WOMEN

4.1 Menstrual Cycle

It is well established that a menstrual cycle is regulated by HPG axis. However, studies also highlight a pivotal role of OT in menstrual cycle. For instance, increased brain OT level can increase GnRH secretion, specifically at pre-ovulation stage (101, 102). Consistently, plasma OT is significantly higher during ovulatory phases than the luteal phase in ovulating women (103). In rats, c-Fos expression in the SON is significantly higher and OT neurons-associated astrocytic processes retract significantly during the proestrus (104, 105). These findings support functional activation of OT neurons and increased OT release before ovulation. Thus, the stimulatory effect of OT on GnRH secretion can promote luteinizing hormone (LH) secretion, facilitate ovulation and prepare uterus environment for pregnancy.

By contrast, exogenous OT can shorten postpartum estrous interval but triggering a delay in ovulation while a higher dose of OT could stimulate the growth of small, medium, and total follicles in postpartum buffaloes (106). Estradiol level increase is correlated with OT release from the pituitary and causes more luteal OT secretion (107). Thus, OT secretion and estrogen release before luteal phase can form a positive feedback loop and they together facilitate ovulation (**Figure 2A**).

During pregnancy, persistent release of uterine progesterone and estrogen interrupts the periodical activity of the HPG axis. Moreover, increased progesterone also inhibits OT release (108). They together cause the cessation of menstrual cycles. Following parturition, breastfeeding delays the resumption of normal ovarian cycles by disrupting the pattern of pulsatile release of GnRH from the hypothalamus. Intermittent bolus release of OT during suckling, its disruption of normal interaction of OT with the HPG axis and inhibition of energy intake also play a key role in lactation-associated amenorrhea (92). When suckling stimulus declines near weanling, preovulatory LH surge restores and ovulation takes place with the formation of a corpus luteum of variable normality.

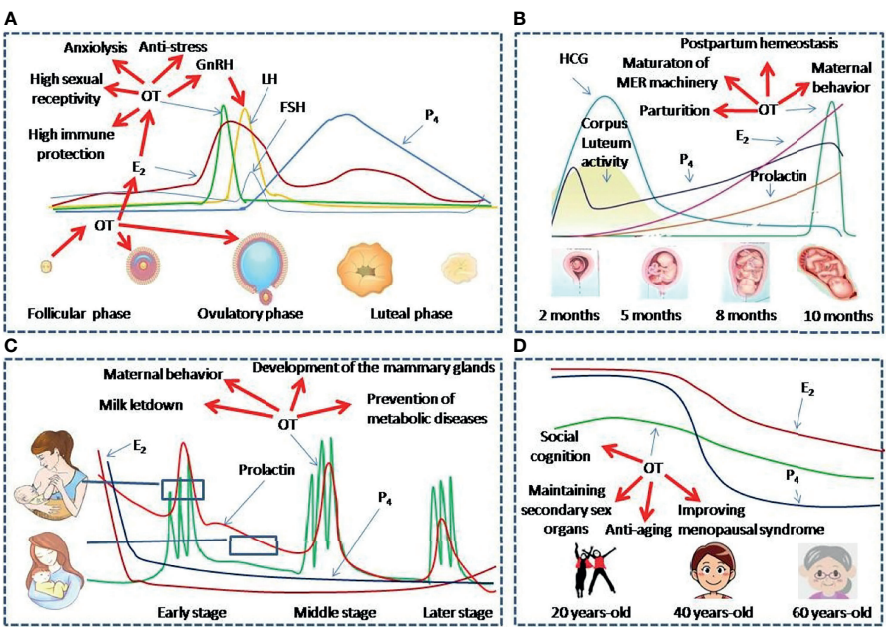

FIGURE 2 | OT functions at different reproductive stages of women. **(A–D)** Panels show levels and functions of OT and associated reproductive hormones during Menstruation **(A)**, Pregnancy and parturition **(B)**, Lactation **(C)** and Peri-menopausal stage **(D)**, respectively. E2, estradiol; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; HCG, Human Chorionic Gonadotropin; LH, Luteinizing hormone; MER, the milk-ejection reflex; P4, progesterone.

By contrast, women with a history of primary or secondary dysmenorrhea have lower blood OT concentrations during menses, which is associated with worse menstrual pain and pain-related behavior (61). In women with early life sexual abuse, OT can positively modulate menstruation-related mood disorders (109). In addition, OT can enhance conditioned safety memory (73), elicit maternal behavior towards alien pups in virgin females (110), alleviate chronic pelvic pain (111) and rehabilitate anorexia nervosa (112). Thus, OT extensively modulates mental and physical activities and menstruation-associated diseases.

4.2 Pregnancy and Parturition

4.2.1 OT and Pregnancy

During pregnancy as marked by the production of human chorionic gonadotropin, OT production increases gradually and prepares for the demands of parturition and breastfeeding (**Figure 2B**). As reported, gestation gradually increases OT synthesis and OTR expressions in magnocellular OT neurons in the SON and PVN and in forebrain neurons, such as the mPOA (34). However, OT release from OT neurons does not increase during pregnancy until the time shortly before parturition. This is clearly beneficial for avoiding abortion during pregnancy. By contrast, blockade of OTR during mid-late gestation delays OT release and causes hypogalactia during lactation (113). Thus, the development of OT/OTR signaling is an adaptive response for maintaining the safety of pregnancy. However, increased OT synthesis and preterm OT release in the hypothalamus are necessary for the maturation of hypothalamic machinery that allows OT to be released in bolus intermittently during parturition and lactation (113). Thus, OT actions during pregnancy highly match peri-partum physiological demands.

4.2.2 OT and Parturition

Shortly before the parturition, progesterone inhibition of OT neuronal excitability *via* endogenous opioids and GABA is weakened (108), which allows OT release in bolus in response to extension of cervix. The mechanical stretch of the cervix can activate magnocellular OT neurons in burst-like firing activity, which determines a bolus release of OT from the posterior pituitary (114). OT further initiates a self-sustaining cycle of uterine contractions until fetus is expelled. In this process, uterine OT release is also increased, which intensifies circulating OT-evoked uterine contraction by increasing prostaglandin F production. As a result, parturition is accelerated (115) and postpartum hemorrhage is reduced (116). Thus, OT is an essential factor for natural delivery.

Notably, nocturnal and pulsatile OT release often occurs at the end of parturition. For example, plasma OT and nocturnal uterine activity in the dams increase progressively during late pregnancy and delivery in rhesus monkeys (117). This is associated with the effect of light/darkness on the pulsatile OT release (118), which determines the high incidence of parturition during night.

4.3 Lactation

In all mammals, OT is a hormone necessary for mothers to nurse their offspring through the milk-ejection reflex. Clearly, in OT-knockout mice, the pups cannot obtain milk from the mother because the dams fail to eject milk for the offspring to obtain through suckling the nipples (119). Consistently, conditioned OTR knockout dams experience high rate of pup mortality (120).

Successful breastfeeding depends on coordinated activities of numerous humoral factors. For instance, the increased hypothalamic OT release during suckling promotes prolactin secretion from the anterior pituitary. They together make milk production and ejection available for the baby during breastfeeding (121). This effect is different from the increased prolactin during pregnancy that acts to maintain the corpus luteum of the ovary and helps to sustain pregnancy but has no direct association with OT release (122). Under physiological condition and normal nutrition, breastfeeding in normally developed women relies on the milk-ejection reflex. In this reflex, suckling stimulation at the nipples activates OT neurons, causes OT release in a bolus into the blood, and results in the ejection of milk from the teat (**Figure 2C**). The activation of OT neurons appears as intermittently recurrent and simultaneous increase in the firing rate in a large pool of OT neurons over several seconds, which causes the bolus release of OT at OTergic neural terminals in the posterior pituitary (44). Without this synchronized burst firing of OT neurons, OT release from one or a few of OT neurons or only one side of the hypothalamus cannot trigger full milk ejections (123, 124). In the synchronized activation of OT neurons, simultaneous retraction of astrocytic processes from OT neurons, shared synapses, increased gap junctional coupling and cellular apposition are logically important contributors (44). However, for coordinated inter-nuclear burst synchrony among OT neurons, a synchronized signal from the mammillary body complex in the ventroposterior hypothalamus is the most important event. This is because disruption of it but not other brain regions disrupts inter-nuclear burst synchrony (125, 126).

Under the drive of exogenous factors including synaptic input, astrocytic plasticity and interneuronal interactions (**Figure 1**), a series of OTR-associated signaling events are activated during suckling. These events include mobilization of $\beta\gamma$ subunits (16), induction of cyclooxygenase 2 and prostaglandin production (21), phosphorylation of extracellular signal-regulated protein kinase 1 and 2 (22), activation of protein kinase A (13, 127), and filamentous actin reorganization (21, 22) among many others (44). These factors express in a different spatiotemporal order to modulate ion channel activity on the membrane (128), such as hyperpolarization-activated cyclic nucleotide-gated channel 3 (13, 23), and thus trigger burst firing in OT neurons and OT release. Nevertheless, detailed neural circuit between OT neurons and their regulatory network and the cellular mechanisms underlying burst firing remain to be explored.

About lactation-associated health issue, it has been extensively accepted that normal breastfeeding can reduce the incidence of postpartum depression, maternal obesity, diabetes, and even breast cancer. OT is necessary for these benefits of breastfeeding. As revealed in animal study, maternal behavior in OT knockout mice is incomplete (83). Lack of suckling stimulation and bolus OT release during lactation can decrease maternal blood OT levels and

cause hypogalactia and maternal depression (13, 95). By contrast, IAO can largely restore maternal behavior and lactation performance as stated above. Importantly, OT can also suppress breast cancer. This is because OT can reduce the oxidative stress of the mammary glands and the occurrence of pre-cancer lesions in the mammary glands (129). Moreover, OT can down-regulate the NF-κB and up-regulation of miR-195. These molecules can promote cell apoptosis, inhibit cell proliferation and consequently, decrease the mammary tumor volume and weight (130). Thus, OT is not only essential for normal breastfeeding but also for the long-term health benefits of women.

4.4 Menopause

Menopause is the end of women's menstrual cycles following decrease of reproductive hormones. From the middle age, blood OT levels decrease gradually (131). Decreased levels of OT can largely account for diminished sexual ability and vagal activity and reduced estrogen levels (**Figure 2D**).

4.4.1 Sex Organs and Bone Metabolism

Menopausal atrophy of accessory reproductive organs is a common sign of reduced ovary functions. However, topical OT application can reverse vaginal atrophy (132, 133). Similarly, a high proportion of women develop osteoporosis after menopause, which increases the incidence of bone fractures. In animals, ovariectomy elicits bone loss and increased bone marrow adiposity (134). Administration of OT can normalize ovariectomy-induced osteopenia in mice by restoring osteoblast/osteoclast cross talk *via* the receptor activator of nuclear factor-κB ligand/osteoprotegerin axis (135). Thus, OT is a potential treatment of menopausal osteoporosis.

4.4.2 Body Mass

Weight gain in menopausal women has been frequently reported (136). Obesity and menopause are independent negative predictors of plasma OT levels (131). Daily administration of OT can normalize body weight and intraabdominal fat depots in ovariectomized mice. This effect is mediated by inhibition of adipocyte precursor's differentiation with a tendency to lower adipocyte size by shifting fuel utilization favoring lipid oxidation (135). In peri- and postmenopausal female rats, intraperitoneally administering OT also reduces serum triglyceride and low-density lipoprotein-cholesterol levels in naturally premenopausal or menopausal rats (137). Thus, OT can be a preventive factor of postmenopausal obesity, diabetes and the associated cardiovascular diseases (46).

4.4.3 Cardiovascular Effects

Cardiovascular diseases increase dramatically in postmenopausal women. This is also associated with reduction of ovary functions and its influence on OT secretion. When estrogen production decreases, its activation of estrogen receptors on preautonomic PVN OT neurons is also weakened. Resultantly, OT regulation of HPG axis and baroreflex is weakened. This has been shown in ovariectomized rats (138). By contrast, OT can protect the cardiovascular system by maintaining cardiovascular integrity, suppressing atherosclerotic alterations and coronary artery disease, inhibiting metabolic disorders, inflammation and apoptosis and promoting regeneration and repair injuries (46).

As a whole, reduced OT secretion during aging can causes cardiovascular disease, osteoporosis, urinary incontinence, sexual malfunction, obesity, low metabolism after menopause. Thus, improvement of OT secretion can be an important strategy of anti-aging in women.

5 CONCLUSION

OT system can extensively modulate women's physiology, particularly the cognitive and reproductive functions. While OT has common effects on the mental and physical activities of both men and women, different OT levels and OTR expressions at different reproductive stages regulate women's reproductive activities differently. The influence of OT on women's health mainly manifests as its modulation of menstrual cycle, pregnancy, production, lactation and menopause. Under pathological conditions, abnormality in OT secretion and/or OTR expressions can cause a series of female-specific diseases. Thus, further study on OT involvement in women's health and disease is warranted. These studies should focus on women-specific mental and physical effects of OT and the underlying mechanisms, particularly during different stages of reproduction.

AUTHOR CONTRIBUTIONS

NL and MM: Conceived the study, wrote, revised and edited the text. HY and LH: Discussed the key contents, revised text and drew the figures. All authors contributed to the article and approved the submitted version.

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Apelin and Apelin Receptor in Follicular Granulosa Cells of Buffalo Ovaries: Expression and Regulation of Steroidogenesis

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Apelin (APLN), as a ligand for APJ (an orphan G-protein-coupled receptor), is an adipokine with pleiotropic effects in many physiological processes of the body. It has an important role in the control of reproduction particularly in females (mainly in control of ovarian function). This study was carried out to investigate the mRNA and protein amounts of APLN/APJ in granulose cells (GCs) of ovarian follicles with small (SF), medium (MF), and large (LF) sizes of buffalo (*Bubalus bubalis*) and the effect of IGF1 and follicle-stimulating hormone (FSH) on the expression levels of APLN/APJ. In addition, we evaluated the effect of various doses of APLN (isoforms -13 and -17) singly or in combination with IGF1 and FSH on estradiol (E2) and progesterone (P4) secretion in GCs. The mRNA and protein abundance of APLN was the highest in GCs of LF while the APJ expression enhanced with follicle enlargement in GCs (p-value <0.01). IGF1 and FSH elevated the mRNA and protein amounts of APLN and FSH, and IGF1 increased the expression of APJ in buffalo GCs (p-value <0.01). Both isoforms of APLN (-13/-17) singly or in the presence of IGF1 or FSH increased the secretion of E2 and P4 with or without preincubation of cells with APJ antagonist (ML221 10 μM), although we had some variation in the effects. Concurrently, APLN-13/-17 significantly increased the mRNA and protein expression of CYP19A1 and StAR (p-value <0.01). ML221 substantially diminished the secretion of E2 and P4 and also the expression of CY19A1 and StAR in buffalo GCs (p-value <0.01). We also revealed that APLN-13/-17 (10^{-9} M), singly or in response to IGF1 and FSH, increased the production of E2 and P4 in different times of stimulation. In conclusion, APLN may play a crucial role in steroidogenesis and follicular development in ovarian GCs of buffalo.

Keywords: apelin, apelin receptor, estradiol, progesterone, granulosa cells, buffalo

INTRODUCTION

As the largest endocrine gland, adipose tissue secretes different bioactive peptides that are mainly named adipokines (1). Apelin (APLN) is a new adipokine derived from preproapelin with 77 amino acids. Then, a 55-amino-acid fragment is derived from preproapelin and, subsequently, smaller bioactive isoforms such as APLN-36, APLN-17, and APLN-13 as well as the pyroglutamyl form of APLN -13 are produced [(Pyr-APLN-13; (2)]. The smaller isoforms ((Pyr) APLN-13 and -17) are more active and frequent in the circulation (3). APLN is initially detected in bovine stomach extracts as an internal ligand for APJ (an orphan G protein-coupled receptor) that is analogous to the angiotensin II type 1 receptor [AT1; (4)]. APJ can connect to various APLN isoforms with different affinities and switch on numerous signaling pathways resulting in divergent consequences in the body (5). In addition to adipose tissue, both APLN and APJ are extensively spread in the body and produced at different abundances in almost all tissues, particularly in the brain, heart, blood vessels, spleen, lung, intestine, breast, and reproductive tract (5–7). Furthermore, APLN and APJ are involved in several different basic biological processes, such as cell proliferation, cardiovascular function, angiogenesis, food intake, fluid homeostasis, and regulation of energy metabolism (5, 8–10). They have also been detected in the reproductive organs as well as in nucleus of the hypothalamus (the arcuate, supraoptic, and paraventricular hypothalamic), addressing their key roles in the regulation of reproduction (11). Shimizu etal. (12) showed that APJ is expressed in the granulosa cells (GCs) of the bovine follicles. They also suggested that APLN and APJ are partly responsible for the selection and dominance of follicles in the bovine species. The steroidogenic roles of APLN and APJ have been reported in different species such as rats, humans, cattle, sheep, and pigs (13–17). In cattle GCs, APJ but not APLN mRNA was expressed (12). Moreover, APLN and APJ were immunolocalized in porcine GCs and their expression were rather than theca cells (15). Furthermore, APLN and APJ expression was detected in large luteal cells in addition to GCs in sheep (17). Some literature showed that APLN singly or with IGF1 and FSH has some effects on the secretion of progesterone (P4) in cattle (16) and porcine (15). It exerts its effects through various signaling pathways. APLN also led to an increase of the basal levels of estrogen (E2) and P4 *via* AMPKα activation and enhanced the concentration of HSD3B protein, and it decreased the IGF1- and FSH-induced steroid secretion in human and porcine GCs (14–16).

The literature shows that ovarian factors can influence the expressions of APLN and APJ. For instance, IGF1 increased the APLN expression; however, it diminished the mRNA expression of APJ (16). P4 and FSH enhanced the expression of APJ in bovine GCs, while LH affected the expression of both APLN and APJ in cultured TCs (12). E2 causes the gene expressions of APLN and APJ in follicles, indicating the correlation of these genes with angiogenesis during follicle maturation and corpus luteum development in the bovine ovary. APLN and APJ have been involved in the atresia of follicles, the temporary luteal phase after ovulation, and the luteolysis process of the corpus luteum (13). In addition, elevated levels of APJ in atretic follicles were correlated with follicular atresia originating from GC apoptosis in bovine (12). In addition, Roche etal. (16) revealed that APLN and its receptor are expressed in GCs and oocytes in cattle.

Nevertheless, the expression and the possible roles of the APLN and APJ system have not been studied in the GCs of buffalo ovaries. Accordingly, the current study was aimed to investigate the expression of APLN and APJ in GCs of ovarian follicles and the *in vitro* effects of APLN on E2 and P4 production along with the associated molecular mechanisms in ovarian GCs of buffalos.

MATERIALS AND METHODS

Reagents and Ethical Statement

All chemicals and media used in the current study were obtained from Sigma-Aldrich (St. Louis, MO, USA) if not otherwise specified. Total approaches of experiments were performed by the Animal Ethics Committee of the Guangxi Buffalo Research Institute and were implemented with the ethical regulations of animal research by this committee.

Hormones and Antibodies

Recombinant porcine FSH, recombinant human IGF1 (ab270062), APLN-13 (ab141010), and APLN-17 (ab141011) were purchased from Abcam. Also, APLN (ab141011), CYP19A1 (ab18995), StAR (ab96637), and actin (ab8226) antibodies were obtained from Abcam. APJ (20341-1-AP), anti-mouse IgG (SA00001-1), and anti-rabbit IgG (SA00001-2) were obtained from Proteintech (Wuhan, China). Primary and secondary antibodies were used at 1: 500 to 1:1,000 and 1/3,000 for Western blotting, respectively.

Follicle Collection and Granulosa Cell Culture

The obtained buffalo ovaries from a regional slaughterhouse were transferred to the laboratory on ice within 2 h after slaughter. The buffalos were originated from different farms in Guangxi province, China. During transportation, the ovaries were kept in phosphate-buffered saline (PBS) containing 0.06 mg/ml penicillin and 0.05 mg/ml streptomycin at nearly 30°C–35°C. In the laboratory, the ovaries were properly washed with physiological saline solution.

To evaluate the effect of the APLN on follicle steroidogenesis, a GC culture model was established. Therefore, all the intact (adequate circularized with clear follicular fluid and, wall) and detectable follicles were extracted by a 17-gauge needle linked to a 10-ml syringe. The extracts with PBS were moved to a 60-mm dish in a sterilized environment, and all cumulus–oocyte complexes were taken out. The residuals were centrifuged in 15-ml conical tubes at 700 g for 5 min. Then, GCs were resuspended in Dulbecco's modified Eagle medium (DMEM) medium consisting of 10% fetal bovine serum (FBS) and a solution of antibiotics and antimycotic (penicillin 100 U/ml, streptomycin 100 mg/ml, amphotericin B 0.25 mg/ml). Trypan blue exclusion dye was used for assessment of cell viability and count which was higher than 80%. Afterward, the cells were cultivated in a 24/48-well plate at 37.5°C in a humidified CO_2 (5%) incubator and included relatively 1.5 × 105 live cells per well. Following the attachment and growth of cells with about 75%–80%

confluence) for 48 h, they were supplemented with fresh media (FBS free) having various concentrations (10^{-9}, 10^{-8}, and 10^{-6} M) of recombinant APLN-13 or -17 singly or at the concentration of 10^{-9} with porcine FSH (10^{-8} M) or human recombinant IGF-I (10^{-8} M) and were kept for 48 h. Control cells were multiplied in the same circumstances as other cells except for the addition of the peptides. Six replicates were tested for each experimental condition. The spent media were accumulated after 48 h and reserved for E2 and P4 assay, and RNA and protein were extracted from cells.

To compare APLN and APJ expression in different groups of follicles, they were assigned into three different classes according to diameter (3–5 mm, small (SF); 6–9 mm, medium (MF); and >9 mm, large (LF)). In each group of follicles, GCs were collected for mRNA and protein extraction and promptly frozen in liquid nitrogen and stored at -80°C.

Total RNA Extraction, cDNA Synthesis

Total RNA was extracted from GCs of follicles using TRIZOL reagent by the manufacturer's instruction and a fixed quantity of RNA (100 ng) was straightly reverse-transcribed into a 20-µl first-strand cDNA using a PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time, Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions.

Quantitative Real-Time PCR Analysis

Rt-qPCR was done in a whole amount of 20 µl, having the same disseminated cDNA (100 ng), 10 mM each of the forward and reverse primers, and 10 µl of 2× SYBR Green Master Mix (SYBR® Premix Ex Taq™ II (Tli RNaseH Plus, Takara, Japan). The reactions were done in triplicate for all genes of interest and were run on the LightCycler 480 system (Roche Diagnostics, Basel, Switzerland) under the subsequent conditions: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. β-Actin and RPS15 were adopted as the internal control (reference genes) to normalize the relative gene expression levels. All reactions were carried out in triplicate. The expression abundances of genes were evaluated with the $2^{-\Delta\Delta CT}$ method described previously by Livak and Schmittgen (18) concerning the housekeeping genes. The specifications of the genes of interest and the primer pairs used in the study are provided in **Table 1**.

Western Blot Analysis

Total proteins were obtained from cultured GCs of different experiments by lysing in RIPA buffer with PMSF (R0010; Solarbio, Beijing, China) at 4°C for 30 min followed by collection and centrifugation at 12,000 rpm for 5 min at 4°C. The pellet was eliminated, and lysates were mitigated with 6× protein loading buffer (DL101-02; TransGen, Beijing, China) and heated to 100°C for 5 min. Following chilling at refrigerator temperature, the samples were stored at -80°C for Western blotting on ice afterward. Western blotting was started by loading the samples on a 12% gradient polyacrylamide gel (P0012AC; Beyotime, Shanghai, China) and then handing it over to a PVDF membrane (ISEQ00011; Millipore, Shanghai, China), followed by blocking in 8% (wt/vol) Difco Skim Milk in Tris-buffered saline including 0.1% (vol/vol) Tween-20 (TBST) for 2 h. The membrane was incubated with monoclonal anti-β-catenin antibody, diluted 1:500 in blocking buffer, whereas, as the molecular weights of proteins (APLN with 9 kDa, APJ with 68 kDa, StAR with 32 kDa, and CYP19A1 with 58 kDa) and β-actin (43 kDa) were different, each half was marked with a separate primary antibody. For other proteins, the membrane was maintained with their primary antibodies, diluted 1:500 to 1:1,000 in blocking buffer, and incubated overnight at 4°C. After four washes, 10 min each, with TBST, membranes were incubated for 1 h at 37°C with goat anti-mouse IgG (SA00001-1, Proteintech, China) for beta-actin and APLN, APJ, StAR, and CYP19A1 with goat anti-rabbit IgG (SA00001-2, Proteintech, China). The membranes were washed four more times in TBST for 10 min each, then developed using ECL Plus (P0018, Beyotime, China) followed by detection with a multifunction imager (Syngene, Cambridge, UK). The strength of each band was normalized to beta-actin expression. Western blots were conducted three times.

Steroid ELISA Assay

Progesterone concentrations were estimated in serum-free medium from buffalo GCs after 48 h of culture using ELISA kits supplied by Miebiao Biology, China. Cells were plated in 24- or 48-well plates (10^5 live cells/well), and six or eight replicates were examined for each tested condition (rh APLN-13/-17, IGF1, FSH) with or without (W/O) ML221 for each culture. The results were represented as the levels of the steroid (ng/mL). The intra- and inter-assay coefficients of variation (CV) for E2

TABLE 1 | Gene, primer sequence (5′–3′), and developing fragment size.

Gene	Primer sequence (5′–3′)	Amplicon size (bp)	Accession no.
β-Actin	F: TCTCACGGAGCGTGGCTACAG R: CTGCTCGAAGTCCAGGGCCACGTA	100	NM_001290932.1
RPL15	F: TGGGCTACAAGGCCAAACAA R: GCTTCGAGCAAA CTTGAGCTGG	140	MG969348
APLN	F: AAGGCACCATCCGATACCTG R: ATGGGACCCTTGTGGGAGA	106	(16)
APJ	F: TCTGGGCCACCTACACCTAT R: ACGCTGGCGTACATGTTG	100	(16)
CYP19A1	F: CGTCCTGGTCACCCTTCT R: ACGCACCGACCTTGCAA	57	(19)
StAR	F: CTGCGTGGATTAACCAGGTTCG R: CCAGCTCTTGGTCGCTGTAGAG	84	(19)

and P4 were lower than 10%. Results are shown as mean ± SEM. Data were collected from three separate cultures.

Statistical Analysis

All the data have been shown as mean ± SEM. One-way (**Figures 1**, **2**, **4**, **5**) or two-way (**Figures 3**, **6**–**8**) ANOVA followed by Duncan's multiple-range test was used to test the differences among groups by GLM procedure of SAS software (Version 9.4). A significant difference of p-value < 0.05 was taken into account. The data were evaluated for normality and homogeneity of variance among the treatments.

RESULTS

Expression of APLN and APJ in the GCs of Follicles With Different Sizes

The expression levels of APLN and APJ in GCs of small (SF), medium (MF), and large (LF) size follicles are presented in **Figure 1**. To assess the relative mRNA expression levels of APLN and APJ in ovarian GCs, two cDNA fragments of 106 and 100 bp were amplified by qRT-PCR corresponding to APLN and APJ, respectively. The analysis of APLN transcript by qRT-PCR showed a varied expression level during the follicular phase. The mRNA expression of APLN significantly (p-value <0.01) increased in GCs of LFs compared to those of SFs or MFs. Its mRNA expression was not significantly different in GCs of Sf and MF, although its expression numerically declined in GCs of Mfs (**Figure 1A**). The expression of APJ in GCs significantly increased with an increase in the size of the follicles (**Figure 1B**; p-value <0.01). The APJ transcriptional abundance was the lowest in SFs and the highest in LFs. The presence of APLN and APJ proteins in GCs of different sizes of buffalo follicles was confirmed by Western blot. The immunoblot bands of APLN (9 kD), APJ (68 kD), and β-actin (42 kD) are evident in **Figure 1**. The protein expressions of APLN and APJ (ratio of optical density of APLN or APJ proteins/optical density of b-actin protein) in GCs were similar to the relative mRNA expressions.

Effect of IGF1 and FSH on the Transcript Abundances of APLN and APJ in Buffalo GCs

The effects of IGF1 and FSH on APLN and APJ expression are shown in **Figure 2**. The effects of IGF1 and FSH as two major hormones influencing the multiplication and differentiation of

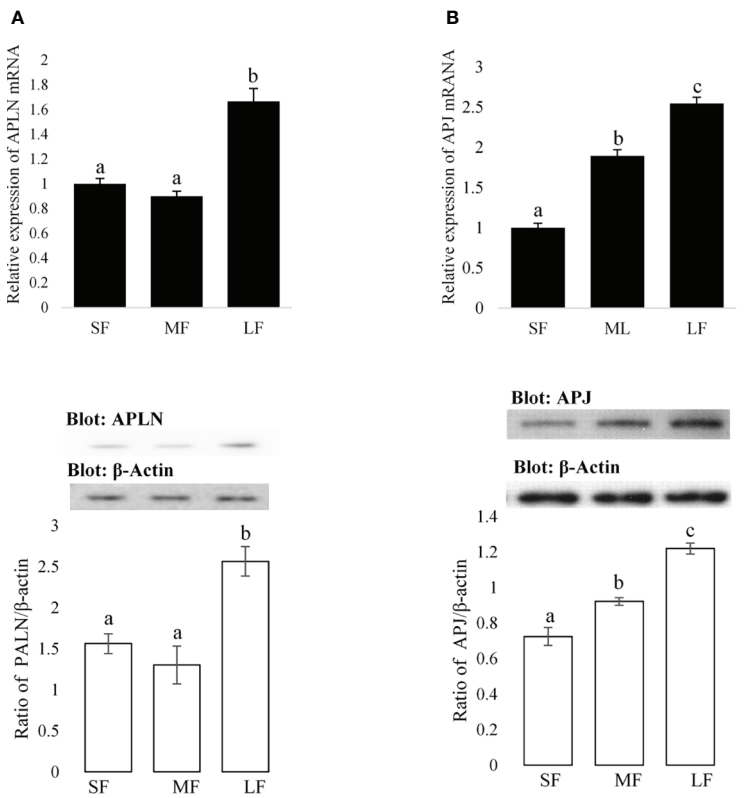

FIGURE 1 | Expression amounts of the APLN and APJ in ovarian GCs of buffalo. Protein and mRNA expression of APLN **(A)** and APJ **(B)** was assessed by quantitative real-time PCR and Western blot in GCs from small (SF), medium (MF), and large (LF) follicles. β-Actin and RPL15 were used as housekeeping genes for qPCR and β-actin as a loading control for Western blot. A pool of 15 follicles for each group was used for the study of APLN and APJ protein and mRNA expression. Then, GC protein extracts were immunoblotted by APLN and APJ antibodies. The membranes were then reprobed by an anti-β-actin antibody with the same amount of protein loading for confirmation. By the evaluation of blots, the ratios of APLN and APJ to -β-actin were calculated. Statistical analyses were done for APLN and APJ, and the results are presented as the mean ± SEM. The different letters on the bars show significant differences at a p-value < 0.01.

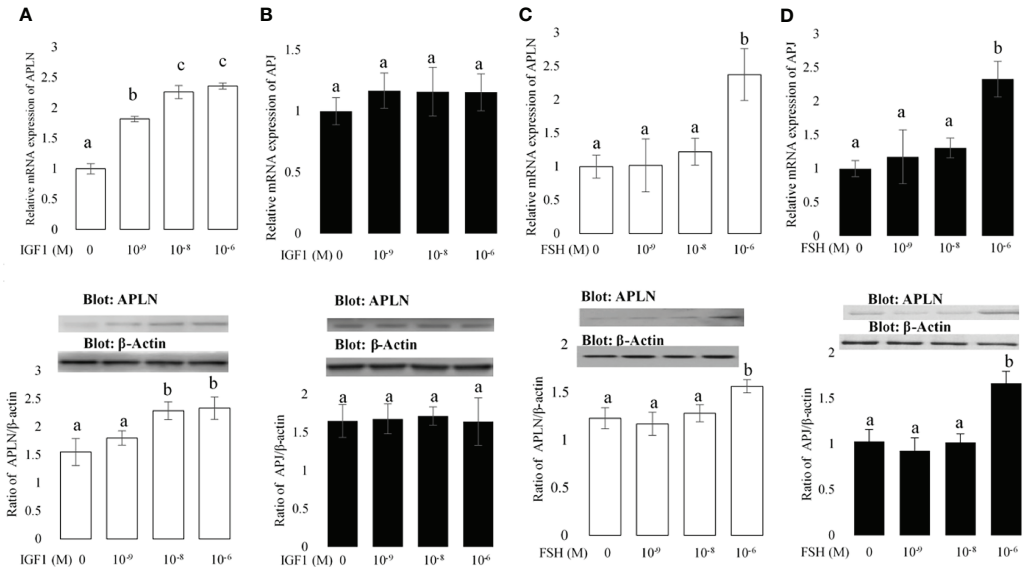

FIGURE 2 | The effects of different concentrations of IGF1 and FSH on the mRNA and protein expression amounts of APLN **(A, C)** and APJ **(B, D)** in buffalo GCs. qPCR was used to assess APLN and APLJ mRNA expression in buffalo GCs. Fresh cells were seeded in a DMEM (with 10% FBS) and after 48 h; culture continues in free FBS media containing IGF1 or FSH with concentrations 0, 10^{-9}, 10^{-8}, and 10^{-6} M for an additional 48 h. GC protein extracts were immunoblotted by APLN and APJ antibodies. The membranes were then reprobed by an anti-β-actin antibody with the same amount of protein loading for confirmation. By the evaluation of blots, the ratios of APLN and APJ to -β-actin were calculated. Statistical analyses were done, and the results are presented as the mean ± SEM. The different letters on the bars show significant differences at a p-value < 0.05.

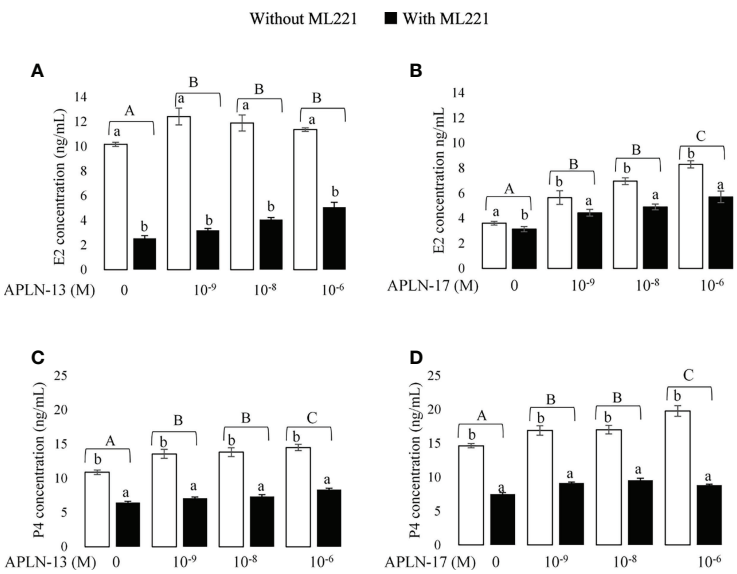

FIGURE 3 | The effect of APLN-13/-17 on E2 and P4 synthesis in buffalo GCs. Cells were seeded in DMEM (with 10% FBS) for 48h in and then in free serum DMEM medium containing different concentrations of APLN-13/-17 (0, 10^{-9}, 10^{-8}, and 10^{-6} M) for 48 h in the presence or absence of APJ antagonist [ML221 (10 μM)]. After the collection, the culture medium was examined for E2 and P4 by ELISA. Results are means + SEM of 6 replicates. Different capital letters show a significant effect of treatments on E2 [APLN-13 **(A)**, APLN-17 **(B)**] and P4 [APLN-13 **(C)**, APLN-17 **(D)**], and lowercase letters indicate a significant effect of ML221 treatment. The different letters on the bars show significant differences at a p-value < 0.01.

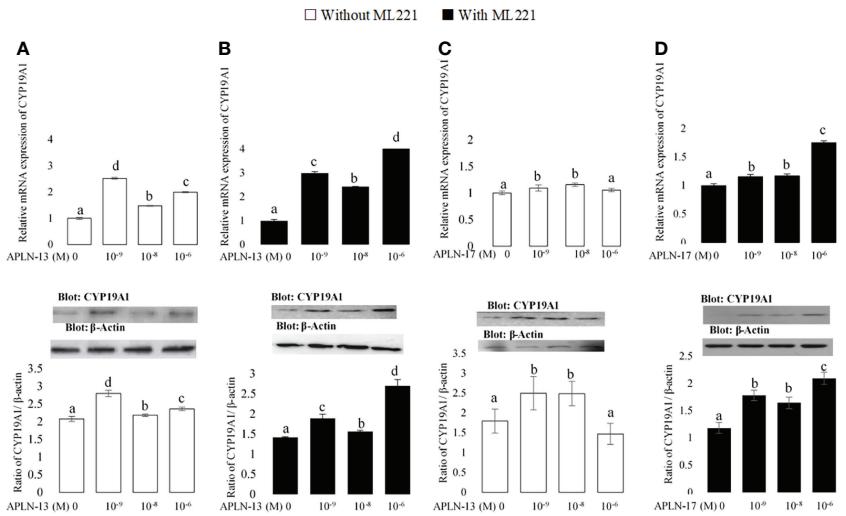

FIGURE 4 | The protein and mRNA expression of CYP19A1 in the buffalo GCs treated by different doses of APLN-13/-17 without **(A, B)** or with **(C, D)** preincubation of cells with APJ antagonist [ML221 (10 μM)]. GCs were seeded in DMEM (with 10% FBS) for 48 h in and then in free serum DMEM medium containing different concentrations of APLN-13/-17 (0, 10^{-9}, 10^{-8}, and 10^{-6} M) for 48 h in the presence or absence of APJ antagonist [ML221 (10 μM)]. Following the total protein and mRNA extraction from the treated buffalo GCs, they were used to assess the expression levels of CYP19A1 by Western blot and quantitative real time-PCR, respectively. β-Actin and RPL15 expression levels in the same mRNA samples were used to normalize the mRNA expression of CYP19A1. The upper and lower parts of the panels show the relative levels of mRNA and illustrative immunoblots, respectively. In this regard, GC protein extracts were immunoblotted by the CYP19A1 antibody. The membranes were then reprobed by an anti-β-actin antibody with the same amount of protein loading for confirmation. By the evaluation of blots, the ratio of CYP19A1 to β-actin was calculated. Statistical analyses were done, and the results are presented as the mean ± SEM. The different letters on the bars show significant differences at a p-value < 0.05.

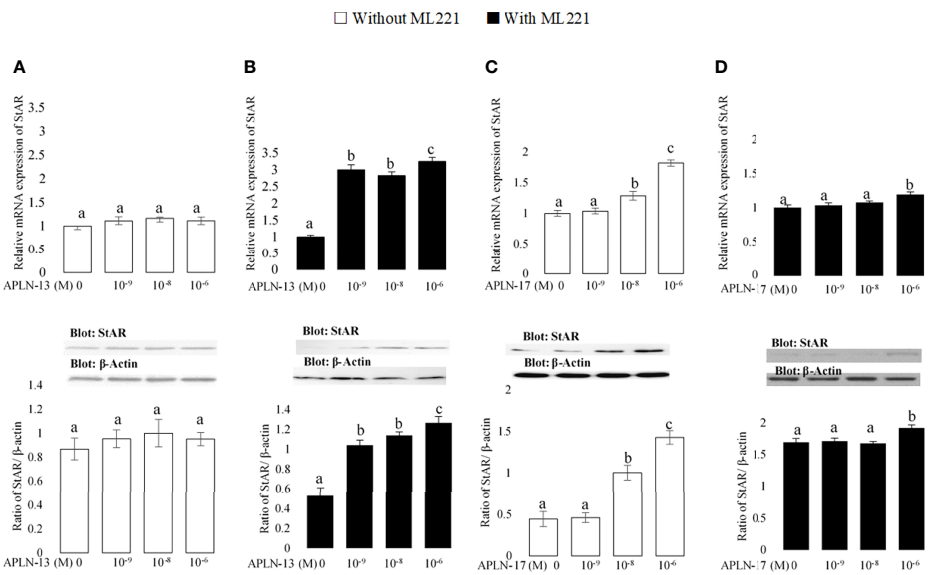

FIGURE 5 | The protein and mRNA expression of StAR in the buffalo GCs treated by different doses of APLN-13/-17 without **(A, B)** or with **(C, D)** preincubation of cells with APJ antagonist (ML221 (10 μM)). GCs were seeded in DMEM (with 10% FBS) for 48 h in and then in free serum DMEM medium containing different concentrations of APLN-13/-17 (0, 10^{-9}, 10^{-8}, and 10^{-6} M) for 48 h in the presence or absence of APJ antagonist [ML221 (10 μM)]. Following the total protein and mRNA extraction from the treated buffalo GCs, they were used to assess the expression levels of StAR by Western blot and quantitative real time-PCR, respectively. β-Actin and RPL15 expression levels in the same mRNA samples were used to normalize the mRNA expression of StAR. The upper and lower parts of the panels show the relative levels of mRNA and illustrative immunoblots, respectively. In this regard, GC protein extracts were immunoblotted by the StAR antibody. The membranes were then reprobed by an anti-β-actin antibody with the same amount of protein loading for confirmation. By the evaluation of blots, the ratio of StAR to β-actin was calculated. Statistical analyses were done, and the results are presented as the mean ± SEM. The different letters on the bars show significant differences at a p-value < 0.05.

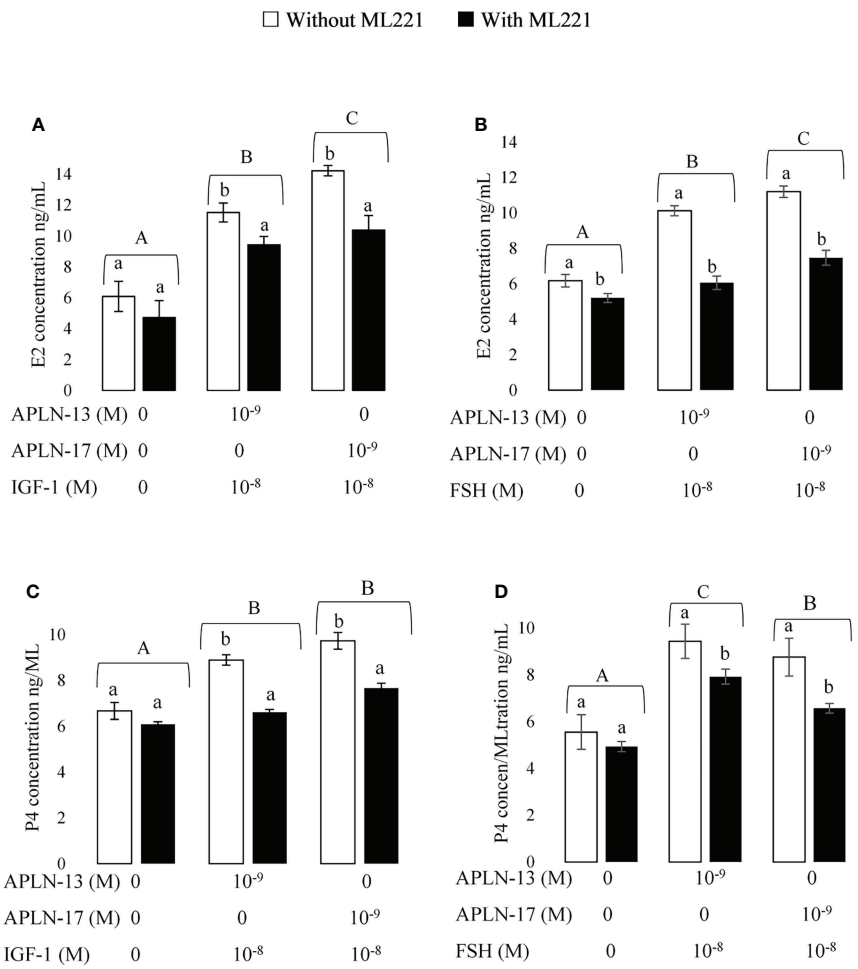

FIGURE 6 | The concentration of E2 **(A,B)**; and P4 **(C,D)** in spent media of buffalo GC culture. GCs were seeded in DMEM (with 10% FBS) for 48 h in and then in free serum DMEM medium solely or containing APLN-13/-17 at the dose 10^{-9} M or in combination with IGF1 (10^{-8}) or FSH (10^{-8}) for 48 h in the presence or absence of ML221 (10 μM). After the collection, the culture medium was examined for E2 and P4 by ELISA. Results are means + SEM of 6 replicates. Different capital letters show a significant effect of treatments on E2 [APLN-13/-17 with IGF1 **(A)**, APLN-13/-17 with IGF1 **(B)**] and P4 [APLN-13/-17 with IGF1 **(C)**, APLN-13/-17 with IGF1 **(D)**] and lowercase letters indicate a significant effect of ML221 treatment.

ovarian cells at concentrations of 0, 10^{-9}, 10^{-8}, and 10^{-6} were studied on the expression of APLN and APJ in buffalo GCs. The results of qPCR showed that all doses of IGF1 enhanced the expression of APLN (**Figure 2A**); however, the Western blot results did not verify the effect of IGF1 at the dose of 10^{-9} on APLN level. Moreover, IGF1 did not have any effect on the mRNA or protein expression of APJ at all (**Figure 2A**). In addition, FSH at the concentration of 10^{-6} M (not other doses) increased the mRNA expression levels of APLN and APJ (**Figures 2C, D**). These results were verified by immunoblotting of APLN and APJ proteins.

Effect of APLN on E2 and P4 Production in Cultured Buffalo GCs

The concentrations of E2 and P4 in the spent media of GC culture are presented in **Figure 3**. With the aim of detecting the effect of APLN on the production of E2 and P4, buffalo GCs were cultured in the medium containing different levels of APLN-13/-17 (10^{-9}, 10^{-8}, and 10^{-6} M) for 48 h. Moreover, to verify a clear-cut effect of APLN, buffalo GCs were preincubated by the APJ antagonist, ML221 for an hour, in the same conditions outlined above. Results showed that APLN-13 (**Figures 3A, C**) and APLN-17 (**Figures 3B, D**) significantly increased the secretion of E2 and P4. ML221 (APJ antagonist) significantly diminished the secretion of E2 and P4 in buffalo GCs. Moreover, the effect of APLN-13/-17 on the levels of E2 and P4 was not the same in the presence or absence of ML221. Without ML221, APLN-13 had numerically a higher effect on the secretion of E2 at the concentration 10^{-9} M compared to concentrations of 10^{-8} and 10^{-6}M. However, E2 increased by the treatment of APLN-13 or APLN-17 in the presence of ML221 in a dose-dependent manner. Furthermore, P4 concentration increased (p-value < 0.01) by the treatment of buffalo GCs with all concentrations of APLN-13 (W/O ML221). APLN-17 treatment at the concentrations of 10^{-9}, 10^{-8}, and 10^{-6} M enhanced the P4

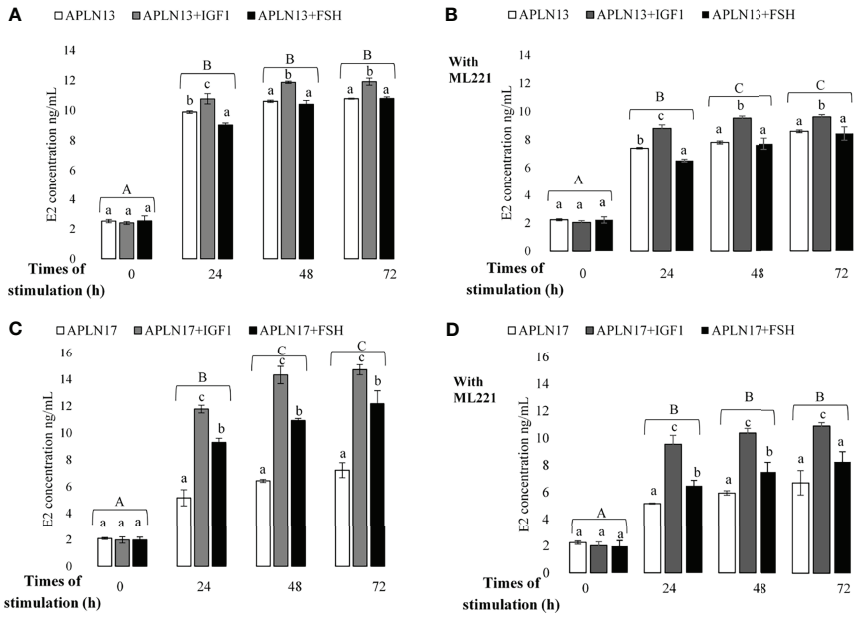

FIGURE 7 | The concentration of E2 in buffalo GCs treated by APLN-13 (10^{-9}; **A, B**) or APLN-17 (10^{-9}; **C, D**) solely or in combination with IGF1 (10^{-8}) and FSH (10^{-8}) in different times of stimulation (0, 24, 48, and 72 h) with **(B, D)** or without **(A, C)** preincubation of cells with ML221 (10 μM). GCs were seeded in DMEM (with 10% FBS) for 48 h in and then in free serum DMEM medium according to the above description. After the collection, the culture medium was examined for E2 and P4 by ELISA. Different capital letters show a significant effect of different times of stimulation by treatments on E2 [without ML221 **(A)**, with ML221 **(B)**] and P4 [without ML221 **(C)**, with ML221 **(D)**] and lowercase letters indicate a significant effect of different treatment (APLN-13/-17 in the presence of IGF1 or FSH). Results are means ± SEM of 6 replicates. The different letters on the bars show significant differences at a p-value < 0.05.

secretion of GCs in the absence of ML221, but the same effects were not seen with ML221. The highest dose of APLN-17 (10^{-6} M) had the greatest effect on P4 secretion without ML221, but it was the lowest by preincubation of GCs with ML221 for an hour.

Effects of APLN on the Transcription Amount of Some Factors Involved in E2 and P4 Production

The effects of APLN-13/-17 on the transcription amount of CYP19A1 as an important steroidogenesis enzyme as well as that of StAR as an important cholesterol transporter were evaluated to better understand their effect on steroidogenesis. As revealed in **Figure 4**, APLN-13 and APLN-17 W/O preincubation of GCs by ML221 for an hour increased the protein and mRNA levels of CYP19A1. Furthermore, APLN-13 (with ML221; **Figure 5B**) significantly increased the protein and mRNA amount of StAR at all treated concentrations but the same effect was not observed in the absence of ML221 (**Figure 5A**). In addition, APLN-17 at the concentrations of 10^{-8} M (without ML221; **Figure 5C**) and 10^{-6} (W/O ML221; **Figures 5C, D**) increased the protein and mRNA levels of StAR.

Effect of APLN on IGF1- and FSH-Stimulated E2 and P4 Secretion

The effects of APLN-13/-17 (10^{-9}M) in combination with IGF1 (10^{-8}M) and FSH (10^{-8}M) W/O preincubation of buffalo GCs by ML221 on the secretion of E2 and P4 are presented in **Figure 6**. The results showed that both APLN-13 and APLN-17 in the presence of IGF1 or FSH increased the concentration of E2 (**Figures 6A, B**). Preincubation of the cells for an hour with ML221 decreased the concentrations of E2 and P4 significantly. In addition, both APLN-13 and APLN-17 in the presence of IGF1 or FSH had a significant effect on the levels of P4 W/O ML221 in buffalo GCs (**Figures 6C, D**).

Effect of APLN Treatment on E2 and P4 Secretion by Buffalo GCs in Response to Various Times of Stimulation by IGF1 and FSH

The effects of APLN-13/-17 (10^{-9} M) on the secretion of E2 and P4 only or in response to various times of stimulation by IGF1 and FSH W/O preincubation of cells with ML221 for an hour are shown in **Figures 7**, **8**. As the results showed, APLN-13/-17 in the presence of IGF1 W/O ML221 had the highest effect on the concentrations of E2 after 24, 48, and 72 h of stimulation (**Figure 7**). APLN-17+FSH had a meaningfully higher effect on the E2 production after 24, 48, and 72 h than APLN-17 W/O ML221 (**Figures 7C, D**). APLN-13 (without ML221; **Figure 8A**) and APLN-17 (W/O ML221; **Figures 8C, D**) had a substantially greater effect on the secretion of P4 than their combination with IGF1 or FSH. Compared to APLN-17 or APLN-17+FSH, APLN-17+IGF1 had the greatest impact on P4 secretion at all times of stimulation (24, 48, and 72 h). Furthermore, the secretion of E2 and P4 was diminished while the buffalo GCs were preincubated

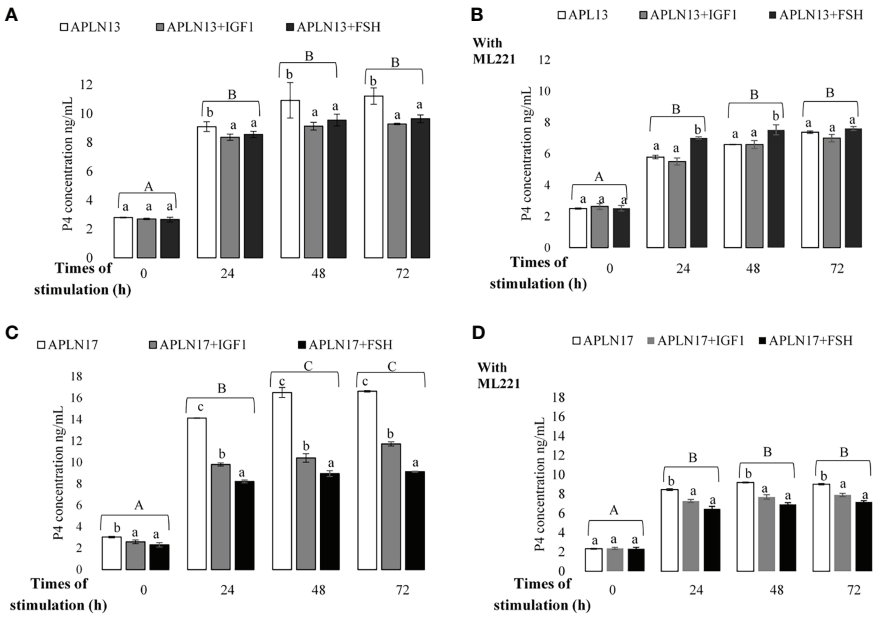

FIGURE 8 | The concentration of P4 in buffalo GCs treated by APLN-13 (10^{-9}; **A, B**) or APLN-17 (10^{-9}; **C, D**) solely or in combination with IGF1 (10^{-8}) and FSH (10^{-8}) in different times of stimulation (0, 24, 48 and, 72 h) with **(B, D)** or without **(A, C)** preincubation of cells with ML221 (10 μM). GCs were seeded in DMEM (with 10% FBS) for 48 h in and then in free serum DMEM medium according to the above description. After the collection, the culture medium was examined for E2 and P4 by ELISA. Different capital letters show a significant effect of different times of stimulation by treatments on E2 (without ML221 **(A)**, with ML221 **(B)**) and P4 (without ML221 **(C)**, with ML221 **(D)**), and lowercase letters indicate a significant effect of different treatment (APLN-13/-17 in the presence of IG F1 or FSH). Results are means ± SEM of 6 replicates. The different letters on the bars show significant differences at a p-value < 0.05.

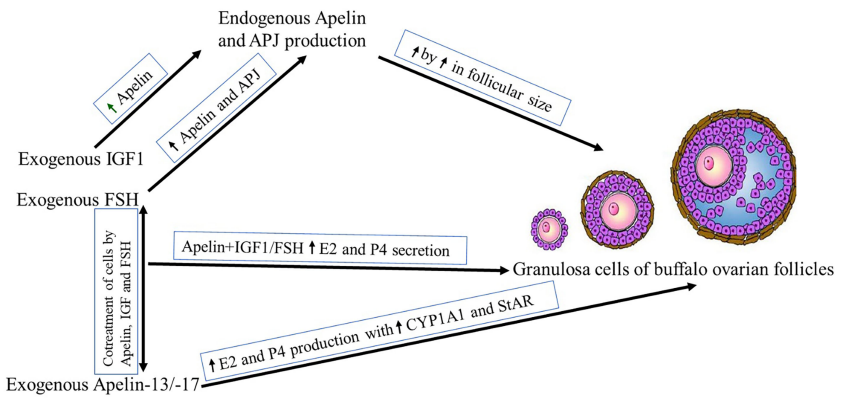

FIGURE 9 | Schematic presentation of the study results. ↑ Stands for the increasing effect.

with ML221 for an hour. In addition, the differences in E2 or P4 production were not statistically different after 48- and 72-h stimulation of cells by different treatments.

DISCUSSION

In the current study, the presence of APLN and its receptor (APJ) was evaluated in GCs of buffalo ovarian follicles with different sizes and the effects of IGF1 and FSH as relevant hormones affecting proliferation and differentiation of ovarian cells, on the expression of APLN/APJ were studied. In addition, the roles of different isoforms of APLN on the E2 and P4 secretion in GCs were investigated (The summarized results are represented in **Figure 9** schematically.). Although the *in vitro* effect of APLN on GCs has previously been declared in various animal species, the protein and mRNA transcript abundances of APLN and its effects on steroidogenesis in GCs have not been addressed in buffalo.

Our results demonstrated that the protein and mRNA of APLN and APJ were expressed in GCs of SF, MF, and LF of buffaloes. The expression of APJ was increased by enlargement of the follicles; however, the expression abundance of APLN was the same in GCs of SF and MF and increased in those of LFs. These results indicated that by the follicular enlargement, the protein expression of APLN significantly enhanced and the highest level of APLN was observed in LFs, whereas the greatest levels of APJ were seen in MFs and LFs. Therefore, the expression of APLN and APJ depends on the ovarian follicle sizes. Similar results were reported by Roche etal. (16) in GCs and oocytes of bovine ovarian follicles even though, unlike our results, the expression of APLN was increased with follicular growth in GCs and oocytes. Likewise, the protein and mRNA transcript levels of APLN/APJ significantly elevated with the follicle augmenting in porcine ovaries (15). Furthermore, Shimizu etal. (12) demonstrated that APLN was not expressed in GCs of bovine ovarian follicles but they showed that APJ mRNA expression increased in line with follicular growth. In other studies, APLN and APJ expressions were detected in GCs of ovarian follicles in sheep (17), pigs (15), and humans (14). The synchronic expression of APLN and APJ in the GCs and other cells of ovarian follicles powerfully implies that APLN may have some paracrine or autocrine roles as a local regulator in GCs, theca cells, and the corpus luteum. The triggering effect of APLN has already been reported on proliferation, migration, and angiogenesis of cells (5, 8, 20, 21); therefore, it can be one of the key factors that have physiological functions in the maturation of follicles. Moreover, according to the reports regarding their expression in different stages of the corpus luteum in porcine (13) and bovine (22), it also influences different actions in the corpus luteum such as vascular formation, maturation, and maintenance (15).

Additionally, hormonal regulation and endogenous hormones can also associate with differences in APLN/APJ expressions in follicles of different sizes because the ovarian conditions change during the ovarian cycle (15).

Furthermore, our results showed that treatment of buffalo GCs with IGF1 at levels of 10^{-9}, 10^{-8}, and 10^{-6} increased the transcript abundance of APLN; however, FSH only at the dose of 10^{-6} enhanced the expression amounts of APLN or APJ. IGF1 did not change the expression levels of APJ in buffalo GCs. Literature shows that the expression of APLN as an adipokine is regulated by various factors in adipose tissue in humans and mice (23, 24). Nonetheless, the reports regarding the effect of different factors on the APLN/APJ expression levels in GCs are contradictory. Roche etal. (14) reported that FSH/LH had no effect on the expression of APLN and APJ, but IGF1 increased the transcript abundance of APJ in human granulosa cells. Shimizu etal. (12) revealed that P4 elevated the mRNA transcript abundance of APJ in the cultured GCs of cattle, and similar to our results, they suggested that FSH triggered the mRNA expression levels of APJ in the bovine GCs. In another study, Roche etal. (16) demonstrated that IGF1 led to an increase in APLN mRNA expression in bovine GCs whereas it diminished the mRNA expression of APJ. These authors also did not find any significant influence of FSH on the mRNA expression amounts of APLN/APJ. Along with some discrepancies with the literature, our findings revealed that APLN in combination with IGF1 and FSH has a positive effect on steroidogenesis in GCs. It shows that these hormones cooperate in the proper functioning of GCs and interactions among them and other hormones secreted from different ovarian cells are meaningful for the appropriate action of all ovarian cells. However, these inconsistent data can be addressed by some differences in culture condition, type of GCs (from primary, middle, or ovulatory follicles), species, study design, and sample size.

Our results also showed that two isoforms of APLN (-13 and -17) increased the concentrations of E2 and P4 production in buffalo GCs. Higher doses had the greater effects on E2 secretion with preincubation of the cells by ML221 for an hour, but the same effects were not observed without it. Likewise, ML221 decreased both E2 and P4 levels in buffalo GCs treated or untreated by APLN13/-17. Rak etal. (15) found that different doses of APLN increased the production of E2 and P4 in porcine GCs. Similar to our results, Roche etal. (16) suggested that treatment of bovine GCs or human GCs (14) with APLN-13/-17 increased the secretion of P4, and preincubation of cells with ML221 decreased the concentration of P4. There is a disagreement among our findings as they suggested that ML221 did not have any effect on P4 secretion when the GCs were not treated with APLN-13 or APLN-17. On the other hand, APLN-13 and APLN-17 (except 10^{-6} M without ML221) at all doses increased the transcription abundance of CYP19A1. Moreover, APLN-13 with ML221 and APLN-17 without ML221 enhanced the expression of StAR protein in buffalo GCs. In agreement with our findings, CYP19A1 expression levels were improved by treatment of porcine GCs with APLN and it was concurrent along with the increasing effect of APLN on E2 and P4. In contrast to these results, Roche etal. (14) revealed that APLN-13/-17 did not have any meaningful influence on the expression of CYP19A1 or StAR; however, they increased the transcription amount of HSD3B in human cultured GCs. In the current study, the specific effect of APLN on E2 and P4 secretion was correlated with the elevation of StAR and CYP19A1 mRNA levels; accordingly, APLN caused an increase in the production of E2, P4, CYP19A1, and StAR in buffalo GCs. CYP19A1 is an important enzyme for the production of E2, and StAR is a cholesterol carrier required for the preparation of the steroidogenesis precursor. It might be mentioned that an increase in P4 secretion could be connected to an increase in StAR expression abundance and an enhancement in E2 secretion was associated with an enhancement in CYP19A1 expression. Thus, APLN could modulate the activity of StAR and CYP19A1 enzymes in GCs of buffalo ovaries.

Additionally, our results revealed that APLN-13/-17 in combination with IGF1 and FSH increased the concentration of E2 and P4 secretion in buffalo GCs. Likewise, their effects diminished when the cells were preincubated with ML221. The findings also showed that APLN-13 or -17 in the presence of IGF1 substantially had the highest effect on E2 secretion after 24, 48, and 72 h of stimulation. These APLN isoforms also had more

effect on the concentration of P4 singly than in the presence of IGF1 or FSH, although APLN-13+FSH with ML221 had a higher effect than APLN-13 or APLN-13+IGF1 in the buffalo GCs. Furthermore, the secretion of E2 and P4 of buffalo GCs was not significantly altered after 48 h of stimulation by different treatments. Roche etal. (16) suggested that APLN elevated E2 and P4 production in human primary GCs and P4 secretion in bovine GCs. These authors also reported that APLN-13 significantly increased P4 secretion W/O IGF1 for 24, 48, and 72 h sequentially, whereas it did not influence the FSH response in line with the time of stimulation in bovine GCs. These results are contrary to our data. In these studies, the P4 concentration increased after 24 h but we did not obtain the same results, and the concentration of P4 and even E2 did not change after 48 or 72 h compared to 24 h of stimulation of buffalo GCs by APLN-13/-17 in combination with IGF1 or FSH. The possible reason to address these discrepancies could be related to species differences and, also, they used primary GCs, whereas we cultivated the GCs from all visible buffalo ovarian follicles. IGF1 is an important endogenous growth factor for the ovaries which have a crucial function in the regulation of follicular development in mammals (25). In such a way, IGF1 triggers GC proliferation and P4 secretion (26). Furthermore, APLN plays a substantial act in energy metabolism and it is an insulin mimic peptide in skeletal muscle. As the structure of insulin and its receptor is similar to IGF1 and IGF1R, APLN could enhance the sensitivity of GCs to IGF1 as already discussed in mouse skeletal muscle; in addition, we discussed the stimulatory effect of APLN on the E2 and P4 secretion, therefore APLN may cooperate with IGF1 or other ovarian hormones in the regulation of steroidogenesis in mammals.

CONCLUSIONS

The results of the current study demonstrated that APLN/APJ is expressed in ovarian GCs of buffalo. A correlation was found between the size of follicles and the expression abundance of APLN and APJ. Our finding also showed that two isoforms of APLN had a stimulatory effect on steroidogenesis and such effect was verified by the specific impact of ML221 as an APJ antagonist. Furthermore, APLN in the presence of IGF1 or FSH, as important hormones influencing the ovarian follicles functions, affected the steroid secretion of buffalo ovarian GCs. The particular effects of different isoforms of APLN on the steroidogenesis of GCs imply that this adipokine has a considerable impact on reproductive functions (especially folliculogenesis) in buffalo (*Bubalus bubalis*), although it needs approval by further studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of the Guangxi Buffalo Research Institute.

AUTHOR CONTRIBUTIONS

BS designed and conducted the study and data analysis and also wrote the manuscript. H-YZ, L-YL, L-PT, X-YM, X-RL, A-QD, YZ, X-HT, C-XH, and Y-YX helped in a few practical lab works. J-HS was the project leader. All authors contributed to the article and approved the submitted version.

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REVIEW
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Estrogenic Pastures: A Source of Endocrine Disruption in Sheep Reproduction

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Phytoestrogens can impact on reproductive health due to their structural similarity to estradiol. Initially identified in sheep consuming estrogenic pasture, phytoestrogens are known to influence reproductive capacity in numerous species. Estrogenic pastures continue to persist in sheep production systems, yet there has been little headway in our understanding of the underlying mechanisms that link phytoestrogens with compromised reproduction in sheep. Here we review the known and postulated actions of phytoestrogens on reproduction, with particular focus on competitive binding with nuclear and non-nuclear estrogen receptors, modifications to the epigenome, and the downstream impacts on normal physiological function. The review examines the evidence that phytoestrogens cause reproductive dysfunction in both the sexes, and that outcomes depend on the developmental period when an individual is exposed to phytoestrogen.

Keywords: phytoestrogen, reproduction, endocrine disruptor, clover disease, ruminant

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INTRODUCTION

The endocrine disrupting effects of phytoestrogens has been of emerging interest in both animal production and human health. The full spectrum of effects on reproduction continue to be elucidated. Initially of interest in human reproduction, the impact of phytoestrogens on reproduction is becoming increasingly relevant in livestock production because several key pasture species are estrogenic. The effects of estrogenic pasture on livestock reproduction are a considerable economic and animal welfare issue.

Several species of subterranean clover ("sub clovers") were incorporated into Australian livestock grazing systems in the 19th century (1, 2). These sub clovers were widely adopted due to their nutritive value as pasture legumes, their resilient characteristics such as the ability to remain dormant over many seasons, their lower requirement for fertilizer than other pasture species, and their persistence under grazing (1). As a result, sub clover pastures are well integrated into animal production systems in Australia. Many of those species are now recognized to have high levels of the isoflavone formononetin, which is metabolized to equol in the rumen. Equol causes a spectrum of moderate to severe functional and morphological changes to the ewe reproductive tract (3–5) that

has been termed "clover disease". Although it is suspected that clover disease continues to cause fertility and welfare issues in sheep, investigation into it has been dormant since the 1990's.

The female has been the focus of livestock research into phytoestrogen effects on reproduction. Early investigations into the disruptive effects of estrogenic clover described masculinized genitalia, morphological changes to the cervix, and irregularities in the duration of the estrous cycle (3, 4, 6, 7). While there have been reports on the profound effects of phytoestrogens on male fertility in several species (8–10) and *in vitro* (11), there has been very little investigation into reproductive compromise in the ram.

The obvious solution to mitigate clover disease in sheep production is pasture renovation to eliminate the sub clover cultivars that are estrogenic. However, pasture renovation can take several years, and does not guarantee that estrogenic sub clover will not persist or re-emerge, given the resilient characteristics of this species (1).

Though the known presentation of clover disease has changed over time, with reduced severity compared to initial reports (4, 12, 13), an impact on sheep reproduction is likely still present (14). With the expansion of research into the impacts of other endocrine disruptors on reproduction (15, 16), new perspectives emerge on the potential mechanisms behind how estrogenic pasture influences on sheep reproduction. The first step toward prevention of the issue, therefore, is to define the severity, nature, and distribution of clover disease in commercial systems, to establish the mechanistic basis behind the phenomenon, and to increase awareness of the potential impacts of estrogenic sub clover within the industry. Accordingly, this review examines phytoestrogens as endocrine disruptors in sheep reproduction, with reference to known and postulated mechanisms of phytoestrogens in both the sexes.

OVINE CLOVER DISEASE: THE FIRST EVIDENCE FOR PHYTOESTROGENS AS ENDOCRINE DISRUPTORS IN SHEEP

Ovine clover disease was first detected and described in Western Australia in the 1940's (17). Decades later, the disease was attributed to the high formononetin content of several cultivars of *Trifolium subterraneum*, a common pasture legume in sheep production systems in Australia (3, 4, 6, 7, 18). In the rumen, formononetin is converted to the estrogenic metabolite equol, causing masculinized genitalia, morphological changes to the cervix (**Figures 1A, B**), and irregularities in the duration of the estrous cycle (3, 4, 6, 7). These changes are accompanied by a reduction in the fertility rate, an increase in dystocia, an increase in the number of stillborn lambs, and an increase in the incidence of uterine prolapse after parturition (3, 17–19). Though these studies were critical in identifying the role of estrogenic sub clover in the compromise of sheep reproduction, the mechanisms behind the disease are not fully understood. Though the severe manifestations of clover disease appear to have been eradicated, anecdotal reports indicate that more subtle manifestations of sub-fertility have persisted when sheep graze on sub clover cultivars that are estrogenic.

Despite evidence that clover disease remains an issue in Australian sheep production systems (14), there has been little progress toward understanding the disease or developing solutions that will mitigate the impact on ovine fertility. Investigations in other species have revealed that phytoestrogens operate *via* diverse pathways, including competitive binding to nuclear estrogen receptors, that can result in both agonistic and antagonistic actions, as well as by modifying the epigenome. While these studies in other species confirm that exposure to phytoestrogens has detrimental effects on reproduction, particularly during some sensitive developmental periods, there remains a lack of comprehensive evidence on the causal pathways.

A BRIEF OVERVIEW OF PHYTOESTROGENS AND THEIR PREDICTED MODES OF ACTION

New additions continue to be made regularly to the spectrum of plant-derived estrogenic compounds that are known broadly as phytoestrogens. The predominant classes of phytoestrogens are isoflavones (daidzein, formononetin, genistein, glycitein), flavones (luteolin), flavonoids (quercetin, kaempferol), coumestans (coumestrol), stilbenes (resveratrol), and lignans (lariciresinol, matairesinol, pinoresinol, secoisolariciresinol) (20, 21). When they are consumed by an animal, many of these compounds can be further metabolized by the gut microflora into estrogenic metabolites. An example of particular relevance is the estrogenic by-product equol, resulting from the breakdown of formononetin in the mammalian digestive tract (22, 23).

The mechanism(s) that link phytoestrogens and reproduction remain controversial, though several key pathways have been proposed *via* which estrogenic compounds could operate. Perhaps the most discussed pathway is the interaction of phytoestrogens with estrogen receptors, albeit with a far lower binding affinity than the endogenous ligand, 17-β-estradiol (24, 25). In vertebrates, there are two main subtypes of nuclear estrogen receptor, ERα and ERβ (which are comprehensively reviewed by 26). The biological functions of the two receptor subtypes vary, as does their distribution between tissues, between the sexes, and between species (27–29). When they are activated by a ligand, the nuclear receptors ERα and ERβ are thought to act through either direct genomic modulation or indirect signaling. In the former, when ligand binds to the estrogen-receptor, the receptor dimerizes and binds to estrogen response elements in the promoter region of specific genes, thereby stimulating or repressing the transcription of that gene (30, 31). Indirect signaling leads to changes in transcription without a direct interaction with DNA (32, 33). Importantly, phytoestrogens not only competitively bind with ERα and ERβ, but can promote transcription from this interaction (34, 35).

Many of estrogens actions occur after stimulation of ERα. Stimulation of ERα is known to induce cellular proliferation, including the priming of the uterus *via* uterine cell production (36) and cancer progression, particularly in breast tissue, reproductive tissues, and bone (37–39). Estrogen- ERα

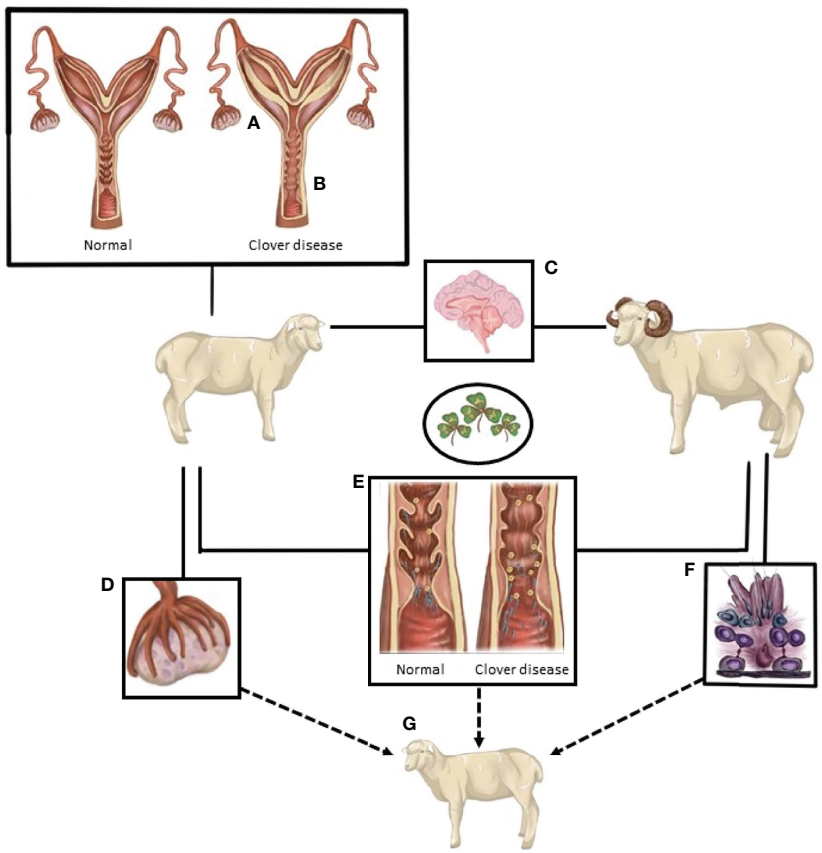

FIGURE 1 | A summary of the known and postulated sites of action of compounds from oestrogenic pasture that lead to compromised sheep reproduction. **(A)** Endometrial thickening/edema. **(B)** Loss of cervical folds. **(C)** Excessive oestrogen-like actions in the neuroendocrine control of reproduction. **(D)** Follicle development, quality and potentially ovulatory ability are reduced. **(E)** The interaction between spermatozoa and the female tract is altered. Loss of cervical crypts, changes in mucus composition, and consistency and changes in the female immune response hinder sperm navigation of the female tract. **(F)** Sperm production and quality is potentially reduced. **(G)** Exposure of both male and female gametes, and the ovine embryo, may cause differential developmental programming of the subsequent generation.

binding is also able to induce estrogen-neuroendocrine feedback in the hypothalamus (40, 41), and increase transcription in most cell types (42). While ERβ is often co-expressed with ERα (26, 43), the stimulation of ERβ is thought to oppose the actions of ERα on gene expression (26, 31, 44–46). Thus, the outcome of estrogen stimulation for a particular cell will depend on the relative expression of ERα and ERβ. Both subtypes are involved in glucose (47–49) and lipid (50, 51) homeostasis in the brain, liver, pancreas, heart, and skeletal muscle (52). Most phytoestrogens have a higher binding affinity for ERβ than ERα (53), though that bias does not clearly translate to predictable physiological outcomes *in vivo* in mammalian systems. Notably, the expression of both receptor subtypes is increased by increased estrogen exposure (26), which may be a crucial mechanism behind the cumulative pathology resulting from long-term phytoestrogen exposure (3, 6). In the context of estrogenic pasture, an increase in receptor expression could underly the clinical manifestations, whereby long-term phytoestrogen exposure confers permanent infertility in the ewe, rather than the transient infertility that is observed with short-term exposure.

In addition to actions mediated by ERα and ERβ, phytoestrogens can interact with the G protein-coupled estrogen receptor (GPER). While ERα and ERβ are nuclear receptors, and therefore require their ligands to access the nucleus, GPER is a membrane associated receptor that is distributed across a multitude of tissues, at least in humans and rodents (54–57). When it binds ligand, GPER operates through non-genomic signaling, a comprehensive discussion of which can be found in a recent review by Luo and Liu (54). Of note, GPER is present in the reproductive tissues of both sexes, including breast tissue (58–60), the testis (61), prostate (62), ovary (63, 64), and endometrium (65, 66). Exogenous estrogenic compounds, including genistein, bisphenol A, and zearalenone, have a similar binding affinity for GPER as they do for the nuclear estrogen receptors (67). Of interest,

some phytoestrogens, including equol and genistein, have been reported to have a greater binding affinity for GPER compared to 17β-estradiol (68). Thus, these extra-nuclear estrogen receptors may be a significant pathway through which exogenous estrogens can exert endocrine-disruptive effects through non-genomic, or indirect transcriptional pathways.

Phytoestrogens could also alter reproductive function *via* an indirect effect on genome transcription (69) that is often referred to as organizational effects. Modifications to the epigenome, such as DNA methylation, non-coding RNA's, and histone modification, can influence the expression of the genome without altering the DNA sequence (70, 71). There is evidence that endocrine disrupting agents, and specifically exogenous estrogenic compounds, alter the epigenome in several tissues. For example, in neonatal rodents, coumestrol, genistein, and the estrogenic metabolite equol induce hypermethylation in several regions of the genome, including tissue-specific alterations in the uterus, kidney, and pancreas (72–76), as well as broadly increasing DNA methylation in the epigenome (73), in proto-oncogenes (72), and in dermal tissue (74). In addition to the endocrine disrupting actions of phytoestrogens, it is likely that *in utero* exposure to estrogenic compounds is detrimental to reproductive outcomes, given that gestation is an important period of epigenetic remodeling. Phytoestrogens can cross the blood-placenta barrier and infiltrate fetal tissues, including the brain (77, 78). To date, the phenotypic consequences of the epigenetic effects of phytoestrogen exposure have not been investigated.

It is therefore possible that epigenetic modifications that follow phytoestrogen exposure will have biologically relevant effects on the ovine reproductive system. Epigenetic changes that are induced by environmental factors, such as by assisted reproductive technologies and climate, are known to modify sperm function (79, 80), reproductive success in both male and female (81, 82), and disease onset (83) across a range of mammalian species. Moreover, these modifications can persist through generations (83, 84). Targeted and broad epigenetic modifications can influence the clinical severity of other reproductive pathologies, such as varicocele (85), polycystic ovary syndrome (86), and endometriosis (87). Theoretically, epigenetic changes in the reproductive system of sheep after exposure to phyto-estrogens could be at least partially responsible for the wide variation in clinical manifestations of clover disease, and could account for the persistence of flock sub-fertility even after sheep are removed from clover pasture.

Thus, the effects of phytoestrogens can be exerted *via* several mechanisms and pathways. An understanding of the systemic and direct mechanisms that lead from phytoestrogen exposure to reproductive pathology will be essential to find solutions. The developmental period during which an individual is exposed to phytoestrogens is likely critical in the extent of reproductive dysfunction. Developmentally sensitive periods include uterine life, neonatal life, prior to and during puberty, and times when estrogen has an essential biological role, such as during the estrous cycle.

THE TIMING OF PHYTOESTROGEN EXPOSURE IS CRITICAL FOR REPRODUCTIVE OUTCOMES

Maternal Exposure to Phytoestrogens During Pregnancy, in the Neonate, and Prior to the Onset of Puberty

Beneficial and detrimental outcomes on female fertility after exposure to phytoestrogens have been reported. That apparent contradiction is probably due to the timing, duration, and level of phytoestrogen exposure, with consequences dependent on whether the individual is exposed *in utero*, during the neonatal and prepubertal periods, or during adult life.

The widespread use of soy-based infant formula, which is now known to contain physiologically relevant concentrations of the isoflavones genistein and genistin, has stimulated research on phytoestrogen exposure during key developmental periods in humans, and to a much larger extent in rodents (88, 89). Infants that consume soy-based formula have circulating levels of genistein that are 13,000 to 68,000-fold higher than the normal biological concentration of estradiol in non-exposed infants in the same age bracket (88). While the long term effects of phytoestrogen exposure in human infants are not known, *in utero* and neonatal exposure to genistein in rodents induces morphological alterations in the reproductive tract, including precocious vaginal opening (90), ovarian follicle atresia (91), increased uterine fluid content (92), and hyperplasia of the endometrium (92–94).

Given those outcomes in humans and rodents, it is plausible that phytoestrogen exposure during the pre-pubertal period could alter the onset of puberty in the ewe-lamb. The onset of puberty in the ewe requires considerable modulation of the hypothalamic-hypophyseal-gonadal axis and is subject to complex interactions between endocrine, metabolic, and genetic pathways (95–97). Prior to the change in amplitude and frequency of the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus that defines the initiation of ovine puberty, the hypothalamus of the pre-pubertal ewe is highly sensitive to negative feedback from estradiol. Estradiol, produced primarily from the pre-pubertal ovary, inhibits GnRH secretion and the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH), without which ovulation does not occur. Phytoestrogens, such as equol, may exert a similar inhibitory effect in the ewe-lamb, particularly when it is present in combination with other factors that are known to delay puberty onset, such as poor nutrition, inadequate exposure to short-day photoperiod patterns, or a lack of social interaction (98).

Comparatively, in juvenile rodents, the effects of phytoestrogens on markers of puberty onset appear to be inconsistent, and depend on dosage (99, 100). At concentrations comparable with low level dietary exposure, genistein induces hyperplasia of mammary tissue in the pre-pubertal rat, but at concentrations comparable with high level dietary exposure reduce both alveolar development (99, 100) and

the sensitivity of mammary tissue/gland to estradiol (101). In rodents, in cases where sexual maturity is advanced by phytoestrogen exposure, the estrous cycles are very irregular at the commencement of puberty (102–104). While the mechanism that leads to the oligomenorrhea is not known, several studies support the notion that pre-pubertal exposure to estrogen-like compounds, including genistein, alters hypothalamic kisspeptin expression and leads to a lower density of kisspeptin expressing neurons and fibers in the anteroventral periventricular and arcuate nuclei (102, 105). Kisspeptin has a well-established role in the timing of both puberty and the estrous cycle, playing a critical role in estrogen feedback to the hypothalamus. Because GnRH neurons do not express estrogen receptors, the mechanism of steroid feedback remained a mystery until the discovery of kisspeptin (106). The discovery that kisspeptin neurons do express estrogen receptors, and that substances released from kisspeptin neurons (including kisspeptin, neurokinin B, and dynorphin) can stimulate GnRH neurons, provided a mechanism for steroid feedback. Any lowering of the activity of the kisspeptin signaling pathway can compromise the normal steroid feedback to GnRH neurons (107–111). Theoretically, a decrease in the stimulation of GnRH neurons by kisspeptin could account for the delayed pubertal onset and impaired ovulation that is observed after neonatal phytoestrogen exposure (4, 102, 112–116). Investigation of the neuronal circuitry that leads to GnRH release in phytoestrogen-exposed ewe lambs may shed further light on not only whether puberty is physiologically delayed, but also whether there are more permanent effects on the hypothalamic-pituitary-gonadal axis.

In rats, phytoestrogen exposure during the fetal and early neonatal stage is associated with long term alterations to the estrous cycle after the exposed individuals reach puberty, with prolonged suppression of ovarian cycling (117) and inhibition of LH secretion (118). Neonatal exposure to genistein might create a more hostile uterine environment that is less capable of supporting embryo implantation (103, 119). In addition to these reproductive consequences, *in utero* exposure to estrogenic compounds also impairs immune function in rats (120), increases the incidence of cancerous lesions in the uterus (121, 122), and impairs the development of estrogen-sensitive organs, particular secondary sex organs (123, 124).

Phytoestrogens Have Conflicting Effects on Reproductive Function in Adult Females

The effects of phytoestrogen exposure in the adult female remain unclear, with a spectrum of effects reported across species, ranging from beneficial to injurious. Adult mice exposed to estrogen-like compounds, for example, develop irregular estrous cycles, with more atretic follicles and an absence of *corpora lutea* (90). In women, dietary isoflavone intake is associated with an abnormally short luteal phase (125). More broadly across species, phytoestrogens have been linked to reproductive abnormalities, including an increase in uterine weight in cheetahs (126), reduced fertility in rhinoceros (127), and altered ova composition and estrous cyclicity in several bird species (112). In the context of clover disease, morphological changes in the follicles, along with reduced fertility, have been observed in mature ewes grazing estrogenic pasture, as outlined further below. Briefly, exposed ewes present excessive numbers of small to medium ovarian follicles with inadequate antrum formation, increased uterine fluid and subacute inflammation of the endometrium, and a reduction in the depth of cervical crypts and cervical squamous metaplasia (3, 6, 13, 128).

In contrast, in adult humans, isoflavone has been reported to have beneficial effects on fertility. In preconception cohort studies, higher intake of isoflavone was associated with improved fecundity and fertility (129, 130) and the re-instatement of ovulation in anovulatory cycles (131). In women with polycystic ovarian syndrome, supplementation with phytoestrogen extract of *Cimicifuga racimosa* increased plasma progesterone and endometrial thickness, suggesting that the phytoestrogens facilitated ovulation (132). It should be noted that the metabolites that are produced from phytoestrogens vary between ruminant and monogastric species, which may partly account for some of the species-specific outcomes of exposure to phytoestrogens. Several isoflavones, and their metabolites, have been suggested to have antioxidant effects and to mitigate inflammation (for an extensive review, see 133). These properties are likely responsible for the beneficial effects of isoflavones in cancer models (134) and in some cases, improvements in fertility. Reactive oxygen species (ROS) have a dichotomous role in reproduction, whereby particular levels of ROS are necessary for the processes of oocyte maturation, ovulation (135), and for spermatozoa to undergo capacitation and the acrosome reaction (136). Yet excessive levels of ROS are detrimental to gamete function and quality (136–138). In many cases, supplementation with antioxidants can improve gamete function and fertility *in vivo* (139, 140) and *in vitro* (141–144). Because phytoestrogens are known to reduce pro-inflammatory cytokines in experimental models of encephalitis (145, 146), any modulation of the immune response by phytoestrogens could either promote or inhibit normal reproductive function. In the female for example, non-specific inflammation can alter endometrial receptivity, impair tissue repair and remodeling, and affect trophoblast-endometrial interaction (147, 148).

A Meta-Analysis of the Effect of Phytoestrogen Exposure on Ovulation in Sexually Mature Females

It is clear that the impact of phytoestrogens on reproduction varies between studies, and that that variability is probably caused by differences in the dose of phytoestrogens and the timing of delivery. To tease apart the factors that determine the outcomes of phytoestrogen exposure, we performed a meta-analysis to clarify the impact of phytoestrogens on ovulation. Our logic was that ovulation is a key determinant of female reproductive performance. Major databases such as Pubmed, Cochrane Library, Google Scholar, and ResearchGate were searched using combinations of the following keywords: phytoestrogens, ovulation, ovary, sheep, clover, clover disease, isoflavones, coumestans, soy (i.e., not all terms were included in every search). The search included articles with at least an

TABLE 1 | Inclusion and exclusion criteria used in the meta-analysis.

Inclusion criteria
The phytoestrogen was a flavonoid
Ovulation was assessed by visualization of the corpus luteum/palpation (for mammals)
The route of administration was oral
Exclusion criteria
There was no control group
The ovulation was assessed only by an increase in plasma progesterone
Numerical outcome data were not provided
The phytoestrogens were administered by injection

abstract in English. The end date of the database search was October 2020. Twenty-five studies were initially selected when the abstract indicated that the data would be relevant. Seven studies were selected for analysis after we applied the inclusion and exclusion criteria that are listed in **Table 1**, and the information and data extracted are listed in **Table 2**.

Statistical Analysis

The results of each study were considered as binary (ovulation or no ovulation), and the odds ratio was used as the measure of effect size. A random effect model, with the DerSimonian-Laird (DSL) method (155), was used to evaluate the effect of phytoestrogens on ovulation occurrence because the conditions differed between studies. R studio software (156) was used for this meta-analysis, with the packages *rmeta* and *meta* and the function *meta.DSL*. For odds ratios, computations were carried out on a log scale to maintain symmetry in the analysis. Visualization of the results was done with the functions *summary* and *plot*, which were used to confirm heterogeneity, the individual odds ratios, and confidence intervals. A Funnel plot was created to evaluate publication bias, with a test for funnel plot asymmetry, based on rank correlation or linear regression methods (function *metabias*).

Results

The number of animals in the studies ranged from 4 to 99. Laparoscopy was the most common method used to assess whether ovulation had occurred [one outcome per study, with the exception of Smith et al. (114)]. Four of the studies were conducted in sheep, three of which were conducted in Western Australia (7, 114, 149) and the fourth in Egypt (150). There was no bias across the included studies (P = 0.8349, **Figure 2**).

The results exhibited a large heterogeneity between studies (**Figure 2**), which can be primarily explained by differences in study design, including mainly species and sample size. However, the Woolf's test for heterogeneity (X^2) returned a p-value of 0.3157, and a between-studies variance of 0.23. Confidence intervals were notably wide, particularly for the studies that had fewer subjects in each experimental group (**Figure 2**).

Conclusions

It can be concluded that, depending on species, there remain contradictions around the impact of phytoestrogens on ovulation. It may be proposed that phytoestrogens inhibit ovulation in sheep, because that outcome seemed to emerge clearly from the data in the largest and the most statistically powerful of the studies. Interestingly, other phytoestrogens, non-flavonoids, have been reported to induce ovulation in women with polycystic ovarian syndrome or with anovulatory cycles (132). The impact of phytoestrogen on ovulation will be clarified only by further studies that are designed to control other external factors that can affect ovulation, such as metabolic status (157, 158).

TABLE 2 | A summary of characteristics for studies included in the meta-analysis.

(First author, year)	Abbreviation	Treatment		Control		Detection of ovulation	Species
		Diet	Number of subjects	Diet	Number of subjects		
(Adams et al., 1979) (149)	ADAMS79B	Pasture of Yarloop subterranean clover for 3 years (isoflavones)	99	Non-estrogenic pasture	78	Laparoscopy	Sheep (Merino)
(Smith et al., 1979) (114)	SMITH79A	Lucerne pasture (coumestrol) for 2 months	80	Non-estrogenic grass pasture	80	Laparoscopy	Sheep (Perendale)
	SMITH79C	Pelleted lucerne (coumestrol) for 3 months	49	Pelleted non-estrogenic lucerne	49	Laparoscopy	Sheep (Perendale)
(Hashem and Sallam, 2012) (150)	HASHEM12	Berseem clover pasture for 50 days	6	Corn silage	6	Transrectal ultrasonography	Sheep (Barki X Awassi)
(Adams et al., 1981) (151)	ADAMS81	Yarloop subterranean clover pasture for 3 years (isoflavones)	49	Non-estrogenic pasture	53	Laparoscopy	Sheep (Merino)
(Santhosh et al., 2006) (152)	SANTHOSH06	*Rhaphidophora pertusa* (flavonoid) covering one estrous cycle	4	Rice gruel during entirety of estrous cycle	12	Rectal palpation	Dairy cows
(Bennetau-Pelissero et al., 2001) (153)	BENNETEAU01	Genistein-enriched diet for one year	19	Normal diet	19	Histology of ovary	Rainbow trout
(Li et al., 2014) (154)	RONG14	Genistein-enriched diet from weaning to week 7	6	Normal diet	6	Histology of ovary	Mice (C57BL/6J)

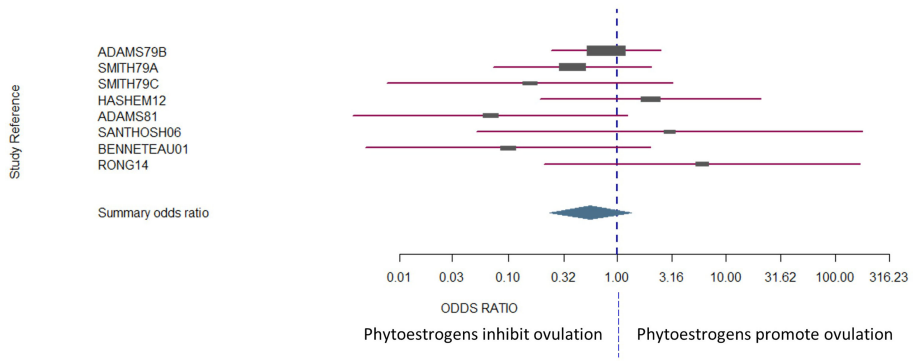

FIGURE 2 | The confidence interval for each study is given by a horizontal line, and the point estimate is given by a square whose height is inversely proportional to the standard error of the estimate. The summary odds ratio is represented by a diamond with horizontal limits at the confidence limits and width inversely proportional to its standard error (R documentation). An odds ratio higher than 1 means that the treatment is more effective than the control and vice versa (whereby 1 means null effect). In the present analysis, the positioning of the summary odds ratio shows that phytoestrogen exposure in sexually mature females inhibits ovulation.

Male Reproductive Function

Estrogen was once considered a female hormone, but evidence over the past few decades has shown that it is critical for normal male reproductive development and function. In fact, estrogen appears to be essential for normal male reproductive function because mice with the ERα gene knocked out are sterile (159–161). Negative feedback from estrogen contributes to the activity of the hypothalamic-pituitary axis (162), and estrogen signaling plays a role in fluid reabsorption in the epididymis (163). Less is known about the effect of exogenous estrogenic compounds in the male than in the female, though there are several potential mechanisms for these agents to impact on the spermatogenic cycle. In primates and mice, ERβ is present in Leydig cells, Sertoli cells, and in the nuclei of epithelial and stromal cells throughout the reproductive tract, while ERα seems primarily to be present in the accessory sex glands and efferent ductules (164, 165). The disruption of ERα in male mice results in compromised dynamics of fluid resorption in the efferent ductules, an increase in the secretion of chloride ions into seminal fluid, an increase in abnormal sperm morphology, an inhibition of sodium transport in the testis and ultimately reduced fertility (159, 160, 163). Phytoestrogens that can bind to ER receptors would therefore be expected to alter testicular function, sperm production, and sperm quality.

In man, phytoestrogen exposure has a spectrum of effects (**Table 3**). Although the effects are generally more subtle than the marked changes in females (4, 120, 126), those effects again depend on timing, with exposure during sensitive developmental periods, particularly *in utero*, leading to compromised reproductive function. Maternal isoflavone intake has been linked to hypospadias in male infants (182, 183) and lower testicular steroidogenic activity in neonatal rats (178). Similarly, as for the female, in the male conflicting results have been reported for the effects of phytoestrogen exposure during adult life. Several studies have been reported no effect in humans (166, 170), rodents (184–186), and rabbits (187), while other studies in the same species have associated phytoestrogen exposure during adult life with decreased sperm production, lower blood testosterone concentration, and reduced testicular weight (167, 168, 173, 174, 188, 189). These conflicting results can probably be attributed to several differences between studies, including phytoestrogen dosage, route of administration, length of exposure and, in the case of human studies, demographic biases including variation in age, ethnicity, and systemic health. Moreover, none of the studies reported the concentration of phytoestrogens in the seminal plasma. Thus it is not clear to what degree these compounds can infiltrate the reproductive tract or whether other indirect pathways are involved.

While the impact of phytoestrogen exposure on male reproduction is poorly understood, *in vitro* work suggests several isoflavones and flavonoids can influence the functionality of spermatozoa. The exposure of ram spermatozoa to physiological concentrations of equol *in vitro* decreases sperm motility, increases ROS, increases membrane fluidity, and increases DNA fragmentation (11). Genistein (8, 190, 191) and myricetin (192) have been reported to cause premature capacitation and acrosome loss in human, boar, and mouse spermatozoa, and inhibit the acrosome reaction in bull spermatozoa. In contrast, other studies have reported that the inclusion of isoflavones *in vitro* improves sperm viability and function (193–195). One possible confounding factor is that several isoflavones, including the widely tested genistein, can act as an antioxidant and mitigate oxidative stress (196, 197), thus improving sperm function. It is possible that the benefits of the antioxidant action can, at certain concentrations, mitigate any negative impacts from the estrogen-like actions of phytoestrogens.

TABLE 3 | The effect of phytoestrogen exposure on male reproductive function across several species and routes of exposure.

Species	Phyto-estrogen or source	Route	Period of exposure	Effects on sperm production and quality	Other changes to reproductive capacity	Effects on endocrinology	Reference
Human	Soy	Dietary	Adult	No effect	No effect	No effect	(166)
				Decreased concentration	No effect	N/A	(167)
				No effect	No effect	Decreased testosterone	(168)
	Daidzein, genistein	Dietary	Adult	Low motile sperm count	N/A	N/A	(169)
				Decreased concentration and motility, increased abnormalities	N/A	Increased infertility	(10)
		Oral	Adult	No effect	No effect	No effect	(170)
		Intraperitoneal injection of mother	Fetal	Decreased concentration	Low epididymal density and quality, deteriorated testicular architecture, reduced total pups sired	Decreased testosterone	(171)
	Genistein	Dietary	Birth to adulthood	Reduced cauda epididymis sperm reserve	Penis underdevelopment, lower epididymal weight	Decreased testosterone	(9)
			Neonate, adult	N/A	Abnormal testis, increased inflammation of testis, increased rates of infertility.	N/A	(172)
			Conception to adulthood	Decreased concentration (epididymal)	Reduced haploid germ cells in testis, decreased size of seminal vesicle	N/A	(173)
	Soy	Dietary	Adult	Increased abnormalities in sperm morphology	N/A	N/A	(174)
	Thai Mucuna seed (isoflavone)	Dietary	Adult	Increased sperm concentration	N/A	N/A	(175)
Rodent	Pueraria mirifica (isoflavone)	Dietary	Adult	N/A	Lipid peroxidation of epididymal sperm was significantly increased	Disrupted steroid regulation of epidydimis, significantly reduced fecundity	(176)
	Genistein,	Oral	Neonate	No change	No change	No change	(103)
	daidzein, glycitein	Dietary	Adult	Decrease in the weights of the left testicle, seminal vesicle, sperm count	Decreased sperm motility	Decreased testosterone hormone, no change in plasma estradiol	(177)
	Lignans	Dietary	Adult	Increased sperm concentration	N/A	Leydig cell number increase	
	Isoflavones (soy)	Dietary	Fetal	Increased proliferation of Leydig cells	N/A	Reduced steroidogenesis in adulthood	(178)
		Dietary	Fetal	No effect on gametogenesis	N/A	No impact on testosterone	(179)
Rabbit	Soy, lignans	Dietary	Adult	Decreased sperm concentration	N/A	Decreased libido, testosterone, seminal plasma fructose. No effects on number of offspring	(180)
Bat	Coumestrol	Dietary	Adult	N/A	N/A	Increased testicular weight, loss of typical histological structure of testis	(181)

N/A is specified in cases where the parameter was not measured in that study.

CLOVER DISEASE: HAVE WE OVERLOOKED A DETRIMENTAL IMPACT ON THE INTERACTION BETWEEN SPERMATOZOA AND THE FEMALE REPRODUCTIVE TRACT?

Despite its categorization as a female oriented issue, mounting evidence suggests that the subfertility and infertility that is associated with clover disease is likely far more complex. Since a 1960's report that wethers on estrogenic pasture exhibited mammary development, lactation, and pathology of the bulbourethral glands (198), there is little investigation into the impact of phytoestrogens on ram reproduction. Similarly, there is no information on how ram spermatozoa perform in the female tract after phytoestrogen exposure in either, or both, sexes. The profound effects of phytoestrogens on male fertility in other species (**Table 3**) suggests strongly that phytoestrogen impact on ram reproductive function may be a critically overlooked component of clover disease. Of note, human males exposed to estrogenic compounds at relatively high dosages or duration have smaller seminiferous tubules and

impaired spermatogenesis (199). Roosters and rats exposed to estrogenic compounds have lower testosterone production (200, 201), more oxidative stress, and reduced sperm function (5). In the boar, estrogenic compounds cause premature capacitation (202) and a stronger immune response to spermatozoa in the female tract (203).

We propose that exposure to phytoestrogens could compromise both the male and the female, as well as interactions between spermatozoa and the female tract, creating conditions within the female reproductive system that are hostile, more so than usual, to spermatozoa. The exposure to exogenous estrogens could drastically impair the ability of spermatozoa to traverse the female tract and successfully fertilize, which could partially account for the poor fertility in sheep that graze estrogenic pasture (4, 204). The addition of physiological concentrations of equol to ram spermatozoa *in vitro* negatively alters sperm function, with decreased sperm motility, increased ROS, increased membrane fluidity, and greater DNA fragmentation compared to non-exposed ram spermatozoa (11).

One obvious factor that will influence the interaction between spermatozoa and the ewe reproductive tract is the morphological changes to the female reproductive tract that are observed after phytoestrogen exposure (3, 6, 128). Prolonged grazing of estrogenic pasture has been found to induce a loss of crypts and folds in the cervix of ewes (4, 6, 13). The crypts of the ovine cervix represent "privileged pathways", potential routes where conditions of cervical mucous flow, composition, and viscosity are conducive to the successful progression of spermatozoa toward the oocyte (205, 206). Thus, the morphological changes that are observed in female tract and in the sperm physiology after exposure to phytoestrogens could synergize to compromise the ability of spermatozoa to traverse the female reproductive tract.

The ability of spermatozoa to navigate the female reproductive tract is not merely a function of motility, but rather involves dynamic interactions between the male gamete and the tract environment. Particularly noteworthy in the context of reproduction after animals are exposed estrogenic compounds, is the role of ovarian steroids in the production and composition of cervical mucous, and in the immune response that is stimulated by the presence of the incoming spermatozoa (205, 207). Exogenous hormones, such as those that are used to synchronize the estrous cycle of ewes, are known to vastly alter the composition of cervical mucous thereby impairing the function of spermatozoa, leading to a reduction in fertility (205, 207–210). Infertility in ewes that graze sub clover species that are estrogenic could be partially due to changes in cervical mucous that are induced by phytoestrogens. During the follicular phase, the viscosity of the cervical mucous decreases in response to rising estrogen concentrations, lowering the barrier to sperm migration through the female reproductive tract. That change optimizes the conditions in preparation for ovulation and potential insemination (205, 207). Very early studies in sheep showed that the viscosity of mucus was lower after chronic exposure to estradiol and in ewes with clover disease (12, 211). However, the lower mucus viscosity in ewes with clover disease was also correlated with reduced fertility (212). This suggests that a factor other than viscosity, such as the biochemical composition of mucus following long term phytoestrogen exposure, is less favorable to sperm transport.

While exogenous estrogen is known to alter the female immune response and the composition of seminal plasma in the male (163), it is not known if phytoestrogens elicit similar effects. When seminal plasma enters the female tract immune cells are recruited and activated, releasing cytokines and chemokines that play a role in the sperm selection process in the female tract (213). In mammals, the magnitude of this immune response varies in response to endogenous estrogen (203), exogenous hormones (214), and specifically in sheep, between breeds (205). There are reports of the production of anti-sperm antibodies in the female reproductive tract after insemination, though a predominant focus of immune studies has been the known immunogenic properties of seminal plasma (215, 216). Because some of the constituents of seminal plasma have an immunomodulatory effect (215, 217), anything that influences the composition of the seminal plasma could affect the female immune response, with an impact on sperm survival. Greater numbers of leukocytes in cervical mucus increases sperm capacitation and the acrosome reaction (218). Because leukocytes produce ROS at far higher levels than do spermatozoa (219), the production of ROS might be the underlying mechanism for the promotion of these events. It will be worthwhile to investigate whether phytoestrogens alter the composition of seminal plasma, and if that then modulates the immune response that is mounted by the female after copulation.

In summary, the combination of loss of privileged pathways for sperm in the cervix, changes in the composition of cervical mucous, and changes in the immune response of the female to spermatozoa all represent possible mechanisms that could contribute to the fertility issues that occur in sheep when they graze sub clover (**Figure 1**). Investigation is warranted into male-female gamete interactions after either sex is exposed to phytoestrogens.

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE MANAGEMENT OF OVINE CLOVER DISEASE

There are two clear rationale for further research into clover disease; there are clear agricultural benefits to be gained, and this syndrome in sheep presents a unique opportunity to develop a model of the reproductive consequences of phytoestrogen exposure, particularly during critical developmental periods. The review of the known literature points logically to several potential sites of action, and mechanisms of action, of phytoestrogens on sheep reproduction. This includes estrogen receptor binding, genomic and epigenetic alterations and immune modulation, at sites including the reproductive tract, brain and gametes of

both the male and female. It is likely that a systemic interplay between those factors is responsible for the spectrum of pathological changes that are observed in ovine clover disease, though that itself requires clarification. In our view, it is necessary not only to understand the consequences of exposure to phytoestrogens on gamete production in both sexes, but also to understand how that exposure alters the interaction of the gametes in the female reproductive tract. An interesting avenue of research would be to explore the physiological and genetic basis of individual susceptibility to phytoestrogens. The identification and strategic breeding of resilient animals is a logical start to address the issue, as well as the removal from breeding of persistently sub-fertile individuals and the early identification of susceptible flocks. Those strategies could be used in parallel with pasture renovation to eliminate estrogenic clover.

AUTHOR CONTRIBUTIONS

KP drafted, edited and wrote the final manuscript. FC conducted the meta-analysis. DB and JS reviewed, edited and contributed to the manuscript. All authors approved the final version of the manuscript.

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Placental Abnormalities and Placenta-Related Complications Following *In-Vitro* Fertilization: Based on National Hospitalized Data in China

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Introduction: Emerging evidence has shown that *in-vitro* fertilization (IVF) is associated with higher risks of certain placental abnormalities or complications, such as placental abruption, preeclampsia, and preterm birth. However, there is a lack of large population-based analysis focusing on placental abnormalities or complications following IVF treatment. This study aimed to estimate the absolute risk of placental abnormalities or complications during IVF-conceived pregnancy.

Methods: We conducted a retrospective cohort study of 16 535 852 singleton pregnancies with delivery outcomes in China between 2013 and 2018, based on the Hospital Quality Monitoring System databases. Main outcomes included placental abnormalities (placenta previa, placental abruption, placenta accrete, and abnormal morphology of placenta) and placenta-related complications (gestational hypertension, preeclampsia, eclampsia, preterm birth, fetal distress, and fetal growth restriction (FGR)). Poisson regression modeling with restricted cubic splines of exact maternal age was used to estimate the absolute risk in both the IVF and non-IVF groups.

Results: The IVF group (n = 183 059) was more likely than the non-IVF group (n = 16 352 793) to present placenta previa (aRR: 1.87 [1.83–1.91]), placental abruption (aRR: 1.16 [1.11–1.21]), placenta accrete (aRR: 2.00 [1.96–2.04]), abnormal morphology of placenta (aRR: 2.12 [2.07 to 2.16]), gestational hypertension (aRR: 1.55 [1.51–1.59]), preeclampsia (aRR: 1.54 [1.51–1.57]), preterm birth (aRR: 1.48 [1.46–1.51]), fetal distress (aRR: 1.39 [1.37–1.42]), and FGR (aRR: 1.36 [1.30–1.42]), but no significant difference in eclampsia (aRR: 0.91 [0.80–1.04]) was found. The absolute risk of each outcome with increasing maternal age in both the IVF and non-IVF group presented two patterns: an upward curve showing in placenta previa, placenta accreta, abnormal morphology of placenta, and

gestational hypertension; and a J-shape curve showing in placental abruption, preeclampsia, eclampsia, preterm birth, fetal distress, and FGR.

Conclusion: IVF is an independent risk factor for placental abnormalities and placental-related complications, and the risk is associated with maternal age. Further research is needed to evaluate the long-term placenta-related chronic diseases of IVF patients and their offspring.

Keywords: *in-vitro* fertilization, placental abnormality, placenta-related complication, maternal complication, neonatal complication

INTRODUCTION

The placenta is the first organ to form during mammalian embryogenesis. As the vascular interface between maternal and fetal circulation systems, the placenta plays a crucial role in nutrient, gas, immune, and toxic substances transfers or barriers between mothers and fetuses (1). Hence, placental development and function are essential for developing mammalian embryos in the uterine environment. However, if the placental development or function is impaired, or the capacity to adapt to adverse environmental exposures is exceeded, intrauterine fetal development and long-term health in childhood and even adulthood may be compromised. Such short- and/or long-term adverse outcomes include: i. placental abnormalities, such as placenta previa, placental abruption, placenta accreta, and abnormal placental morphogenesis; ii. placenta-related complications during prenatal and perinatal periods, such as preeclampsia, fetal growth restriction, preterm birth, fetal distress, and fetal growth restriction (FGR) (2, 3); and iii. placenta-related chronic diseases in adulthood, such as cardiovascular disease, diabetes, and obesity (4). Therefore, the placenta has been considered as a key predictor of maternal and offspring's health.

Over the past four decades, there has been a growing appreciation that *in-vitro* fertilization (IVF) has helped millions of infertile couples worldwide to have babies. The latest estimate of global utilization is 2.8 million IVF cycles and 0.9 million IVF babies in 2012 (5). In 2016, China reported 0.9 million IVF cycles and 0.3 million IVF babies, which accounted for 1.6% of the total live births (6). The embryo implantation and development, which is mainly dependent on the placental formation and function, is critical to the success of IVF. Some previous studies reported that IVF-conceived pregnancies had thicker and heavier placentas, higher placental weight/birthweight ratio, decreased apical microvilli, and increased multiple vacuoles compared with spontaneous pregnancies, suggesting maternofetal traffic downregulation following IVF treatment (7, 8). Blastocyst malrotation at implantation in IVF treatment may lead to low-lying placenta, placenta previa, and velamentous insertion of the umbilical cord (9). Furthermore, IVF has been associated with higher risks of certain placental abnormalities or complications, such as placental abruption, preeclampsia, and preterm birth (10–12). However, there is a lack of population-based analysis focusing on placental abnormalities or complications after IVF treatment.

Based on a retrospective analysis of 17 million hospitalized deliveries between 2013 and 2018 in China, this study aimed to estimate the absolute risk of placental abnormalities or complications following IVF treatment to provide evidence for medical professionals to make optimal decisions in clinical management, thereby improving maternal and offspring's safety and health.

METHODS

Study Design and Population

This was a retrospective cohort study of 17 million hospitalized delivery cases in 1853 maternity hospitals between 2013 and 2018 in all 31 provinces of Mainland China. 17 540 380 hospitalized delivery cases were retrieved from the Hospital Quality Monitoring System (HQMS) databases, established and formally run by National Health Commission of China since 2013. After excluding 1 004 528 (5.72%) cases with unknown or non-Chinese nationality (100 560 cases), permanent residents in Hong Kong, Macao, and Taiwan (1 030 cases), missing value of maternal age (63 309 cases), maternal age below 20 years (254 297 cases) or beyond 49 years (30 835), and multiple pregnancies (554 497 cases), there were 16 535 852 eligible cases included in the analysis, including 183 059 IVF-conceived pregnancies (the IVF group) and 16 352 793 spontaneous pregnancies (the non-IVF group) (**Figure 1**).

Variables

In the HQMS database, the standardized electronic hospitalized discharge records (also known as "first page of electronic medical record") were automatically collected from each hospital. All diagnoses and operating procedures were coded by using the International Classification of Diseases -10th Revision (ICD-10) and the International Classification of Diseases -9th Revision, Clinical Modification (ICD-9-CM) (**Table S1**) (13, 14). In this study, we mainly used three categories of variables for each pregnant woman (1): general characteristics: geographical region (eastern, central, or western), year of discharge, maternal age at delivery, and ethnicity (Han or Minority) (2); chronic diseases before pregnancy: chronic hypertension, diabetes, thyroid diseases, anemia, circulatory diseases and other chronic conditions (including coagulation disorders, kidney diseases, diseases of connective tissues, respiratory diseases, and

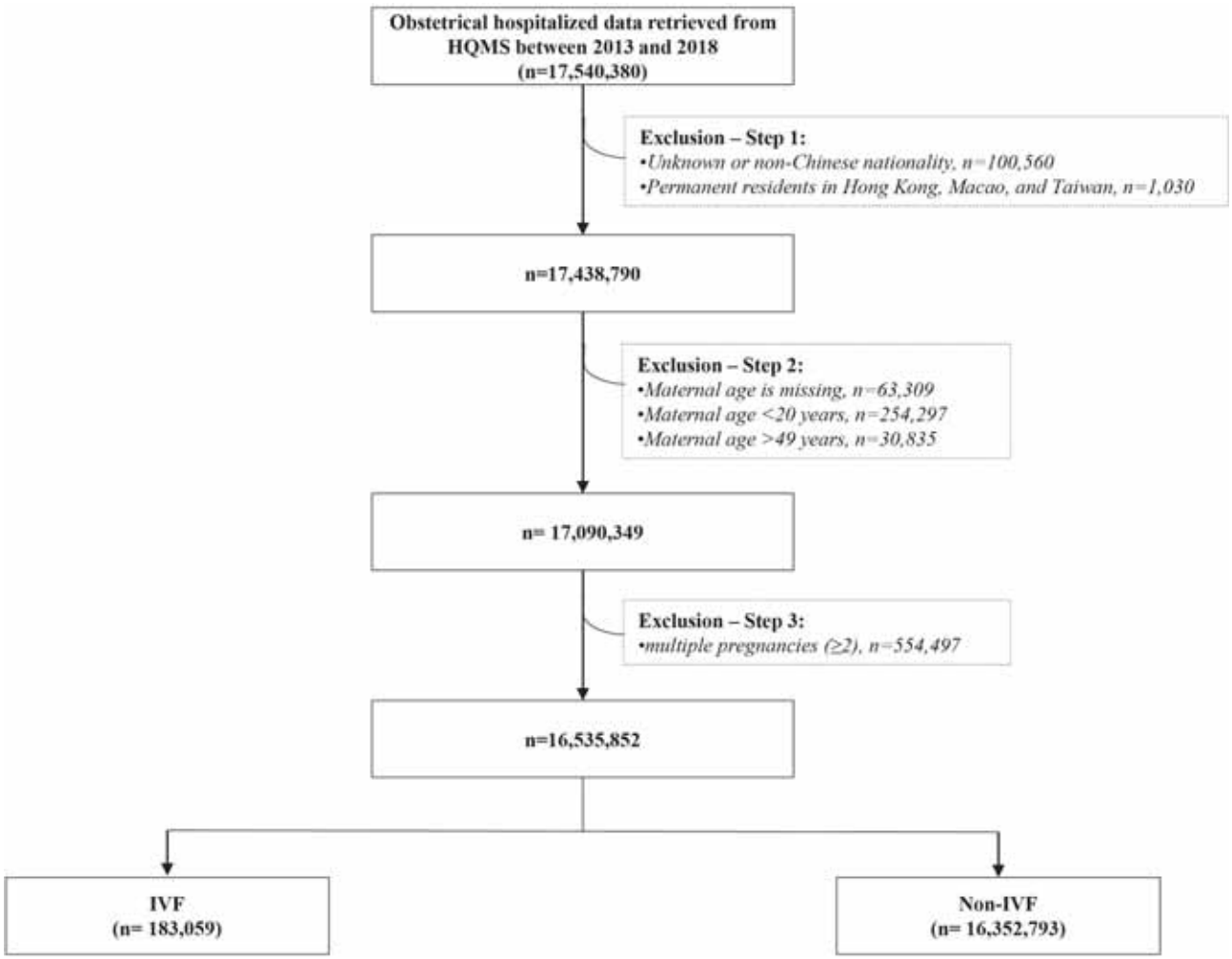

FIGURE 1 | The flowchart of eligible study population in this study IVF, *in vitro* fertilization; Non-IVF, non-*in vitro* fertilization.

digestive system diseases) (3); placental abnormalities: placenta previa, placental abruption, placenta accrete, and abnormal morphology of placenta (4); placenta-related complications: gestational hypertension, preeclampsia, eclampsia, preterm birth, fetal distress, and fetal growth restriction (FGR).

Statistical Analysis

Chi-squared test for categorical variables and Student's t-test for continuous variables were performed to compare the differences of general characteristics between the IVF group and the non-IVF group. Considering maternal age usually presents an independent, nonlinear association with many maternal and neonatal complications (15), we performed Poisson regression models with restricted cubic splines of exact maternal age to allow the most flexible characterization of the nonlinear association between maternal age and each placenta-related outcome. For each restricted cubic spline of maternal age, the number of knots placed at the default percentiles was identified by the principle of minimized Akaike Information Criterion (AIC) (16, 17). For each placenta-related outcome, we performed two Poisson regression models: an unadjusted model with only maternal age spline terms to examine crude relative risk (RR) and 95% confidence interval (CI) of IVF vs. non-IVF (2); a multivariable model adjusted by general characteristics and chronic diseases before pregnancy to examine adjusted RR (aRR_1) and 95% CI of IVF vs. non-IVF. Finally, we used the multivariable model to calculate the predicted absolute risk (probability and 95% CI). We then graphically presented them to visually illustrate the absolute risk of each placenta-related complication by maternal age. All analyses were conducted using the SAS 9.0 software (18). A two-tailed P value < 0.05 was considered statistically significant. Sensitivity analyses of this study were done by comparing the results of two Poisson regression models (unadjusted model and multivariable model) with restricted cubic splines of exact maternal age to assess for confounding from general characteristics, chronic diseases before pregnancy, and year-by-year maternal age. We also performed an additional sensitivity analysis by using logistic

regression models with restricted cubic splines of exact maternal age, with adjustment for geographic region, maternal age, year, ethnicity, and chronic diseases before pregnancy.

RESULTS

In this study, there were 183 059 pregnancies in the IVF group (1.1%) and 16 352 793 pregnancies in the non-IVF group (98.9%). The differences in general characteristics between the IVF and non-IVF groups were statistically significant (**Table 1**). The proportion of pregnant women with advanced maternal age (≥35 years) was markedly higher in the IVF group (31.9%) than that of the non-IVF group (13.7%). Compared with the non-IVF group, the IVF group was more likely to have chronic diseases before pregnancy, including chronic hypertension, diabetes, thyroid diseases, anemia, circulatory diseases, and other chronic conditions. Thus, these general characteristics of the study population would be adjusted in the multivariable modeling in order to control confounding bias.

After adjusting for general characteristics and chronic diseases before pregnancy with restricted cubic splines of exact maternal age (**Table 2**), the IVF group was more likely than the non-IVF group to present each outcome of placental abnormalities: placenta previa (4.8% vs 2.0%; aRR=1.87, 95% CI: 1.83 to 1.91), placental abruption (1.0% vs 0.8%; aRR=1.16, 95% CI: 1.11 to 1.21), placenta accrete (4.7% vs 2.0%; aRR=2.00, 95% CI: 1.96 to 2.04), abnormal morphology of placenta (4.5% vs 1.8%; aRR=2.12, 95% CI: 2.07 to 2.16) (**Table S2**).

Except for eclampsia, the IVF group was more likely to present all the other placenta-related complications than the non-IVF group: gestational hypertension (3.3% vs 1.7%; aRR_1 = 1.55, 95% CI: 1.51 to 1.59), preeclampsia (5.5% vs 2.7%; aRR_1 = 1.54, 95% CI: 1.51 to 1.57), preterm birth (8.2% vs 4.9%; aRR_1 = 1.48, 95% CI: 1.46 to 1.51), fetal distress (8.0% vs 6.1%; aRR_1 = 1.39, 95% CI: 1.37 to 1.42), and FGR (1.2% vs 0.8%; aRR_1 = 1.36, 95% CI: 1.30 to 1.42) (**Table 2**). After further adjusting for the four placental abnormalities above, the IVF group continued to present a higher risk of these placenta-related complications comparing to the non-IVF group: gestational hypertension (aRR_2 = 1.54, 95% CI: 1.50 to 1.58), preeclampsia (aRR_2 = 1.52, 95% CI: 1.49 to 1.55), preterm birth (aRR_2 = 1.34, 95% CI: 1.32 to 1.36), fetal distress (aRR_2 = 1.38, 95% CI: 1.36 to 1.40), and FGR (aRR_2 = 1.26, 95% CI: 1.21 to 1.32) (**Table 2**).

Figure 2 shows the absolute risk and 95% CI of each outcome by maternal age in both the IVF and non-IVF groups. It can be shown that the IVF group had a higher absolute risk of each outcome (except for eclampsia) at each maternal age than the non-IVF group, and the gaps between the two groups demonstrated a growing trend with the increase of maternal age. Furthermore, the curves can be mainly concluded in two patterns. The first pattern was an upward curve, with absolute

TABLE 1 | The general characteristics of study population in this study.

General characteristics	Total (n = 16,535,852)	IVF (n = 183,059)	non-IVF (n = 16,352,793)	*P*-value
Geographical area				
Eastern	8462641 (51.2)	101253 (55.3)	8361388 (51.1)	<0.001
Central	3852875 (23.3)	43965 (24.0)	3808910 (23.3)	<0.001
Western	4220336 (25.5)	37841 (20.7)	4182495 (25.6)	<0.001
Year				
2013	751997 (4.5)	5477 (3.0)	746520 (4.6)	<0.001
2014	1438167 (8.7)	9791 (5.4)	1428376 (8.7)	<0.001
2015	1327773 (8)	13383 (7.3)	1314390 (8.0)	<0.001
2016	4370506 (26.4)	41992 (22.9)	4328514 (26.5)	<0.001
2017	4451031 (26.9)	47874 (26.2)	4403157 (26.9)	<0.001
2018	4196378 (25.4)	64542 (35.3)	4131836 (25.3)	<0.001
Maternal age at birth, Mean (SD)	29.20 (4.6)	32.51 (4.5)	29.16 (4.6)	<0.001
20-24	2419814 (14.6)	4859 (2.7)	2414955 (14.8)	<0.001
25-29	7184983 (43.5)	43033 (23.5)	7141950 (43.7)	<0.001
30-34	4641920 (28.1)	76727 (41.9)	4565193 (27.9)	<0.001
35-39	1857960 (11.2)	46330 (25.3)	1811630 (11.1)	<0.001
40-44	402996 (2.4)	10990 (6.0)	392006 (2.4)	<0.001
45-49	28179 (0.2)	1120 (0.6)	27059 (0.2)	<0.001
Ethnicity				
Minority	1515065 (9.2)	16366 (8.9)	1498699 (9.2)	<0.001
Han	14856494 (89.8)	165736 (90.5)	14690758 (89.8)	<0.001
Unknown	164293 (1.0)	957 (0.5)	163336 (1.0)	<0.001
Chronic diseases before pregnancy				
Chronic hypertension	133051 (0.8)	2713 (1.5)	130338 (0.8)	<0.001
Diabetes	158200 (1.0)	3822 (2.1)	154378 (0.9)	<0.001
Thyroid diseases	640093 (3.9)	13441 (7.3)	626652 (3.8)	<0.001
Anemia	2589684 (15.7)	29171 (15.9)	2560513 (15.7)	0.0012
Circulatory diseases	167004 (1.0)	2665 (1.5)	164339 (1.0)	<0.001
*Other chronic conditions	734827 (4.4)	11226 (6.1)	723601 (4.4)	<0.001

* *"Other chronic conditions" included coagulation disorders, kidney diseases, diseases of connective tissues, diseases of respiratory system, and diseases of digestive system.*

TABLE 2 | Associations of IVF with placental abnormalities and placenta-related complications.

	Total (n = 16,535,852)	IVF (n = 183,059)	non-IVF (n = 16,352,793)	RR (95% CI)	aRR (95% CI)
Placental abnormalities					
Placenta previa	328773 (1.99)	8865 (4.8)	319908 (2.0)	**2.48 (2.42 to 2.53)**	**1.87 (1.83 to 1.91)**
Placental abruption	127427 (0.77)	1853 (1.0)	125574 (0.8)	**1.32 (1.26 to 1.38)**	**1.16 (1.11 to 1.21)**
Placenta accreta	326875 (1.98)	8554 (4.7)	318321 (2.0)	**2.40 (2.35 to 2.45)**	**2.00 (1.96 to 2.04)**
Abnormal morphology of placenta	308626 (1.87)	8231 (4.5)	300395 (1.8)	**2.45 (2.40 to 2.50)**	**2.12 (2.07 to 2.16)**
Placenta-related complications					
Gestational hypertension	276136 (1.67)	5962 (3.3)	270174 (1.7)	**1.97 (1.92 to 2.02)**	**1.55 (1.51 to 1.59)**
Preeclampsia	456977 (2.76)	10082 (5.5)	446895 (2.7)	**2.02 (1.98 to 2.05)**	**1.54 (1.51 to 1.57)**
Eclampsia	20621 (0.12)	226 (0.1)	20395 (0.1)	0.99 (0.87 to 1.13)	0.91 (0.80 to 1.04)
Preterm birth	808063 (4.89)	15066 (8.2)	792997 (4.9)	**1.70 (1.67 to 1.72)**	**1.48 (1.46 to 1.51)**
Fetal distress	1010414 (6.11)	14627 (8.0)	995787 (6.1)	**1.31 (1.29 to 1.33)**	**1.39 (1.37 to 1.42)**
FGR	134722 (0.81)	2205 (1.2)	132517 (0.8)	**1.49 (1.43 to 1.55)**	**1.36 (1.30 to 1.42)**

IVF, in vitro fertilization; Non-IVF, non-in vitro fertilization; FGR, fetal growth restriction. RR, relative risk; aRR, adjusted relative risk. CI, confidence interval. RR were calculated in Poisson regression modelling with restricted cubic splines of exact maternal age. aRR were calculated in Poisson regression modelling with restricted cubic splines of exact maternal age, with adjustment for geographic region, maternal age, year, ethnicity, and chronic diseases before pregnancy.
The bold values means has statistically significant.

risk continuing to increase with maternal age, including placenta previa, placenta accreta, abnormal morphology of placenta, and gestational hypertension. The second pattern was a J-shape curve that the absolute risk dropped at first and then increased with maternal age, including placental abruption (the inflection point is 25 years), preeclampsia (26 years), eclampsia (28 years), preterm birth (27 years), fetal distress (35 years), and FGR (28 years). The sensitivity analysis showed that different statistical approaches did not substantially affect the estimates (**Table S3**).

DISCUSSION

On the basis of national hospitalized data in China, we found that IVF-conceived pregnancies had a higher risk of placenta-related adverse outcomes than non-IVF pregnancies, including four categories of placental abnormalities (placenta previa, placental abruption, placenta accrete, and abnormal morphology of placenta) and five categories of placenta-related complications (gestational hypertension, preeclampsia, preterm birth, fetal distress, and FGR), but no significant association with eclampsia was found. In both the IVF and non-IVF groups, the absolute risk of each outcome was associated with increasing maternal age, presenting two patterns: an upward curve showing in placenta previa, placenta accreta, abnormal morphology of placenta, and gestational hypertension; and a J-shape curve showing in placental abruption, preeclampsia, eclampsia, preterm birth, fetal distress, and FGR.

The findings of this study demonstrated that IVF is an independent risk factor for placenta previa, placental

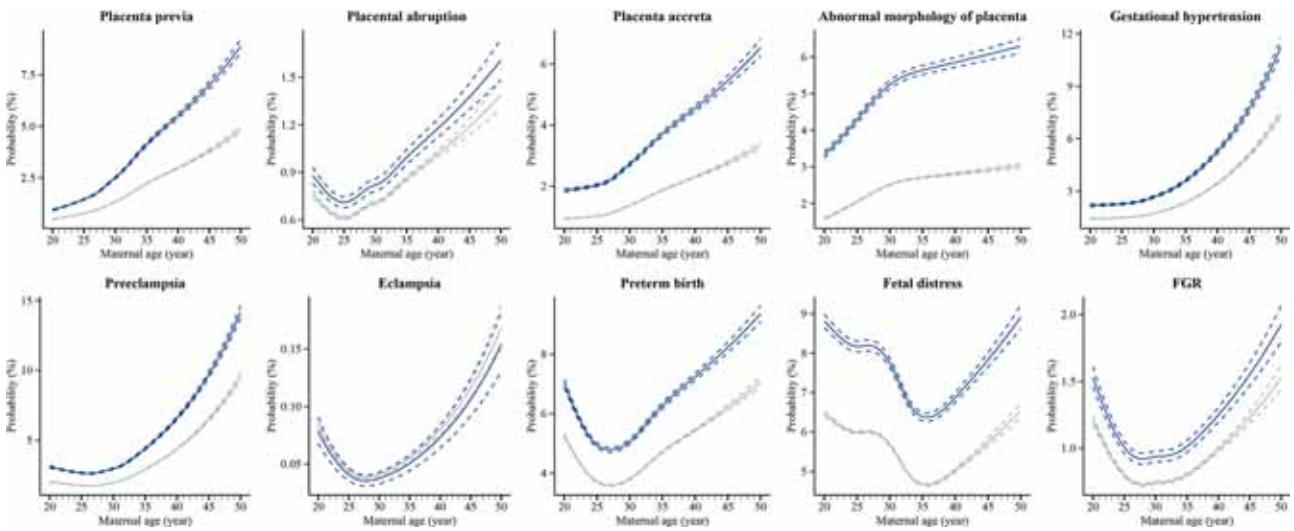

FIGURE 2 | Estimated absolute risks of placental abnormalities and placenta-related complications at each maternal age in IVF group and non-IVF group IVF, *in vitro* fertilization; Non-IVF, non-*in vitro* fertilization; FGR, fetal growth restriction. Estimated absolute risks (probabilities) were calculated by using Poisson regression modelling with restricted cubic splines of exact maternal age, with adjustment for geographic region, maternal age, year, ethnicity, and chronic diseases before pregnancy.

abruption, placental accreta, and abnormal morphology of placenta after adjusting for factors such as maternal age at birth, geographical area, ethnicity, the year of IVF implementation, and chronic diseases before pregnancy, which is consistent with the previous studies. In earlier epidemiologic studies and systematic reviews, IVF is a risk factor of placenta previa (19–22) and placental abruption (23, 24). IVF singleton pregnancies are associated with a higher incidence of placenta accreta and velamentous placenta (25–28). Moreover, we also noted a greater risk for developing gestational hypertension, preeclampsia, preterm birth, fetal distress, and FGR among the IVF group, which has been documented in other studies (29–31). While there is a scientific consensus about the link between IVF and placental abnormalities or complications, current evidence remains fragmented. Our study generated integrated evidence from a large population-based analysis to confirm the hypothesis that IVF is associated with placenta-related adverse outcomes.

We further analyzed the relationship between each placenta-related adverse outcome and maternal age. We detected that the IVF group had a higher absolute risk at each maternal age than the non-IVF group. The absolute risk of each outcome with increasing maternal age in both the IVF and non-IVF groups presented in two dominant patterns. The first was an upward curve indicating that the absolute risk increases with maternal age. The second was a J-shape curve indicating that the absolute risk is higher at the younger age interval (20-24 years or below) and advanced age interval (35 years or above). These findings illustrated that the optimal age for a woman to be pregnant, whether conceived spontaneously or by IVF treatment, is 25-34 years, which has been well established in many pieces of literature (32–35). Meanwhile, many studies have attempted to analyze the biological plausibility of maternal age-related higher absolute risk of adverse obstetrical and perinatal outcomes, including placental abnormalities or complications. Firstly, it is clear that advanced maternal age is an independent strong risk factor, since the majority of adverse outcomes can be explained by the physio-pathological changes in the female reproductive apparatus that come with aging and its inherent comorbidities (36). Placenta senescence is characterized by reduced telomerase activity and aging suppressor expression (37), increased DNA damage and DNA oxidation{Biron-Shental, 2010 #140}{Biron-Shental, 2010 #140}, and increased expression of senescent biomarkers. In recent years, with the rapid increase of the number of older pregnancies, more studies have focused on the relationship between critical placental aging regulation related proteins and placental adverse outcomes. One study showed that advanced maternal age pregnancy can result in reduced α-Klotho (a well-known antiaging protein) expression in placental trophoblasts, which ultimately lead to placental malformation and adverse perinatal outcomes, possibly through the reduction in the transcription of cell adhesion molecule genes (38). In addition, age-related risks of some adverse pregnancy and birth outcomes for women in younger age groups cannot be overlooked. Although more evidence from the perspective of physiological and pathological mechanism is needed to explain and distinguish why some adverse consequences are positively related to young pregnancy while others are negatively related, absolute risk at the younger age interval can be attributed to unwanted pregnancy, inadequate prenatal care, low income and more social factors to some extent (15). In conclusion, our findings provided compelling evidence for both the reproductive and obstetrical practitioners to focus on the prevention of placenta-related adverse outcomes, particularly for women with younger or advanced maternal age who are undergoing IVF.

As for the location and morphology of the placenta, previous investigations have reported that ovulation induction was associated with an increased risk of placental abruption (39). In addition, *in vitro*-fertilized embryos are transferred to the uterine cavity by the transcervical route using a catheter. This procedure may induce uterine contraction, possibly due to the release of prostaglandins after mechanical stimulation of the internal cervical, which could modify the physiological interaction between the embryo and the endometrium and/or intrauterine blood flow, thereby affecting the embryo implantation process (9). IVF covers a variety of complex operations, including ovulation induction, egg retrieval, fertilization method (conventional IVF or intracytoplasmic sperm injection), type of embryo transfer (fresh embryo or frozen embryo, cleavage-stage embryos or blastocysts), and other operations such as maturation of oocytes *in vitro* and preimplantation genetic test (40). These IVF-related operations may have independent or additive effects on the occurrence of placental structure and function abnormalities compared with spontaneous pregnancy, illuminating the importance of examining the complex relationships between IVF-related operations and the described adverse perinatal outcomes.

While our study was not designed to explain the mechanisms through which IVF may adversely impact placental development, emerging studies are searching for the answers since placental abnormalities are associated with pregnancy-related complications, such as preeclampsia, preterm delivery, and intrauterine growth restriction (2). Evidence suggested that while IVF treatment does not have an adverse effect on the chromosomal constitution of fetal and placental lineages (41), 3405 differentially regulated genes were found to be significantly dysregulated from four human placental villi from first-trimester samples obtained from patients undergoing IVF treatment due to oviductal factors (42). The genes were involved in more than 50 biological processes and pathways, including coagulation cascades, immune response, transmembrane signaling, metabolism, cell cycle, stress control, invasion, and vascularization, whose disruption can cause detrimental outcomes (42). One study showed that IVF treatment could alter the placental phospholipid profiles, affecting the flexibility, fluidity, and function of transporting proteins in the membrane (43). Such alternations can then lead to placental complications. In addition to the genetic perturbations, epigenetic alterations, such as changes in DNA methylation patterns, were detected in the placenta and extraembryonic tissues in IVF-conceived mice, leading to nutrient transport and amino acid metabolism dysregulation (44). This could result in compensatory placenta enlargement to meet fetal nutrient requirements at the end of gestation, which in turn may increase the risk of abnormal

placental location (45). As the precise underlying mechanisms that explain the impact of IVF on placental development and function remain to be elucidated, the need for continued exploration to accrue insights on placental complications after IVF treatment has become clearer.

China is currently the country with the largest capacity for ART services globally (6). In light of the relaxing population policy, more potential infertile couples in China might have birth plans and thus pose more disease burden after IVF pregnancy. Both the public health and clinical researchers will put more emphasis on the considerable impact of IVF treatment on the short- or long-term health outcomes of pregnant women, fetuses, and offspring. Our study generates preliminary evidence about the risk of placental abnormalities and placenta-related complications following IVF treatment, prompting IVF specialists to heed the disease susceptibility associated with IVF operations and procedures to mitigate relevant disease risk. The current study's findings also underline the importance of gestation period management strategies of obstetric complications for obstetric clinicians, with special attention to women with younger or advanced maternal age undergoing IVF, from the perspective of maternal and child health.

LIMITATIONS OF THE STUDY

This study has some limitations that should be cautiously considered when interpreting the results. First of all, while we attempted to control for several factors, the HQMS database lacks several key obstetric risk factors, such as maternal health-related information before and during pregnancy (e.g., smoking, drinking, or medication use), paternal characteristics, and family history, which may also be confounding factors of the outcome variables. In addition, IVF treatment involves a variety of medications or surgical procedures. Thus, it is necessary to further analyze the impact of specific operations or medication use on the risk of placenta-related outcomes. Last, longitudinal cohort studies are warranted to trace the incidence of placenta-related chronic diseases and examine the long-term health outcomes of IVF patients and their offspring.

CONCLUSION

IVF is an independent risk factor for placental abnormalities and placental-related complications, and the risk is associated with maternal age. It is imperative for the reproductive and obstetric practitioners to carefully consider the impact of IVF treatment on maternal complications and perinatal health outcomes and continue to evaluate the long-term health impact of IVF to ensure the safety and health of the mother and child.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Peking University Third Hospital Medical Science Research Ethics Committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JQ and YW conceived and designed the study. FK, YF, HS and YW did the statistical analyses and drafted the manuscript. RL and YZ contributed to the interpretation of the data. All authors reviewed and revised the manuscripts and supplementary files. All authors read the final manuscript and approved submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.924070/full#supplementary-material

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Kisspeptin in the Prediction of Pregnancy Complications

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Kisspeptin and its receptor are central to reproductive health acting as key regulators of the reproductive endocrine axis in humans. Kisspeptin is most widely recognised as a regulator of gonadotrophin releasing hormone (GnRH) neuronal function. However, recent evidence has demonstrated that kisspeptin and its receptor also play a fundamental role during pregnancy in the regulation of placentation. Kisspeptin is abundantly expressed in syncytiotrophoblasts, and its receptor in both cyto- and syncytio-trophoblasts. Circulating levels of kisspeptin rise dramatically during healthy pregnancy, which have been proposed as having potential as a biomarker of placental function. Indeed, alterations in kisspeptin levels are associated with an increased risk of adverse maternal and foetal complications. This review summarises data evaluating kisspeptin's role as a putative biomarker of pregnancy complications including miscarriage, ectopic pregnancy (EP), preterm birth (PTB), foetal growth restriction (FGR), hypertensive disorders of pregnancy (HDP), pre-eclampsia (PE), gestational diabetes mellitus (GDM), and gestational trophoblastic disease (GTD).

Keywords: gestational trophoblastic disease (GTD), gestational diabetes mellitus (GDM), pre-ecalmpsia (PET), foetal growth restriction (FGR), hypertensive disorders of pregnancy (HDP), preterm (birth), miscarriage, kisspeptin

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INTRODUCTION

Kisspeptin is best known for its role as a hypothalamic neuropeptide that regulates gonadotrophin releasing hormone (GnRH) secretion (1). Indeed, early studies showed that inactivating variants of the kisspeptin receptor result in pubertal failure due to hypogonadotrophic hypogonadism, confirming the importance of kisspeptin signalling to reproductive health (2, 3).

During pregnancy, kisspeptin is produced in large amounts by the placenta and thus there is significant interest in evaluating its potential as a novel marker of pregnancy complications (4). Kisspeptin is a peptide encoded by the *KISS-1* gene that binds to a G-protein coupled kisspeptin receptor (*KISS-1R*, previously known as the orphan receptor GPR54) (5). Kisspeptin levels in the circulation are several hundred fold higher during healthy pregnancy compared to the non-pregnant state (6, 7). This review will summarise data evaluating kisspeptin's role as a putative biomarker of pregnancy complications including miscarriage, ectopic pregnancy (EP), preterm birth (PTB), foetal growth restriction (FGR), hypertensive disorders of pregnancy (HDP), pre-eclampsia (PE), gestational diabetes mellitus (GDM), and gestational trophoblastic disease (GTD).

KISSPEPTIN

The gene encoding kisspeptin (*KISS-1*) was first identified in 1996 as a metastasis tumour-suppressor gene in malignant melanoma cell lines and its peptide product was initially termed 'metastin' (8). Subsequently, it became known as kisspeptin in homage to its discovery in Hershey, Pennsylvania, USA, the hometown of the famous chocolate Hershey's kisses (8). The *KISS-1* gene, located on chromosome 1q32, encodes a 145 amino acid prepropeptide that is post-translationally cleaved into biologically active kisspeptin peptides of different amino acid lengths indicated by their suffix: e.g. kisspeptin -54, -14, -13, and -10 (5, 9, 10). All of these peptides bind and activate the kisspeptin receptor through their shared C-terminal region decapeptide motif (Arg-Phe-NH_2) (5, 10). Kisspeptin is expressed in multiple tissues including the hypothalamus, limbic system, gonads, pancreas, and liver, but is particularly abundant in the placenta, and thus is believed to play an important role in pregnancy (10, 11).

KISSPEPTIN IN HEALTHY PREGNANCY

Kisspeptin plays a key role in implantation and decidualisation. Kisspeptin promotes embryo attachment to the endometrium through interaction with cell adhesion molecules, and stimulates stromal decidualisation by up-regulating leukaemia inhibitory factor (LIF) (12) **(Figure 1)**. Kisspeptin also attenuates the excessive migration and invasion of trophoblasts through inhibition of the matrix metalloproteinases (MMP) 2 and 9 (13–15). Kisspeptin may also impact angiogenesis and uterine spiral artery modelling (16–18). A further relevant mechanism of kisspeptin in pregnancy relates to the maternal immune tolerance needed to avoid foetal rejection. Indeed, *in vitro* incubation with kisspeptin at levels corresponding to those found in pregnancy, results in increased differentiation of human naive T cells into T-regulatory cells (19).

The placenta is considered the main source of kisspeptin during pregnancy and the *KISS-1*/*Kiss-1* gene is expressed in syncytiotrophoblasts, whereas its receptor is expressed in both cytotrophoblasts and syncytiotrophoblasts (12) **(Table 1)**. Expression of kisspeptin and its receptor is high during early pregnancy and declines as the placenta matures, thus highlighting kisspeptin's role in placentation (14). Interestingly, circulating kisspeptin levels increase linearly with advancing gestation and kisspeptin-54 immunoreactivity dramatically rises from 1230 pmol/L during the first trimester to 9590 pmol/L during the third trimester and returns to non-pregnant levels (<100 pmol/L) soon after birth (8 pmol/L) (6, 7, 20).

Circulating kisspeptin levels are affected by several variables in healthy pregnancy (20). Whilst gestational and maternal age are associated with raised kisspeptin levels, Afro-Caribbean ethnicity, smoking during pregnancy, and high body mass index (BMI) are associated with reduced kisspeptin levels (20). Additionally, kisspeptin levels have been shown to be lower in serum compared to plasma samples, and are influenced by pre-analytical factors such as collection tube type, processing time and time to sample storage (49).

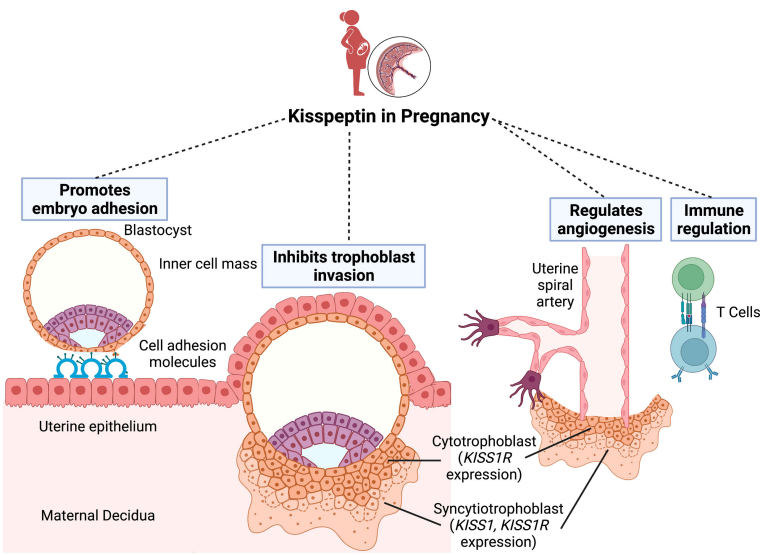

FIGURE 1 | The role of kisspeptin in embryo implantation. Successful implantation requires communication between the blastocyst and a receptive uterine epithelium. Kisspeptin initially promotes embryo attachment to the endometrial epithelium through interaction with cell adhesion molecules. Once the blastocyst penetrates the epithelium, the trophoblast cells differentiate into the inner cytotrophoblast and outer syncytiotrophoblast cells. Whilst the cytotrophoblast cells express the kisspeptin receptor (*KISS-1R*), the syncytiotrophoblast cells express both *KISS-1R* and the kisspeptin gene (*KISS-1*). Kisspeptin subsequently regulates implantation by inhibiting excessive trophoblast invasion into the endometrium. Finally, kisspeptin also has roles in uterine spiral artery remodelling and immune regulation to avoid maternal foetal rejection. Figure created with BioRender.com.

TABLE 1 | Summary of Kisspeptin gene, receptor and circulating levels in different pregnancy states.

Pregnancy state	KISS-1 expression	KISS-1 receptor expression	Circulating Kisspeptin levels
Healthy Pregnancy	Increased in first trimester (12) - Villous cytotrophoblasts	Increased in first trimester (12) - Villous cytotrophoblasts - Syncytiotrophoblasts - Extravillous cells	Increase linearly with pregnancy progression (20)
Miscarriage	Reduced (11) - Trophoblasts	No difference in women with recurrent pregnancy loss (11)	Reduced (20–25)
Ectopic pregnancy	Reduced (26) - Embryonic tissue	NA	Reduced (25, 26) No difference (20)
Preterm Birth	Increased (27) - Placental tissue	NA	No difference (27, 28) (unadjusted KP higher in late first trimester) (28)
Foetal Growth Restriction	NA	NA	Reduced (28–31)
Pre-Eclampsia	Increased (32–35) Increased (EPE) (36) No difference (LPE) (36) Decreased (37, 38) - Placental tissue	Increased (35, 37) No difference (32) - Placental tissue	Reduced in PE: 1st trimester (29, 39), 2nd trimester (40–42), 3rd trimester (32, 40–43) Reduced in EPE 9-13 wks (28) No difference in PE (36, 44) Increased in LPE 9-13 wks (28) No difference in PIH (40, 44) No difference in HDP: 1st and 2nd trimesters (28) Increased in HDP: 3rd trimester (28)
Gestational Diabetes	Increased (35, 45) - Syncytiotrophoblasts - Cytotrophoblasts	Increased (35, 45) - Syncytiotrophoblasts - Cytotrophoblast	No difference (46) Reduced (28, 40, 47)
Gestational Trophoblastic disease	Molar pregnancy: No difference (38, 48) Choriocarcinoma: Decreased (38, 48)	Molar pregnancy: No difference (38, 48) Choriocarcinoma: Decreased (38, 48)	Choriocarcinoma: Increased (7)

EPE, early onset pre-eclampsia; HDP, hypertensive disorders of pregnancy; KP, kisspeptin; LPE, late onset pre-eclampsia; NA, not applicable; PE, pre-eclampsia; PIH, pregnancy induced hypertension.

KISSPEPTIN IN PREGNANCY COMPLICATIONS

1. Kisspeptin in Miscarriage

Miscarriage is the spontaneous loss of an intrauterine pregnancy before 24 weeks of gestation and affects 1 in 5 clinical pregnancies (50). Miscarriage predominantly occurs during the first trimester of pregnancy and the majority of early miscarriages are due to a genetic abnormality of the developing embryo, however other causes include endocrine, anatomical, and immunological factors (51).

Miscarriage diagnosis can be challenging as often a pregnancy is failing for a time before pregnancy loss has conclusively been confirmed. This uncertainty can exacerbate the psychological burden related to investigating possible miscarriage, with up to 6% of women suffering from moderate-severe depression, 17% from moderate-severe anxiety and 18% from post-traumatic stress disorder (52). To date, there is no clinical predictor of miscarriage, however recent data demonstrates a potential for kisspeptin as a biomarker of miscarriage.

Kisspeptin levels (adjusted for gestation) are markedly reduced by 60-79% in women with miscarriage compared to healthy pregnancy (20–25) **(Table 2.1)**. Above average levels, when corrected for gestational age, are reassuring with a <1% chance of miscarriage (20), whereas kisspeptin levels 95% lower than the median for that gestation are associated with up to an 85% chance of miscarriage. Concordantly, *KISS-1* expression is decreased in the placentae of women with recurrent spontaneous abortion compared to those who undergo voluntary termination of pregnancy (11). Furthermore, whilst kisspeptin's high diagnostic performance for identifying miscarriage is maintained in late-first trimester pregnancies (>8 weeks of gestation), that of β-human chorionic gonadotrophin (β-hCG) worsens (20). Thus, the combination of both kisspeptin and β-hCG can be used to ensure high diagnostic accuracy at all gestations (AUCROC 0.92, 95% CI 0.89-0.95) (20, 22, 24). Kisspeptin has also been shown to reflect different types of miscarriage, with lower levels reported in complete (no retained products of conception) versus incomplete (retained products of conception) or missed (empty gestational sac or a foetal pole with no heartbeat) miscarriage (20). Additionally, both kisspeptin and β-hCG levels decline with closer proximity to miscarriage confirmation, and therefore repeat measurements every 1-2 weeks could enable further risk-stratification of miscarriage risk in clinical practice (20).

Studies involving women with infertility who undergo assisted reproductive techniques (*in vitro* fertilisation, intracytoplasmic sperm insemination (ICSI) or frozen thawed embryo transfer) have found reduced β-hCG levels in miscarriage compared to controls, but no difference in kisspeptin levels (53, 55). These findings may be due to the very early gestations at which kisspeptin levels were assessed (2-3 weeks following, or even before, pregnancy confirmation) (53, 55). Indeed, kisspeptin may not be expressed in the placenta at high levels prior to 6 weeks of gestation, suggesting that β-hCG levels may be more useful at these very early gestations (26).

2. Kisspeptin in Ectopic Pregnancy

Ectopic pregnancy (EP) affects 2% of pregnancies and occurs when a fertilised ovum implants and develops outside the uterine cavity, most commonly within the fallopian tube (56). EP can result in tubal

TABLE 2 | Circulating Kisspeptin Levels in pregnancy complications.

2.1. KISSPEPTIN IN MISCARRIAGE

Author	Study Design	Cohort	Sample size	Kisspeptin measurement	Kisspeptin and βHCG values	AUCROC
Kavvasoglu (2011) (21)	Prospective Cohort	Pregnant women who delivered to term and miscarriage	Controls 20 Miscarriage 20	Plasma at 7-18 wks GA KP-10 ELISA (Phoenix, Germany)	**Kisspeptin pg/ml (median, min-max)*** Controls: 5,783 (3,168–9,953) Miscarriage: 391 (152–951)	NA
Jayasena (2014) (22)	Prospective Cohort	Asymptomatic pregnant women	Controls 899 Miscarriage 50	Plasma at 7-14 wks GA All KP forms In house RIA	**Kisspeptin MoM (mean ± SD)*** Controls: 1.06 ± 0.42 Miscarriage: 0.42 ± 0.39 **β-hCG MoM (mean ± SD)*** Controls: 1.08 ± 0.47 Miscarriage: 0.69 ± 1.35	**KP** 0.899 **βhCG** 0.775
Mumtaz (2017) (23)	Case-Control	Women with infertility undergoing ICSI treatment	Controls 28 Preclinical abortion 30	Serum before treatment All KP forms ELISA (Kiss-1, China)	**Kisspeptin ng/L (mean ± SEM)*** Controls: 296.23 ± 12 Miscarriage: 215.11 ± 34.14	
Sullivan-Pyke (2018) (24)	Case-Control	Symptomatic pregnant women	Controls 20 Miscarriage 20	Serum at 6-10 wks GA KP-54 ELISA (Peninsula, USA)	**Kisspeptin ng/ml (median, IQR)*** Controls: 1.50 [0.55 – 3.72] Miscarriage: 0.20 [0.07 – 0.37] **β-hCG mIU/mL (median, IQR)*** Controls: 117202 [83975 – 148784] Miscarriage: 4739 [1858 – 8650]	**KP** 0.953 **βhCG** 0.994
Yu (2019) (53)	Case-Control	Women with infertility undergoing IVF/ICSI treatment	Controls 28 Miscarriage 21	Serum at (i) 12 days after blastocyst transfer and (ii) 4 days after pregnancy confirmation All KP forms ELISA (BlueGene, China)	**Kisspeptin** No significant difference between controls and miscarriage **β-hCG*** Significantly lower in miscarriage compared to controls	**KP** (i) 0.63, (ii) 0.76 **βhCG** (i) 0.76, (ii) 0.89
Hu (2019) (12)	Case-Control	Women with infertility undergoing frozen thawed embryo transfer	Controls 47 Miscarriage 28	Serum at (i) 14 days and (ii) 21 days after embryo transfer KP-54, KP-10 RIA (Phoenix, USA)	**Kisspeptin pg/ml (mean ± SD)** Controls: (i) 420.9 ± 201.5, (ii) 730.8 ± 274.4 Miscarriage: (i) 434.9 ± 215.1, (ii) 762.2 ± 210.3 **β-hCG IU/L (mean ± SD)*** Controls: (i) 1791 ± 1730, (ii) 21833 ± 16160 Miscarriage: (i) 777.8 ± 783.8, (ii) 6720 ± 4413	**KP** 0.533 **βhCG** 0.777
Abbara (2021) (20)	Case-Control	Asymptomatic and Symptomatic pregnant women	Controls 265 Miscarriage 95	Plasma every 2 wks between 6-14 wks GA All KP forms In house RIA	**Kisspeptin MoM (median, IQR)*** Controls: 1.00 [0.63–1.31] Miscarriage: 0.21 [0.08–0.47] **β-hCG MoM (median, IQR)*** Controls: 1.00 [0.74–1.32] Miscarriage: 0.30 [0.08–0.64]	**KP** 0.874 **βhCG** 0.859
Gorkem (2021) (54)	Case-Control	Asymptomatic and Symptomatic pregnant women	Controls 30 Miscarriage 30 Threatened miscarriage 30	Serum at 7-9 wks GA KP-54 ELISA (Cloud-Clone Corp, USA)	**Kisspeptin ng/ml (median, IQR)** Controls: 86.7 [69.5-112.4] Miscarriage: 102.5 [79.5-123.5] Threatened miscarriage: 101.7 [85.4-139.4]	NA
Yuksel (2022) (25)	Prospective Case-Control	Symptomatic pregnant women with a pre-diagnosis of EP or miscarriage and healthy pregnancy	Controls 23 Miscarriage 23	Serum at 5-6 wks GA KP form unclear ELISA (Mybiosource, USA)	**Kisspeptin ng/ml (median, min-max)*** Controls: 1.48 (1.29–1.80) Miscarriage: 0.11 (0.08–0.16) **β-hCG mIU/ml (median, min-max)*** Controls: 6151 (576–19,941) Miscarriage: 1771 (98–11,890)	NA

(Continued)

TABLE 2 | Continued; Circulating Kisspeptin Levels in pregnancy complications.

2.2. KISSPEPTIN IN ECTOPIC PREGNANCY

Author	Study Design	Cohort	Sample size	Kisspeptin measurement	Kisspeptin and βHCG values	AUCROC
Romero-Ruiz (2019) (26)	Prospective Case-Control	Women with normal pregnancy that desired VTOP and EP	VTOP 108 EP 45	Plasma at 4-20 wks GA All KP forms In house RIA	**Kisspeptin*** Significantly lower in EP compared to controls at all GA stages **β-hCG*** Significantly lower in EP compared to controls at all GA stages	**KP** 0.909 **βhCG** 0.947
Abbara (2021) (20)	Case-Control	Asymptomatic and Symptomatic pregnant women	VIUP 42 EP 31 FPUL 82 PPUL 8	Plasma every 2 wks between 6-14 wks GA All KP forms In house RIA	**Kisspeptin pmol/L (mean + SEM)** VIUP: 21.6 ± 41. EP: 20.1 ± 10.6 FPUL: 16.9 ± 12.0. PPUL: 21.5 ± 16.0	NA
Yuksel (2022) (25)	Prospective Case-Control	Symptomatic pregnant women with a pre-diagnosis of EP or miscarriage and healthy pregnancy	Controls 23 EP 17	Serum at 5-6 wks GA KP form unclear ELISA (Mybiosource, USA)	**Kisspeptin ng/ml (median, min-max)*** Controls: 1.48 (1.29–1.80). EP: 0.30 (0.22–0.39) **β-hCG mIU/ml (median, min-max)*** Controls: 6151 (576–19,941). EP: 1333 (94–11,600)	NA

2.3. KISSPEPTIN IN HYPERTENSIVE DISORDERS OF PREGNANCY AND PRE-ECLAMPSIA

Author	Study Design	Cohort	Sample size	Kisspeptin measurement	Kisspeptin and βHCG values	AUCROC
Armstrong (2009) (29)	Retrospective Case-Control	Pregnant women with PE and uncomplicated pregnancies	Controls 317 PE 57	Serum at 16-20 wks GA KP-54 In house ELISA	**Kisspeptin pg/ml (median, IQR) *** Controls: 1188 [494 – 2298] PE: 1109 [442 – 3903]	NA
Nijher (2010) (44)	Case-Control	Pregnant women with PE, PIH and uncomplicated pregnancies	Controls 78 PE 9 PIH 78	Plasma at 27-40 wks GA KP-10, KP- 14, KP-54 In house RIA	**Kisspeptin pmol/l (mean ± SE)** Controls: 2878 ± 157 PIH: 2696 ± 299 PE: 3519± 357	NA
Cetcovic (2012) (40)	Prospective Case-Control	Pregnant women with CH, PIH, PE and uncomplicated pregnancies	Controls 25 CH 22 PIH 18 PE 28 EPE 23 LPE 5	Plasma at (i) 21-25 wks and (ii) 32-36 wks GA KP-10, KP- 14, KP-54 Validated RIA (7)	**Kisspeptin nmol/l (mean ± SD)** Controls: (i) 10.33 ± 2.65, (ii) 20.48 ± 7.60 PE: (i) 4.46 ± 3.73, (ii) 16.03 ± 10.09* CH: (i) 3.42 ± 1.04, (ii0 14.14 ± 10.44 * PIH: (i) 8.46 ± 6.24, (ii) 25.68 ± 9.2	NA
Madazli (2012) (39)	Retrospective Case-Control	Pregnant women with PE and uncomplicated pregnancies	Controls 30 PE 31	Plasma at 11-14 wks GA KP form unclear ELISA: (Phoenix, Germany)	**Kisspeptin pmol/l (mean ± SD) *** Controls: 1995 ± 375 PE:1554 ± 385	**KP** 0.797 **PIGF** 0.831
Adali (2012) (43)	Cross-Sectional	Pregnant women with PE (mPE GA 35.4 ± 0.83*, sPE GA 33.09 ± 0.75*) and uncomplicated pregnancies (GA 37.66± 0.39)	Controls 50 mPE 15 sPE 24	Plasma at 33-37 wks GA KP-10, KP- 14, KP-54 ELISA (Phoenix, Germany)	**Kisspeptin ng/ml (mean± SE) *** Controls: 9.69 ± 1.35 mPE: 2.61 ± 0.40 sPE: 1.17 ± 0.24	NA
Logie (2012) (41)	Cross-Sectional	Lean women with healthy pregnancy (controls) and obese women (BMI >40kg/m^2) with uncomplicated pregnancy or PE	Controls 39 Obese (uncomplicated) 112 Obese PE 7	Plasma at (i) 16, (ii) 28, (iii) 36 wks GA KP form unclear ELISA (Phoenix, Germany)	**Kisspeptin at 16 wks pM (mean ± SEM)** Lower in obese PE compared to uncomplicated obese and controls*	**KP** (i) 0·80, (ii) 0·56, (iii) 0·66)
Ziyaraa (2015) (42)	Prospective Case-Control	Pregnant women who completed GA 20 wks with mild and severe EPE and uncomplicated	Controls 40 PE 60 Mild EPE 39 Severe EPE 21	Plasma at (i) 20-27 wks and (ii) 28-40 wks KP-10	**Kisspeptin ng/ml (mean± SEM)** Controls: (i) 2.30 ± 0.51, (ii) 2.95 ± 1.82 Mild EPE: (i) 2.18 ± 0.76, (ii) 2.16 ± 0.48 * Severe EPE: (i) 1.59 ± 0.26 (1^{st}) *, (ii) 2.39	NA

(Continued)

TABLE 2 | Continued; Circulating Kisspeptin Levels in pregnancy complications.

		pregnancies Difference in BMI between the groups *		ELISA (Phoenix, Germany)	± 0.57 Mild vs Severe EPE:(i) *, (ii) (NS)	
Matjila (2016) (32)	Case-Control	Patients with (mean GA 32.95 ± 0.53 *) and without EPE (mean GA 38.03 ± 0.06 *) undergoing elective caesarean delivery	Controls 30 EPE 19	Serum at 32-39wks GA KP-10 ELISA (Phoenix, Germany)	**Kisspeptin ng/ml (mean± SEM) *** Controls: 1.66 ± 0.59 ng/ml PE: 0.58 ± 0.39	NA
Abbara (2022) (28)	Case-Control	Pregnant women with antenatal complications and uncomplicated pregnancies	Controls 265 HDP 32 PE 20 (EPE, LPE) PIH 12	Plasma at (i) <9, (ii) 9-13, (iii) 14-27, (iv) 28-40 wks GA KP-10, KP-14, KP-54 In-house RIA	**Kisspeptin pmol/L (mean± SEM)** HDP Vs Controls No significant difference in (i), (ii), (iii) Higher in HDP than controls (iv) * LPE Vs Controls No significant difference in (i), (iii), (iv) Higher in LPE than controls(ii) * EPE Vs Control No significant difference in (i), (iii), (iv) Lower in EPE than controls(ii) * **Kisspeptin MoM (median) *** Higher in HDP than control pregnancies	NA

2.4. KISSPEPTIN IN GESTATIONAL DIABETES MELLITUS

Author	Study Design	Cohort	Sample size	Kisspeptin measurement	Kisspeptin and βHCG values	AUCROC
Cetcovic (2012) (40)	Prospective Case Control	Pregnant with and without a diagnosis of GDM	Controls 25 GDM 20	Plasma at (i) 21-25 and (ii) 32-36 wks GA KP-10, KP- 14, KP-54 Validated RIA (7)	**Kisspeptin nmol/l (Mean ± SD) *** Controls: (i) 10.33 ± 2.65; (ii) 20.48 ± 7.60 GDM: (i) 4.51 ± 3.18*; (ii) 11.643 ± 7.6 *	NA
Bowe (2019) (47)	Case-Control	Pregnant women with and without a diagnosis of GDM	Controls 62 GDM 26	Plasma at 26-34 wks GA KP form unclear ELISA (Phoenix, Germany)	**Kisspeptin pmol/l (Mean ± SEM) *** Controls: 1270.9 ± 67.1 GDM: 889.9 ± 96.6	NA
Arslan (2020) (46)	Cross-Sectional	Pregnant women with and without a diagnosis of GDM	Controls 82 GDM 76	Serum at 24-28 wks GA KP-54 ELISA (Human KISS-54 kits-Biotek Synergy HT)	**Kisspeptin pmol/l (Mean ± SD)** Controls: 161.3 ± 78.2 GDM: 187.6 ± 132.3 (NS)	NA
Abbara (2022) (28)	Case-Control	Pregnant women with antenatal complications and uncomplicated pregnancies	Controls 265 GDM 35	Plasma at <9, 9-13, 14-27, 28-40 wks GA KP-10, KP-14, KP-54 In-house RIA	**Kisspeptin nmol/l (Median [IQR])** No difference between control and GDM pregnancies in all trimesters **Kisspeptin MoM (median)*** GDM lower than control pregnancies	NA

2.5. KISSPEPTIN IN PRETERM BIRTH

Author	Study Design	Cohort	Sample size	Kisspeptin measurement	Kisspeptin and βHCG values	AUCROC
Torricelli (2008) (27)	Observational	Pregnant women delivering at term (GA 38-40 wks, by SVD or ECS) and preterm (GA 32-34 wks)	Term SVD 15 Term ECS 15 Preterm 10	Plasma at delivery All KP forms ELISA (Phoenix, Germany)	**Kisspeptin ng/ml (mean ± SEM)** Term SVD: 4.332 ± 2.10 Term ECS: 4.021 ± 1.67 Preterm: 4.781 ± 1.51	NA
Abbara (2022) (28)	Case-Control	Pregnant women with uncomplicated pregnancies and preterm birth (GA 24-37wks)	Controls 265 Preterm 11	Plasma at (i) <9, (ii) 9-13, (iii) 14-27, (iv) 28-40 wks GA KP-10, KP-14, KP-54 In-house RIA	**Kisspeptin*** Adjusted KP higher in PTB than controls in all trimesters Unadjusted KP levels in (ii) higher in PTB than controls	NA

(Continued)

TABLE 2 | Continued; Circulating Kisspeptin Levels in pregnancy complications.

2.6. KISSPEPTIN IN FOETAL GROWTH RESTRICTION

Author	Study Design	Cohort	Sample size	Kisspeptin measurement	Kisspeptin and βHCG values	AUCROC
Smets (2008) (30)	Case-Control	Pregnant(women at risk of PE, IUGR and SGA **Birth weight (g)** Controls 3623 ± 334 SGA 2665 ± 369	Controls 31 SGA 31	Plasma at 8-14 wks GA KP-10 Ab RIA (Phoenix, USA)	**Kisspeptin pmol/L (mean ± SD)*** Controls: 2035 ± 1260 IUGR: 1376 ± 1317 **β-hCG pg/ml (mean ± SD)** Controls: 62 ± 56 IUGR: 61 ± 55	NA
Armstrong (2009) (29)	Retrospective case-control	Pregnant women with IUGR and uncomplicated pregnancies **Birth weight (g)** Controls 3496 ± 36.6 IUGR 2307 ± 17.4	Controls 317 IUGR 118	Serum at 16-20 wks GA KP-54 In house ELISA	**Kisspeptin pg/ml (median, IQR)*** Controls: 1188 [494 – 2298] IUGR: 1164 [442 – 3903) **β-hCG MoM (mean ± SEM)** Controls: 0.97 (0.69) [0.20 – 3.19] IUGR: 0.91 (0.74) [0.50 – 3.6]	NA
Khalil (2018) (31)	Case-Control	Pregnant women with PE&IGUR, IUGR and uncomplicated pregnancies that underwent ECS **Birth weight (g)** Controls 3300 ± 110 PE&IUGR 2180 ± 220 IUGR 2280 ± 350	Controls 10 PE&IUGR 10 IUGR 10	Serum at 34-38wks GA KP-10 ELISA (Life span Biosciences)	**Kisspeptin ng/ml (mean ± SD)*** Controls: 2900 ± 600 PE&IUGR: 1640 ± 400 IUGR: 1630 ± 300	NA
Abbara (2022) (28)	Case-Control	Pregnant women with antenatal complications and uncomplicated pregnancies	Controls 265 FGR 17	Plasma at (i) <9, (ii) 9-13, (iii) 14-27, (iv) 28-40 wks GA KP-10, KP-14, KP-54 In-house RIA	**Kisspeptin*** Adjusted KP lower in FGR than controls in all trimesters Unadjusted KP levels in (ii) and (iv) lower in FGR than controls	NA

2.7. KISSPEPTIN IN GESTATIONAL TROPHOBLASTIC DISEASE

Author	Study Design	Cohort	Sample size	Kisspeptin measurement	Kisspeptin and βHCG values	AUCROC
Dhillo (2006) (7)	Case-Control	Healthy pregnant women and women diagnosed with invasive mole undergoing chemotherapy	Controls 26 Invasive mole 11	Plasma at (i) 10 wks GA, (ii) 38 wks GA and (iii) 15 days postpartum and (iv) pre and post chemotherapy for invasive mole KP-10, KP-14, KP-54 In-house RIA	**Kisspeptin pmol/l (mean ± SE)*** Controls 10 wks: 803 ± 13 38 wks: 2,483 ± 302 15 days postpartum: <2 Invasive Mole Pre-chemo: 1,363 ± 1,076 pmol* Post-chemo: <2 **β-hCG U/l (mean ± SE) *** Controls 10 wks: 72,053 ± 10,936 38 wks: 28,818 ± 11,348 Invasive Mole Pre-chemo: 227,191 ± 152,354 Post-chemo: **<**2	NA

BMI, body mass index; CH, chronic pre-existing hypertension; ECS, elective caesarean section; ELISA, enzyme-linked immunosorbent assay; EP, ectopic pregnancy; EPE, early onset pre-eclampsia; FGR, foetal growth retardation; FPUL, failed (negative pregnancy test 2 weeks from follow-up) pregnancy of unknown location; GA, gestational age; GDM, gestational diabetes mellitus; GTD, gestational trophoblastic disease; HDP, hypertensive disorders of pregnancy; ICSI, intracytoplasmic sperm injection; IQR, interquartile range; IUGR, intrauterine growth retardation; IVF, in vitro fertilisation; KP, kisspeptin; LPE, late onset pre-eclampsia; mPE, mild pre-eclampsia; MoM, multiple of the median; NA, not applicable; NS, no statistically significant difference; PE, pre-eclampsia; PIH, pregnancy induced hypertension; PlGF, placenta growth factor; PPUL, persistent (more than three static serial βhCG levels) pregnancy of unknown location; RIA, radioimmunoassay; SD, standard deviation; SEM, standard error of the mean; SGA, small for gestational age baby; sPE, severe pre-eclampsia; SVD; spontaneous vaginal delivery; VIUP, intrauterine pregnancy viable at 12 weeks' gestation; VTOP, voluntary termination of pregnancy; wks, weeks.
**p-values indicate statistically significant difference.*

rupture and accounts for 9-13% of all pregnancy-related deaths in developed countries and can compromise a woman's future fertility (57). EP is currently diagnosed by serial β-hCG measurements in combination with ultrasound, although laparoscopy is often required to provide a definitive diagnosis (58). The sensitivity and specificity of these tests significantly decrease in the case of pregnancies of unknown location (PUL) as false positive or negative diagnoses may occur. This is important as an incorrect diagnosis may lead to termination of a healthy pregnancy (59). Accordingly, different biomarkers have been investigated in an attempt to improve the diagnostic accuracy of EP, including kisspeptin.

Some studies have found that kisspeptin levels in EP are lower than in healthy pregnancy but higher than in miscarriage (25, 26). However, another study demonstrated that kisspeptin levels are not significantly altered between women with viable intrauterine pregnancies (VIUPs) and those with either EP or failing or persistent PUL, after adjusting for confounding variables (20) **(Table 2.2)**. Current evidence remains limited, and larger studies are required to determine kisspeptin's performance as a diagnostic marker in EP at early gestations (<6 weeks).

3. Kisspeptin in Hypertensive Disorders of Pregnancy and Pre-Eclampsia

Hypertensive disorders affect 5% of all pregnancies (60) and include pre-existing chronic hypertension (CH), pregnancy induced hypertension (PIH) and pre-eclampsia (PE). PIH is defined as new onset hypertension (BP ≥140/90mmHg) occurring after 20 weeks of gestation, PE is PIH with proteinuria (urine >3g/24 hours) or significant end-organ dysfunction, and severe PE is the presence of at least one of: hypertension (BP≥160/110 mmHg), visual disturbance, chest pain, dyspnoea, pulmonary oedema, seizures, or neonatal distress (61). PE is further classified, according to the onset of clinical features, into early-onset PE (EPE <34 weeks of gestation) and late-onset PE (LPE ≥34 weeks of gestation). EPE is associated with impaired trophoblast invasion, defective spiral artery remodelling and adverse perinatal complications including IUGR (62). LPE occurs due to hypoxic stress and impaired perfusion but is less likely to compromise foetal growth (63, 64). Currently, PE diagnosis is based on early pregnancy risk factor screening, uterine artery Doppler velocimetry and biomarkers such as PPAP-A or placental growth factor (PlGF) (61). Kisspeptin has been implicated in the pathogenesis of PE through reduced angiogenesis, decreased cytotrophoblast invasion and increased trophoblast apoptosis, and thus could have potential in predicting PE (16–18).

Levels of circulating kisspeptin in HDP vary in the literature, and largely differ according to HDP subtype, severity, and onset (**Table 2.3**). Most of the studies report reduced circulating kisspeptin levels in PE compared to normotensive pregnant controls (29, 32, 40–43, 65) and therefore kisspeptin is considered to reflect placental dysfunction. However, expression of *KISS-1*, which inhibits trophoblast invasion and results in defective transformation of the spiral arteries, is increased in the placentae of PE pregnancies, thus supporting its role in the pathophysiology of PE (32–34, 36, 66) **(Table 1)**. Nonetheless, there are also some reports of decreased *KISS-1* expression in PE placentae (37, 38) **(Table 1)**. Furthermore, evidence suggests that circulating kisspeptin levels decline as the severity of PE increases, which could also reflect reduced placental mass in more severe disease. Indeed, both circulating kisspeptin levels and placental mass is reduced in EPE compared to LPE (28, 42, 67). Additionally, pregnant women with pre-existing hypertension and PE, states associated with a higher burden of disease, have reduced kisspeptin levels compared to PIH (40).

Whilst most studies demonstrate reduced kisspeptin levels in PE, a recent study found that kisspeptin levels are increased in HDP during the third trimester of pregnancy **(Table 2.3)**. However, there was no association between circulating kisspeptin levels and severity of PET (28). It is likely that complexity in the categorisation, severity, and onset of PET, and the need for correction for possible confounders such as BMI and gestational age, could explain differences between kisspeptin levels observed in the current studies. Larger observational studies that are carefully designed to address these and look at each PET-subset throughout pregnancy would therefore be valuable in resolving these inconsistencies.

4. Kisspeptin in Gestational Diabetes Mellitus

During pregnancy a physiological rise in maternal insulin resistance provides glucose to the developing foetus (68, 69). This insulin resistance leads to maternal pancreatic β-cell adaptation and increased insulin secretion. Failure of these changes results in gestational diabetes mellitus (GDM), which affects up to 20% of pregnancies worldwide (70).

Kisspeptin receptors are expressed in pancreatic β-cells (71) and have been implicated in β-cell adaptation during pregnancy. Exogenous kisspeptin administration has variable physiological effects on the glucose-dependent regulation of pancreatic beta-cells. For instance, KISS-1 peptide (KP-145) (71), KP-13 (72), KP-10 (72–74) potentiates glucose-stimulated insulin secretion (GSIS) in animal and human islets *in-vitro*. KP-54 increases GSIS in healthy men following an intravenous glucose tolerance test (IVGTT), which induces high glucose levels (75). On the other hand, Vikam and colleagues have found that KP-13 and KP-54 drives dose-dependent inhibitory effects on insulin secretion in mouse islets in the presence of lower glucose concentrations (2.8-11.1 mmol/l), compared to controls, which is not observed at higher glucose concentrations (76). Furthermore, chronic administration of KP-10 in non-pregnant mice enhances GSIS and improves glucose tolerance (47). Interestingly, hyperlipidaemia, impaired glucose tolerance (IGT) and weight gain develops in *Kiss-1r*-null female mice exclusively, thus suggesting sexual dimorphism in kisspeptin's effects on metabolism and glucose homeostasis (77).

In late gestation murine pregnancy, β-cell specific *Kiss-1r*-knockout models and pharmacological inhibition of *Kiss-1r* leads to reduced GSIS and development of IGT, which is not observed in non-pregnant states or wild-type controls (47). This supports a role for β-cell kisspeptin signalling in the regulation of glucose homeostasis during pregnancy. Loss of kisspeptin signalling in the ß-cell-specific *Kiss-1r*-knockout models also attenuates the increased ß-cell proliferation normally seen during murine

pregnancy when assessed with bromodeoxyuridine (BrdU) labelling. Nonetheless, the levels are not reduced to non-pregnant levels, suggesting contribution of other signals in pancreatic β-cell proliferation during pregnancy (47, 78).

In human pregnancies with GDM, placental *KISS-1* and *KISS-1R* expression is elevated in the third trimester (35, 45) **(Table 1)**, whereas circulating kisspeptin levels have been either lower (40, 47) or not significantly altered (28, 46) **(Table 2.4)**. Finally, Bowe and colleagues have demonstrated a positive correlation between third trimester kisspeptin levels and oral glucose–stimulated insulin levels at 60 minutes (r^2 = 0.18; P < 0.0001) and AUC serum insulin over the OGTT (r^2 = 0.13; P=0.0013) in women with GDM (47).

5. Kisspeptin in Pre-Term Birth

Pre-term birth (PTB) is defined as delivery prior to 37 weeks of gestation and affects 11% of pregnancies (79, 80). Kisspeptin has been proposed to initiate labour through increased oxytocin neuronal firing rate in pregnant rats and thus may play a potential role in PTB (81). Gestation adjusted kisspeptin levels are higher in PTB-affected pregnancies than in control pregnancies during the late-first trimester, with the adjusted odds of PTB being increased by 20% (95% CI, 1-42%) for every 1 nmol/L increase in plasma kisspeptin (28) **(Table 2.5)**. Furthermore, *KISS-1* mRNA expression is higher in preterm placentae than in term placentae delivered vaginally or by Caesarean section thus indicating that increased kisspeptin expression could be involved in the induction of labour (27) **(Table 1)**. However, no alteration in circulating kisspeptin levels have been reported to date during the third trimester between healthy pregnancy and PTB and thus more data is needed to elucidate whether there are changes in kisspeptin levels preceding and around the time of spontaneous labour (27, 28).

6. Kisspeptin in Foetal Growth Restriction

Foetal growth restriction (FGR) encompasses both intrauterine growth restriction (IUGR, foetal weight <10th centile for gestational age with abnormal umbilical artery doppler results) and small for gestation age (SGA, delivery weight <10th percentile for gestational age) (82, 83). FGR is thought to arise from abnormal trophoblast invasion and spiral artery remodelling that limits oxygen supply to the placenta (84, 85). The resulting ischemic injury generates reactive oxygen species which lead to apoptosis and restriction of placental and foetal growth (84, 85). To date, four studies have demonstrated significantly reduced kisspeptin levels in FGR versus healthy pregnancy in all three trimesters (28–31) **(Table 2.6)**. Thus, low circulating kisspeptin levels could reflect low placental mass in pregnancies affected by FGR.

7. Kisspeptin in Gestational Trophoblastic Disease

Gestational trophoblastic disease (GTD) is characterised by an abnormal proliferation of placental tissue and comprises of choriocarcinoma, invasive mole, placental site trophoblastic tumour and epithelioid trophoblastic tumour (86). Molar pregnancy is a benign form of GTD, whereas choriocarcinomas are more aggressive, however both exhibit high β-hCG levels and respond well to chemotherapy (87). Serum β-hCG measurement aids with GTD diagnosis, staging and prognostication before and after chemotherapy (88).

KISS-1 and *KISS-1R* expression is significantly lower in malignant choriocarcinoma cells compared to molar and healthy pregnancies (38, 48) **(Table 1)**. Conversely, circulating kisspeptin levels are elevated in malignant GTD compared to healthy pregnancies but significantly decline following chemotherapy (7) **(Table 2.7)**. The increased circulating kisspeptin levels could reflect an increased malignant trophoblast mass rather than an elevation in cellular *KISS-1* expression (89). Thus, kisspeptin levels can be altered in choriocarcinomas and other GTDs, which is interesting when considering the original identification of *KISS-1* as an anti-metastatic gene.

CONCLUSION

Kisspeptin levels are markedly reduced in miscarriage; and whilst the performance of kisspeptin levels to identify women at high risk of miscarriage is maintained throughout the first trimester, that of β-hCG falls during the latter part of the first trimester. Nevertheless, kisspeptin levels are only mildly elevated at early gestations (< 6 weeks) and therefore can be difficult to detect using current collection and assay methods. Thus, measuring kisspeptin in combination with β-hCG levels could potentially overcome this deficiency at early gestations. Due to the current difficulty in miscarriage diagnosis and the lack of available biomarkers, the high performance of plasma kisspeptin suggests that it has significant potential for further development in this context. Given that kisspeptin has been proposed as a biomarker of healthy placentation, it could potentially be used to recognise late pregnancy complications characterised by abnormal placentation during the first trimester. Regarding HDP, most studies have suggested lower circulating kisspeptin levels but increased placental kisspeptin expression. Kisspeptin levels in pregnancy complications such as PE are confounded by factors such as BMI, disease severity, time of onset, and concomitant FGR, and thus could limit the use of kisspeptin diagnostically.

Overall, current evidence suggests that circulating kisspeptin levels are consistently reduced in miscarriage, EP, FGR, GDM, and increased in PTB and GTD. Larger datasets with adequately sized control cohorts that accurately adjust for gestation, BMI, ethnicity, detailed disease severity phenotype and onset are needed to enable more precise characterisation of the utility of kisspeptin levels in these settings. In summary, circulating kisspeptin is a promising biomarker for early pregnancy loss and further research is needed to assess its potential in other pregnancy complications.

AUTHOR CONTRIBUTIONS

BP, JT wrote the manuscript, designed the figures and tables. AA, WSD, ANC reviewed and edited the manuscript and are the

corresponding authors. All authors have made a substantial, direct and intellectual contribution to the work and approved the manuscript prior to its submission.

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The PNA mouse may be the best animal model of polycystic ovary syndrome

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Polycystic ovary syndrome (PCOS) exerts negative effects on females of childbearing age. It is important to identify more suitable models for fundamental research on PCOS. We evaluated animal models from a novel perspective with the aim of helping researchers select the best model for PCOS. RNA sequencing was performed to investigate the mRNA expression profiles in the ovarian tissues of mice with dehydroepiandrosterone (DHEA) plus high-fat diet (HFD)-induced PCOS. Meanwhile, 14 datasets were obtained from the Gene Expression Omnibus (GEO), including eight studies on humans, three on rats and three on mice, and genes associated with PCOS were obtained from the PCOSKB website. We compared the consistency of each animal model and human PCOS in terms of DEGs and pathway enrichment analysis results. There were 239 DEGs shared between prenatally androgenized (PNA) mice and PCOS patients. Moreover, 1113 genes associated with PCOS from the PCOSKB website were identified among the DEGs of PNA mice. A total of 134 GO and KEGG pathways were shared between PNA mice and PCOS patients. These findings suggest that the PNA mouse model is the best animal model to simulate PCOS.

KEYWORDS

polycystic ovary syndrome (PCOS), bioinformatics, animal model, dehydroepiandrosterone (DHEA), differentially expressed genes (DEG), RNA sequencing (RNA-seq)

Introduction

Polycystic ovary syndrome (PCOS) is an endocrine disorder characterized by hyperandrogenemia, ovulatory dysfunction and metabolic abnormalities and is common among childbearing-aged females (1–4). PCOS has a negative impact on 5-10% of women (2, 5), but its pathogenesis is still unclear (6). Despite the differences in reproductive physiology between experimental animals and humans (7), the limitations related to ethical issues and specifically to obtaining material for research in humans are undeniable. The ease of obtaining and raising experimental animals has also led to a greater use of animal models in basic research on the pathophysiology of PCOS (8, 9).

Various animal models of PCOS have been explored and studied for over 60 years (8). According to the Rotterdam criteria for PCOS diagnosis (2), the main features of PCOS are excess androgens, increased numbers of cystic follicles, and abnormal menstrual cycles (estrous cycles in animal models). The widely used animal models of PCOS include the androgen model (10, 11), estrogen model (12, 13), aromatase inhibitor model (14, 15) and combined models, such as the dehydroepiandrosterone (DHEA) plus high fat diet (HFD)-induced mouse model (16), high-fat high-sugar (HFHS)-induced mouse model (17, 18), etc. Although a variety of PCOS animal models have been established for research purposes, there is still disagreement which model best recapitulates the disease (19, 20). Thus, the choice of an optimal model remains an important issue.

Whether the reproductive characteristics of the model meet the Rotterdam criteria for PCOS diagnosis and their degree of similarity to the characteristics of PCOS patients are the main concerns for researchers in selecting the best model. However, there is still controversy about which model is best because no single animal model exhibits all of the key pathophysiological features of patients with PCOS. Bioinformatics analysis of data from many different studies of PCOS could help us determine how the potential mechanisms cause phenotypic alterations.

Therefore, it is necessary to evaluate PCOS animal models from a novel perspective. In this study, we compared the eight groups of PCOS animal models' data (our data the mRNA expression profile of a DHEA plus HFD-induced PCOS mouse model, and seven groups' data from public databases) with PCOS patients' data to identify the consistency of differentially expressed genes (DEGs) of PCOS and control, respectively. It provides new insights and reliable references for researchers when selecting models.

Materials and methods

The workflow diagram is illustrated in Figure 1.

Animal experiments

All animal studies were approved by the Ethics Committee of Chongqing Medical University. PCOS model

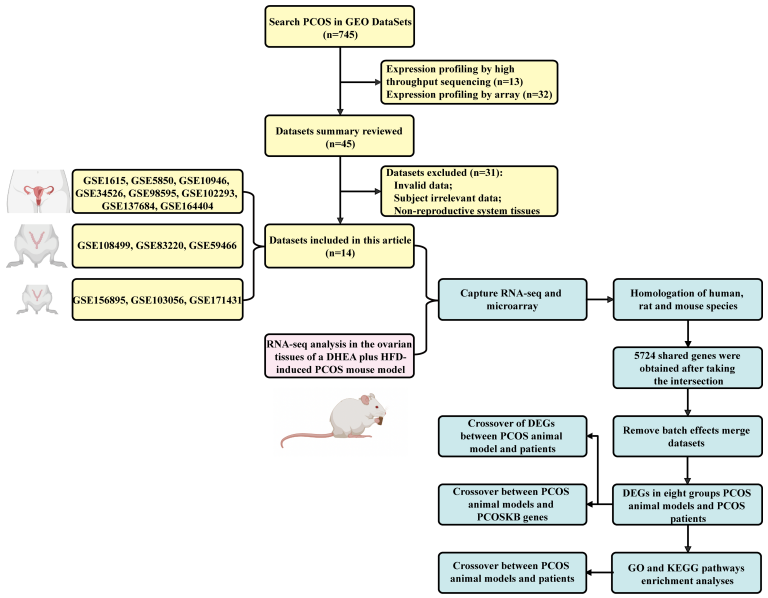

FIGURE 1
The workflow used in this study is illustrated.

mice were established as described by Wang et al. (21). Twenty-one-day-old female C57BL/6J mice were obtained from the animal research center of Chongqing Medical University. All animals were maintained under standard housing conditions (20 °C and 12 h day/night cycles) with free access to water and food according to the institutional guidelines. After four days of acclimatization, the mice were randomly divided into two groups (a control group of 25 mice and a PCOS group of 20 mice). The mice in the PCOS group were injected with DHEA (6 mg/100 g/d) dissolved in 0.1 ml of sesame oil daily and fed a HFD (60% of calories from fat). The control mice were injected with 0.1 ml of sesame oil daily and fed normal chow.

Vaginal smears and estrous cycle determination

The estrous cycle was examined by vaginal smears. Inspections were performed daily at 9:00 a.m. for ten consecutive days before sacrifice. The estrous cycle was determined by analysis of vaginal smears under a microscope. If leukocytes were the predominant cell type, the sample was determined to be in the diestrus stage. If nucleated cells were abundant, the sample was considered to be in the proestrus stage. If cornified squamous epithelial cells were the predominant cell type, the sample was determined to be in the estrus stage. If squamous epithelial cells and leukocytes were abundant, the sample was considered to be in the metestrus stage. Mice already in estrus were sacrificed to eliminate the effect of the estrous cycle on the rest of the experiment.

Hormone assays

The serum testosterone (T) concentrations of the mice were determined using commercial iodine [^{125}I] radioimmunoassay kits (North Institute, Bio-Tech, Beijing, China). The intra- and inter-assay errors among all assays were <10% and 15%, respectively. The sensitivity limit of testosterone was 0.02 ng/mL.

Histological staining

Ovaries were sectioned at 4 μm, with 40 μm discarded between every section, and six sections were collected from each ovary. Sections were stained with hematoxylin and eosin (H&E) according to standard histological procedures and analyzed by conventional light microscopy. The examination was performed by two histologists who were unaware of the source of the material. Follicles were classified according to Kauffman et al. (22).

RNA sequencing analysis in the ovarian tissues of a DHEA plus HFD-induced PCOS mouse model

Ovarian tissues from PCOS and control mice were taken, and total RNA was extracted by the TRIzol method. RNA sequencing (RNA-seq) was conducted by Beijing Allwegene Technology Company Limited (Beijing, China). The cDNA library was constructed by polymerase chain reaction (PCR). RNA-seq was performed using the PE150 sequencing strategy of Illumina's second-generation high-throughput sequencing platform. Poor quality RNA-seq reads and adapters were filtered out. Clean read data were aligned using Tophat2 and Cufflinks software to complete the transcriptome comparison (23).

Acquisition and preparation of data from public databases

Gene expression profiles from PCOS patients were obtained from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/). In this study, the eight datasets from PCOS patients were GSE1615, GSE5850, GSE10946, GSE34526, GSE98595, GSE102293, GSE137684 and GSE168404. Three datasets from rats are GSE108499, GSE83220 and GSEGS59456. Three datasets from mice are GSE156895, GSE103056 and GSE171431. These data are shown in Table 1. Using the exprSet function in the "limma" package (version 3.42.2), the data were normalized to produce the expression matrix. The sample consisted of the PCOS group and the control group. Genes associated with PCOS were obtained from the PCOSKB database (http://pcoskb.bicnirrh.res.in/), a compilation of molecular, biochemical and clinical databases on PCOS (24).

Homologation of human, rat and mouse species

The useMart function in the "biomaRt" R package was used to establish a relationship with the Ensembl BioMart web service, and the datasets were selected by setting the dataset parameter to hsapiens_gene_ensembl, mmusculus_gene_ensembl and rnorvegicus_gene_ensembl (25). The 14 datasets obtained

TABLE 1 Details of microarray and RNA-seq datasets.

Accession ID	Platform	Platform ID	Number of patients	Symbol (N/P)	Organism	Modelling method
GSE1615	Affymetrix HG-U133A	GPL96	9	4/5	Homo sapiens	NA
GSE5850	Affymetrix HG-U133A 2.0	GPL570	12	6/6	Homo sapiens	NA
GSE10946	Affymetrix HG-U133A 2.0	GPL570	23	11/12	Homo sapiens	NA
GSE34526	Affymetrix HG-U133A 2.0	GPL570	10	3/7	Homo sapiens	NA
GSE98595	Affymetrix HG-1_0-st	GPL6244	8	3/5	Homo sapiens	NA
GSE102293	Affymetrix HG-U133A 2.0	GPL570	6	4/2	Homo sapiens	NA
GSE137684	Agilent SurePrint G3 GE 8x60K	GPL17077	12	4/8	Homo sapiens	NA
GSE168404	Illumina HiSeq 2500	GPL16791	10	5/5	Homo sapiens	NA
GSE108499	RiboArray Rat mRNA	GPL24411	12	3/3	Rattus norvegicus	TBT
				3/3		BPA
				3/3		TBT plus BPA
GSE83220	Illumina HiSeq 2500	GPL18694	4	2/2	Rattus norvegicus	HFHS
GSE59456	Affymetrix Rat 230 2.0	GPL1355	8	4/4	Rattus norvegicus	DHT
GSE156895	Illumina HiSeq 2000	GPL13112	5	2/3	Mus musculus	PNA
GSE103056	Affymetrix Mouse 430 2.0	GPL1261	2	1/1	Mus musculus	PNA
GSE171431	Affymetrix MTA-1_0	GPL20258	6	3/3	Mus musculus	DHT
Our data	Illumina NovaSeq	–	6	3/3	Mus musculus	DHEA plus HFD

TBT, tributyltin; BPA, bisphenol A; DHT, dihydrotestosterone; PNA, prenatally androgenized; DHEA, dehydroepiandrosterone; HFD, high fat diet; HFHS, high fat high sugar; NA, not available.

from the GEO database were intersected with our data to obtain the shared genes.

Batch effect removal and consolidation of datasets

The Empirical Bayes (EB) method was used to remove batch effects among different datasets (26). Principal component analysis (PCA) was performed to visualize the results of batch effect removal using EB. Datasets with the same animal model method were merged, while eight datasets of PCOS patients were also merged.

DEGs and pathway enrichment analyzes

For each group of PCOS animal models and PCOS patients, DEGs were screened by comparing the PCOS group with the control group using Student's *t* test. The DEGs were identified according to $P < 0.05$, *t* value > 0 for up-regulated genes and *t* value < 0 for down-regulated genes. To identify the functions of the DEGs, Gene Ontology (GO) enrichment analysis was performed for three categories: biological processes (BP), cellular component (CC) and molecular function (MF). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed to determine the function of DEGs.

Protein–protein interaction network

Protein-protein interaction (PPI) network analysis of the DEGs was conducted using the STRING online biological database (https://string-db.org/) with a threshold value of ≥ 0.4 (medium confidence). PPI networks were constructed and visualized using Cytoscape software (http://cytoscape.org/). The Molecular Complex Detection (MCODE) and CytoHubba plug-ins were utilized to analyze the modules and hub genes of the PPI network in Cytoscape, respectively. The GeneCards database (https://www.genecards.org/) was searched to determine the functions of the hub genes.

Statistical analysis

Student's *t* test and false discovery rate (FDR) were applied in the identification of DEGs. The "sva" package (version 3.34.0) is used for batch effect removal. The "biomaRt" R package (version 2.42.1) was used for homology processing among different species. The "clusterProfiler" R package (version 3.14.3) was used to cluster the DEG enrichment pathways, and the "GOplot" package (version 1.0.2) and "ggplot2" (version 3.3.5) were used to visualize the results of the enrichment analysis. Statistical analysis was performed using R software (version 3.6.3). In the animal experiments, Student's *t* test was used to

analyze the serum testosterone concentration, and GraphPad Prism 9 was used for plotting. All data are presented as the mean ± SD. A two-sided *P* value < 0.05 indicated that the difference was statistically significant.

Results

DHEA plus HFD induced the formation of polycystic ovaries

H&E staining showed increased numbers of atretic and cystic follicles and decreased numbers of corpora lutea in the ovarian tissues of PCOS mice compared with control mice ($P < 0.05$, $P < 0.001$; Supplementary Figures 1A–C). Furthermore, an irregular estrous cycle (Supplementary Figures 1E, F) and elevated serum testosterone levels ($P < 0.001$; Supplementary Figure 1D) were observed in this model compared with the control, which indicated that the PCOS model was successfully constructed. RNA-seq analysis was performed on three ovarian tissue samples in each group of control and PCOS patients, and DEGs were screened out.

Data preprocessing and batch effect removal

The homology analysis of the datasets is shown in Supplementary Table 1, and 5724 shared genes were obtained from the intersection. As shown in Figures 2A, C, E, there were batch effects among the different datasets. The EB method was used to remove batch effects and cluster all samples together, as shown in Figures 2B, D, F. The datasets generated using the same model approach were merged, and the eight datasets from PCOS patients were also merged. Then, data from eight groups of PCOS animal models and PCOS patients were obtained. The model approach, metabolic changes and reproductive abnormalities of the eight groups of PCOS animal models are shown in Table 2.

Intersection of DEGs in PCOS animal models and patients

A total of 791 DEGs, including 509 up-regulated and 282 down-regulated genes, were identified in PCOS patients compared with controls (Supplementary Table 2). The DEGs from the eight groups of animal models of PCOS was compared with those of PCOS patients (Figure 3). The results showed 239 shared genes between prenatally androgenized (PNA) mice and PCOS patients, including 99 upregulated and 77 downregulated DEGs, as shown in Figure 3F. DHT-induced PCOS rats and mice shared fewer DEGs with PCOS patients than PNA mice. The 188 and 107 overlapping genes in DHT-induced PCOS rats and mice are shown in Figures 3E, G respectively. The top five DEGs shared by PNA mice and PCOS patients were *Atg2a*, *Tapbp*, *Tagln*, *P4ha1* and *Amz2*, as illustrated in box plots in Figure 4.

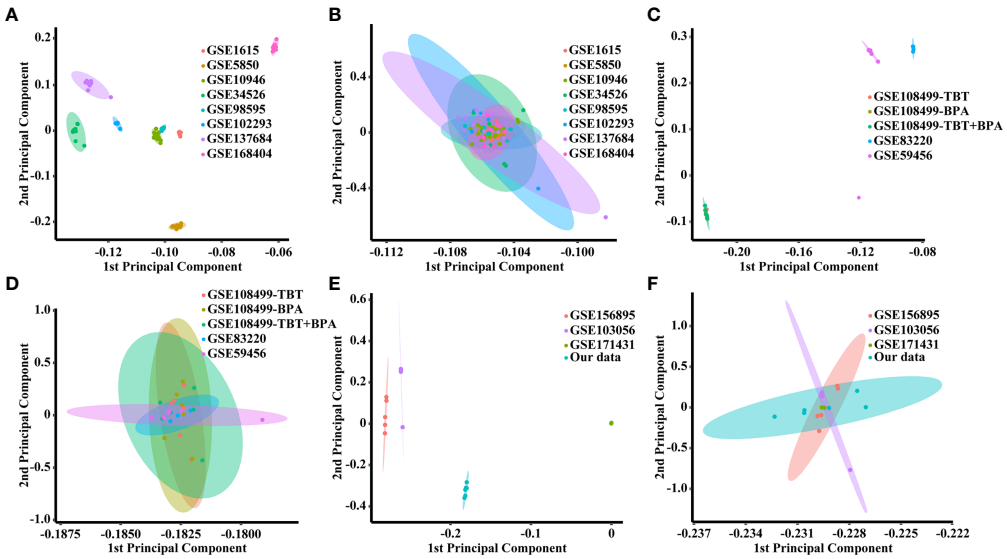

FIGURE 2
PCA score plots for classifying samples from different datasets. **(A)** Human datasets before batch effect removal. **(B)** Human datasets after batch effect removal by the EB method. **(C)** Rat datasets before batch effect removal. **(D)** Rat datasets after batch effect removal by the EB method. **(E)** Mouse datasets before batch effect removal. **(F)** Mouse datasets after batch effect removal by the EB method.

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TABLE 2 Information about the eight groups PCOS animal models.

Method	Specie	Treatment time	Estrus cycle	CL	AF	Atr F	CF	FSH	LH	T	E2	Body weight	Ovary body index	Abnormal metabolism	Reference
TBT	SD rats	16d	irregular	↓	↓	↑	↑	NS	NA	NA	NA	NA	↓	lipid metabolism disorder	(27)
	Wistar dams	30d	irregular	NA	NA	↑	↑	NA	NA	↑	↓	↑	↓	abnormal lipid accumulation	(28)
BPA	SD rats	16d	irregular	↓	↓	↑	↑	NA	NA	NA	NA	NA	↓	lipid metabolism disorder	(27)
	SD rats	10d	irregular	↓	↓	↑	↑	NS	↑	↑	↑	NS	↓	NA	(29)
TBT plus BPA	SD rats	16d	irregular	↓	↓	↑	↑	NA	↑	↑	NA	NA	↓	lipid metabolism disorder	(27)
HFHS	SD rats	11w	irregular	↓	NA	↑	↑	NS	NS	NA	NA	↑	NS	insulin resistance	(18)
	SD rats	14w	irregular	↓	NA	NS	↑	NA	↓	↑	↓	↑	NS	insulin resistance altered steroidogenesis	(17)
DHT	C57BL/6J mice	70d	irregular	↓	↓	↑	↑	↓	↑	NS	↑	↑	NS	hypercholesterolemia	(30)
	SD rats	90d	irregular	↓	↓	↓	↑	↑	↑	↓	↓	↑	↓	insulin resistance	(31)
PNA	ICR mice	gestational days 16–18	irregular	↓	↑	↑	↑	NA	↑	↑	↑	↑	↑	abnormal folate one-carbon metabolism	(32)
	SD rats	gestational days 16–19	irregular	↓	↓	↑	↑	NA	↑	↑	↑	NS	NS	insulin resistance	(33)
DHEA plus HFD	SD rats	20d	irregular	↓	↓	NA	↑	NS	↑	↑	↑	↑	NS	lipid metabolism disorder impaired glucose tolerance	(16)
	C57BL/6J mice	20d	irregular	↓	NA	↑	↑	↑	↑	↑	NS	↑	NS	lipid metabolic disorders	(21)

SD, Sprague-Dawley; CL, corpora lutea; AF, antral follicles; Atr F, atretic follicles; CF, cystic follicles; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; T, testosterone; NA, not available; NS, no significance ; ↓, reduce;↑, increase.

Intersection between DEGs of PCOS animal models and genes from PCOSKB

The above results were confirmed when comparing the PCOS-related genes obtained from the PCOSKB database with the DEGs in the eight groups of PCOS animal models Figure 5. From the PCOSKB website, 9,977 genes associated with PCOS were obtained. Of these PCOS-related genes, 1113 were identified in PNA mice (Figure 5F). The next highest number of overlapping genes were observed in DHT-induced PCOS rats and mice, with 931 and 611 DEGs shared with PCOS-associated genes, respectively (Figures 5E–G).

Intersection of pathway enrichment analyzes in PCOS animal models and patients

A total of 791 DEGs from PCOS patients were selected for GO and KEGG pathway enrichment analyzes, and 185 pathways were obtained. The comparison showed that 134 of the pathways enriched in PNA mice were the same as those enriched in PCOS patients (Figure 6F and Supplementary Table 3). The top ten pathways of PNA in ascending order of FDR value are shown in Figure 7. PCOS patients shared 105 pathways with DHT-induced rats and 40 pathways with DHT-induced mice. The top ten pathways selected from each model are shown in Supplementary Figures 2, 3.

PPI network analysis of DEGs

The PPI networks based on the intersection of DEGs between PCOS animal models and patients were downloaded from the STRING website and visualized using Cytoscape software. The PPI network for the PNA mice is shown in Figure 8A, which was composed of 211 nodes and 492 edges. The top module, with a maximum rating of 7.5, is shown in Figure 8B, created by the MCODE plug-in. The ten most highly connected genes in this PPI network were selected as hub genes (*Cdc6*, *Rpa1*, *Rfc6*, *Mcm5*, *Prim2*, *Rfc2*, *Orc5*, *Psma3*, *Msh2* and *Psmd14*) in Figure 8C. DHT-induced rats and mice were also individually submitted to PPI network analysis in Supplementary Figures 4 and 5, respectively.

Discussion

Although there are many studies involving patients with PCOS, animal models have been used as the basis of

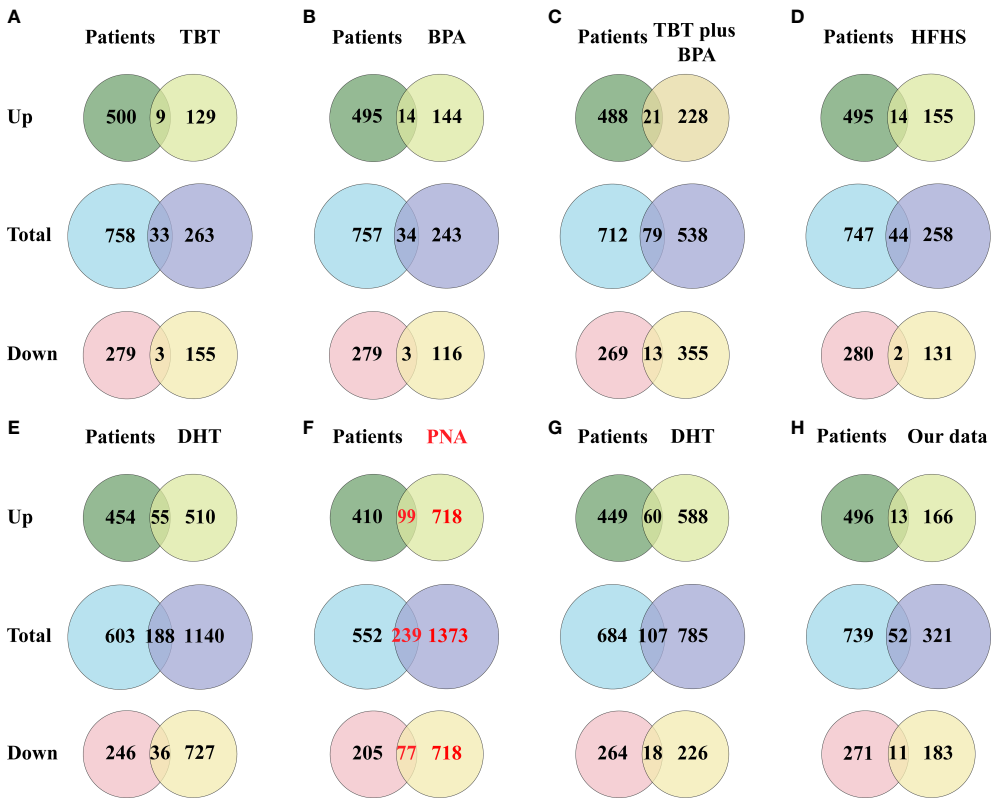

FIGURE 3
Venn diagram showing the intersection of DEGs (including up-regulated genes and down-regulated genes) between the PCOS animal modeland patients. **(A)** DEGs of the TBT-induced PCOS rat model. **(B)** DEGs of the BPA-induced PCOS rat model. **(C)** DEGs of the TBT plus BPAinduced PCOS rat model. **(D)** DEGs of the HFHS-induced PCOS rat model. **(E)** DEGs of the DHT-induced PCOS rat model. **(F)** DEGs of the PNA PCOS mouse model. **(G)** DEGs of the DHT-induced PCOS mouse model. **(H)** DEGs of the DHEA plus HFD-induced PCOS mouse model.

pathophysiological research (8). More than 30 animal models of PCOS have been reported, but most of the models express only certain PCOS phenotypes or exhibit additional relevant features that are generally beyond the scope of the syndrome (9). In our study, the modelling approach, metabolic changes and reproductive abnormalities of the eight models used in PCOS research were taken into consideration (Table 2). Because the DHEA plus HFD-induced animal model is highly similar to PCOS in terms of metabolism and phenotypic characteristics (21), we used this method to establish a PCOS mouse model. We further employed RNA-seq analysis of ovarian tissue from PCOS patients and controls and included these results for analysis together with the seven datasets mentioned above.

We analyzed the consistency between eight PCOS animal models and patients at the mRNA level in terms of DEGs obtained from the GEO database, PCOS-related genes obtained from the PCOSKB website and pathways obtained by GO and KEGG pathway enrichment analyzes. This study confirmed that the PNA mouse model can best simulate patients in terms of DEG and pathway enrichment analyzes, followed by the DHT-induced PCOS rat and mouse models. The PNA model was prepared by subcutaneous or intramuscular injection of testosterone, testosterone propionic acid, DHT, and DHT propionic acid at different doses and different stages of pregnancy, from early-to-mid to late gestation (19), and the offspring rats were used as subjects in this study.

Based on the data of PNA mice in GSE103056 and GSE156895 from the relevant literature (34, 35), the subcutaneous injection of 70 μl of sesame oil alone or containing 350 μg of DHT per day on days 16-18 of gestation in females was used for model induction, and the offspring of the treated mice were considered the target PNA mice. This leads to the conclusion that, among the existing modelling methods, DHT treatment may be the optimal method for constructing rodent models of PCOS. Maternal treatment of study subjects may be a better model than direct DHT-induced PCOS in rats and mice. PCOS is known to have a tendency to run in families (36) and is a highly complicated genetic disorder (37). Hyperandrogenic gestation in the uterus can lead to the development of PCOS in adult offspring (38, 39), which may

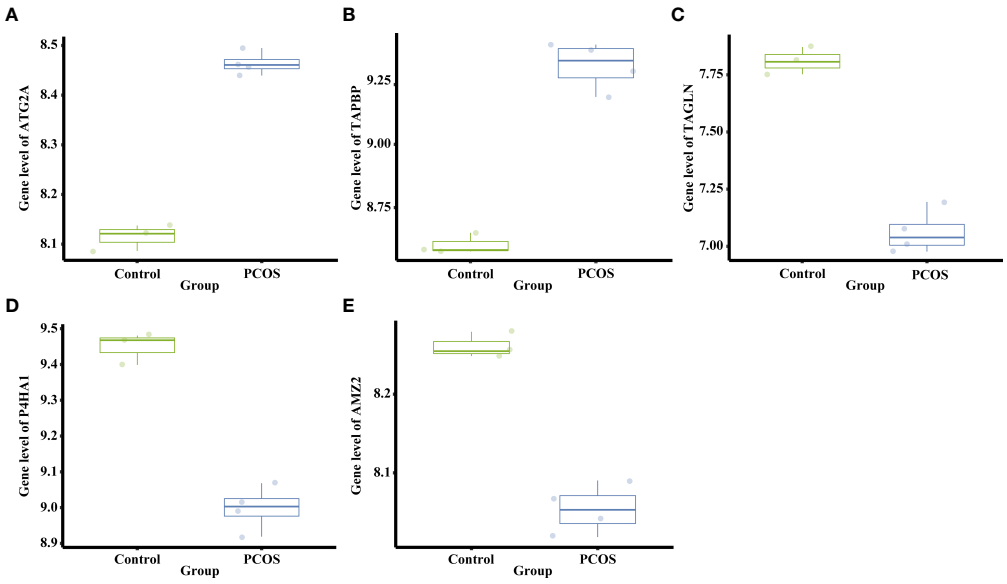

FIGURE 4
Differential mRNA expression of the top five DEGs shared between PNA mice and PCOS patients. **(A)** The gene level of *Atg2a*. **(B)** The gene level of *Tapbp*. **(C)** The gene level of *Tagln*. **(D)** The gene level of *P4ha1*. **(E)** The gene level of *Amz2*.

account for the superiority of PNA mice over DHT-induced rats and mice. This result also suggests that genetic factors influencing PCOS should be considered when selecting an animal model of PCOS, and it also provides clues for subsequent mechanistic studies.

The top ten DEGs of PNA mice, including *Axl, Atg2a, Klhl5, Mafb, Brix, Gpc3, Cdc42ep3, Prkag2, Porcn* and *Haus5*, were associated with autophagy, immunity, cytokinesis, cytoskeleton and inflammation. *Atg2a, TAPBP, Tagln, P4ha1, Amz2, Arhgdib, Cybrd1, Tmem185b, Prim2* and *Ets2* are the top ten DEGs with concordance in PCOS patients and PNA mice. These genes are associated with apoptosis, senescence, inflammation, autophagy and cell signaling. There is evidence that the rate of granular cell apoptosis is significantly increased in the antral follicles of women with PCOS compared to normal controls (40, 41). Apoptosis of follicular granulosa cells may therefore also be involved in the

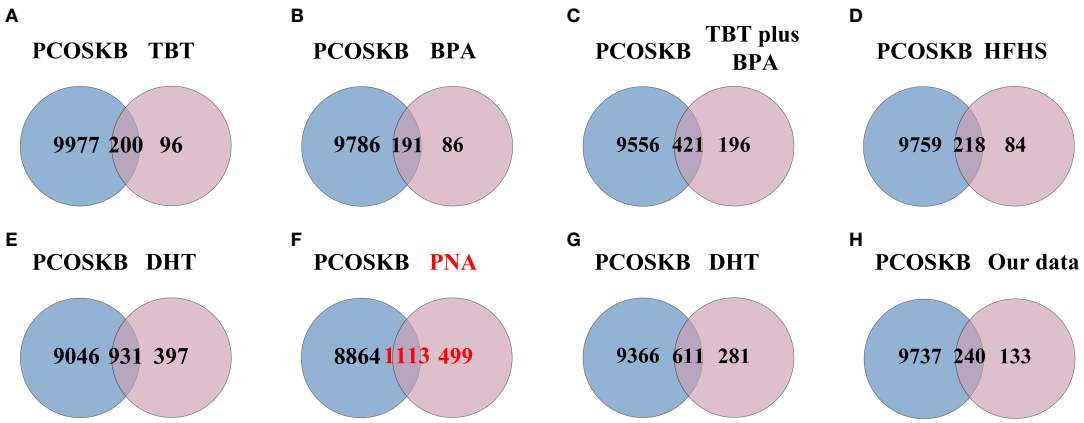

FIGURE 5
Venn diagram showing the intersection of PCOS animal models and genes from PCOSKB. **(A)** DEGs of the TBT-induced PCOS rat model. **(B)** DEGs of the BPA-induced PCOS rat model. **(C)** DEGs of the TBT plus BPA-induced PCOS rat model. **(D)** DEGs of the HFHS-induced PCOS rat model. **(E)** DEGs of the DHT-induced PCOS rat model. **(F)** DEGs of the PNA PCOS mouse model. **(G)** DEGs of the DHT-induced PCOS mouse model. **(H)** DEGs of the DHEA plus HFD-induced PCOS mouse model.

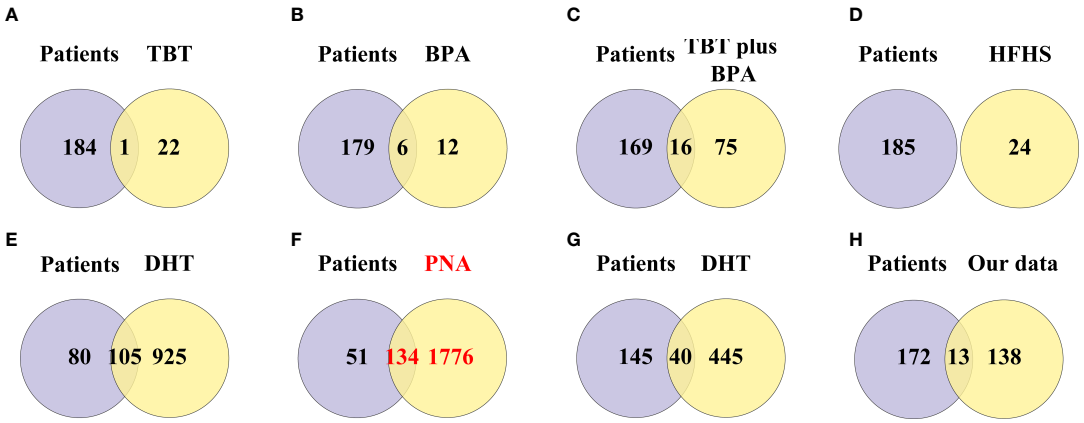

FIGURE 6
Venn diagram showing the intersection of pathway enrichment analyzes between PCOS animal models and patients. **(A)** GO and KEGG terms enriched in the TBT-induced PCOS rat model. **(B)** GO and KEGG terms enriched in the BPA-induced PCOS rat model. **(C)** GO and KEGG terms enriched in the TBT plus BPA-induced PCOS rat model. **(D)** GO and KEGG terms enriched in the HFHS-induced PCOS rat model. **(E)** GO and KEGG terms enriched in the DHT-induced PCOS rat model. **(F)** GO and KEGG terms enriched in the PNA PCOS mouse model. **(G)** GO and KEGG terms enriched in the DHT-induced PCOS mouse model. **(H)** GO and KEGG terms enriched in the DHEA plus HFD-induced PCOS mouse mod.

abnormal development of follicles associated with PCOS (42). PCOS is an inflammatory disease characterized by persistent nonspecific low-grade inflammation (43, 44). It has been shown that dietary induction stimulates the inflammatory response of monocytes in women with PCOS (45). There is a genetic basis for the inflammation observed in PCOS (46). Interestingly, *Atg2a* is shared by PNA mice and PCOS patients, but no studies of this gene relevant to PCOS are available. Meanwhile, *Klhl5, Mafb, Brix, Cdc42ep3, Haus5, TAPBP, Tagln, P4ha1, Arhgdib, Tmem185b, Prim2* and *Ets2* are all mentioned on the PCOSKB website. The GO and KEGG pathway enrichment analyses in the PNA mice showed that the top ten pathways in ascending order of FDR values were associated with chromosomes, lipid metabolism, membranes, and the mitotic cell cycle. A total of 134 GO and KEGG pathways enriched in PNA mice were shared with PCOS patients. The top ten pathways of PNA are shown in Figure 7 and correlate with cell

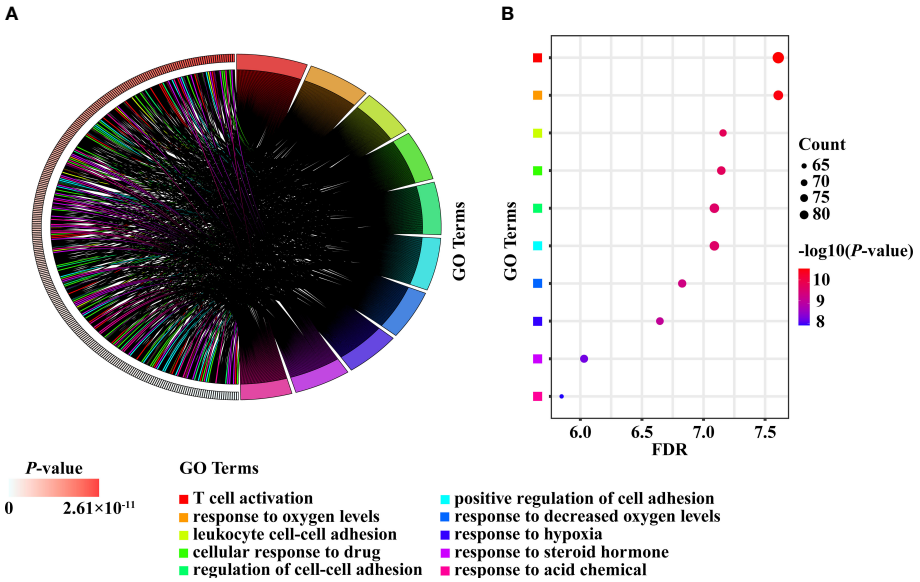

FIGURE 7
The top ten enriched pathways shared between PCOS patients and PNA mice. **(A)** Chord diagram. **(B)** Bubble diagram.

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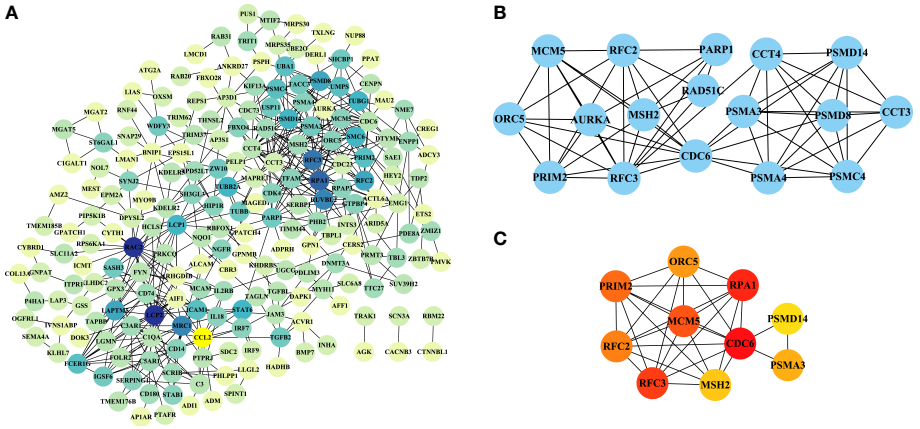

FIGURE 8
PPI network analysis of DEGs in PNA mice. **(A)** A PPI network with 211 nodes and 492 edges was constructed using Cytoscape software. **(B)** The top module results of MCODE PPI analysis. **(C)** The ten genes with the greatest linkage were selected for treatment as hub genes.

adhesion, T cells, hypoxia, steroid hormones and acidic chemicals. *Cdc6*, *Rpa1*, *Mcm5*, *Prim2*, *Orc5* and *Msh2* were associated with DNA replication in the PPI network analysis of PNA mice, which may also explain the greater likelihood of PCOS in the PNA mouse model. There is a chance that PNA mice may share not only reproductive but also immunity and hypoxia-related characteristics with PCOS patients.

Alcam and *Klf15* are in the top ten DEGs of both DHT-induced rat models and PCOS patients. The GeneCards website shows that *Alcam* and *Klf15* are associated with immunity and DNA replication, respectively. *Sgta* also possesses concordance, but studies have shown that *Sgta* is nominally significant (47) and has the potential to modulate androgen receptor signalling (48). In a DHT-induced PCOS rat model, GO and KEGG pathway enrichment analyzes showed that the top ten pathways were associated with metabolic processes, glandular development and intrauterine embryonic development through a variety of substances. As in PCOS patients, the top ten pathways were associated with hypoxia and metabolism of multiple substances. Glandular development and response to steroid hormones are present in both of the abovementioned groups.

Mgst2, Cdkn1b, Tst, Mtmr3, Vdac1, Lpp, Pdhb, Pfkp, Hmga2 and *Bckdhb* are the top ten DEGs in the DHT-induced mouse model, and the GeneCards website shows that they are associated with inflammation, mitochondria and metabolism. One study suggests that *Lpp* may be a new candidate gene for PCOS (49). *Pfkp* was among the top ten DEGs of both the DHT-induced mouse model and PCOS patients. A low level of *Pfkp* expression in cumulus oocyte complexes was found in Chinese patients with PCOS, suggesting a potential link between cumulus oocyte complexes and reduced glycolysis in women with PCOS (50). Among them, *Mgst2, Cdkn1b, Lpp, Pdhb* and *Bckdhb* are all listed on the PCOSKB website. Pathway enrichment analysis in a DHT-induced mouse model revealed that the top ten results were associated with hypoxia, ketones, amino acids, amine metabolism, hematopoietic cell differentiation and regulation. Consistent with that in PCOS patients, the top ten pathways in DHT-induced mice showed correlation with mitochondria, hypoxia, vesicle lumen and amino acid metabolism.

Meanwhile, we observed that the DEGs and the enrichment pathways were different when lean and obese PCOS patients were compared with controls (51). The differentially enriched pathways of lean and obese PCOS patients included mitochondrial gene expression, cell adhesion and signal transduction, cell migration, ubiquitin catabolic process, inflammation and immune response. Our findings differ from those of *Idicula-Thomas* et al. Among them, mitochondria, cell adhesion, immunity and inflammation are consistent, but we also found that in the top ten pathways of the three better models, metabolism of various substances and hypoxia are also very important. This enriches the current understanding of PCOS. Although we did not consider the effect of obesity on PCOS in this study, we believe that this factor should be taken into account when constructing models.

In addition, several novel PCOS loci have been identified in genome-wide association studies (GWASs) in China and Europe (52–55). It has been shown that 14 independent loci, including ERBB4, THADA, and KRR1, are significantly associated with the risk of PCOS, and 11 of these loci may be associated with the endocrine and metabolic pathways in PCOS (56). We looked for novel PCOS loci in the DEGs of eight groups of animal models and found that the trend of *Irf1* expression was the same in the DHT rat model as in humans. The novel *Mapre1* locus showed the same trend in the PNA mouse model and in humans. Therefore, when studying PCOS candidate genes, it is

necessary to compare the models with humans at the mRNA level to select an appropriate model.

However, some limitations exist in our study. First, it is important to mention that the GEO dataset for PCOS includes only rats and mice. There are no reports on other mammalian models, such as primates, which are more similar to humans. Second, the sample size of each model was relatively small; however, more data are expected to be uploaded to these public databases.

Conclusion

In conclusion, the current study shows that among selected mouse and rat models, the PNA mouse model has the best consistency with PCOS patients at the mRNA level. Among the existing modelling methods, treatment with DHT may be the optimal method for constructing rodent models of PCOS. This study provides a new perspective for the evaluation of PCOS models and serves as a reference for researchers to select a more suitable animal model of PCOS.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The datasets used and analyzed during thecurrent study are available in the GEO (https://www.ncbi.nlm.nih.gov/geo/) repository. The dataset of DHEA plus HFD mouse model analyzed are not publicly available as it is still under study but are available from the corresponding author on reasonable request. Requests to access these datasets should be directed to Meijiao Wang, meijiaowang@cqmu.edu.cn.

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Committees of Chongqing Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by The Ethics Committees of Chongqing Medical University.

Author contributions

JR and GT conceived the study. MW performed the animal experiments. JR analyzed the data and wrote the manuscript. WL and XR revised the figures and tables. MW, BX, JT, QP, and YW critically revised the manuscript. BX and MW made significant contributions to the conception and coordination. The authors read and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.950105/full#supplementary-material

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Possible roles of exercise and apelin against pregnancy complications

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The prevalence of maternal obesity during pregnancy is associated with the risk of gestational diabetes, preeclampsia, and cardiomyopathy. Environmental factors such as active lifestyles and apelin may lead to beneficial changes. In rats, apelin and exercise (45 to 65% VO_{2max} for 6 to 9 weeks) during pregnancy increase brown adipose tissue (BAT) proteins such as Cidea, Elovl3, UCP1, PRDM16, and PGC-1α in males and females fetuses, while white adipose tissue (WAT) is reduced. In humans and animals, apelin and exercise stimulate the expression of the glucose transporters (GLUT1/2/4) in the muscle and adipose tissue through the PI3K/Akt and AMPK pathways. Hence, exercise and apelin may are known as regulators of energy metabolism and be anti-obesity and anti-diabetic properties. In mice, exercise also creates a short-term hypoxic environment in the pregnant mother, activating HIF-1, VEGF, and VEGFR, and increasing angiogenesis. Exercise and apelin also increase vasodilation, angiogenesis, and suppression of inflammation through the L-arginine/eNOS/NO pathway in humans. Exercise can stimulate the ACE2-Ang-(1-7)-Mas axis in parallel with inhibiting the ACE-Ang II-AT1 pathway. Exercise and apelin seem to prevent preeclampsia through these processes. In rats, moderate-intensity exercise (60 to 70% VO_{2max} for 8 weeks) and apelin/APJ also may prevent pathological hypertrophy in pregnancy by activating the PI3K/Akt/mTOR/p70S6K pathway, PI3k-Akt-ERK1/2-p70S6K pathway, and the anti-inflammatory cytokine IL-10. Since pre-clinical studies have been more on animal models, future research with scientific guidelines should pay more attention to human specimens. In future research, time factors such as the first, second, and third trimesters of pregnancy and the intensity and duration of exercise are important variables that should be considered to determine the optimal intensity and duration of exercise.

KEYWORDS

exercise, pregnancy, blood pressure, insulin sensitive, myocardial hypertrophy, Preeclampsia

Introduction

Pregnancy is a dynamic and organized process for the development of one or more babies (1). Maternal obesity (body mass index (BMI) of 29 kg/m2 or more) has now become a public health concern that may affect the health of mothers and children as well as their longevity (2, 3). Maternal obesity and a sedentary lifestyle also stop the growth of fetal brown fat by impairing its thermogenesis function in the later life phase (4, 5). Common risks associated with maternal obesity in pregnancy include gestational diabetes, vascular disorders such as preeclampsia, and cardiovascular diseases (3, 6–8). Gestational diabetes or insulin resistance during pregnancy is one of the main obstacles to achieving mother and child health. Approximately 9 to 25 percent of pregnancies worldwide are affected by acute long-term complications of the disease (9). On the other hand, preeclampsia is the most common gestational hypertension, affecting 5 to 7% of pregnant women worldwide. Preeclampsia accounts for 20% of all pregnancy deaths, leading to increased preterm birth and fetal growth retardation (1, 10). Cardiomyopathy during pregnancy is the leading cause of non-obstetric mortality. The most common cardiomyopathies in pregnancy are hypertrophic and dilated cardiomyopathy. Hypertrophic cardiomyopathy most often develops one month before or after childbirth, while dilated cardiomyopathy often develops before or during the second trimester (11, 12). Most serious heart accidents (66%) occur in the prenatal period. Almost half of the serious heart events (49%) are preventable and most preventable serious heart events (74%) depend on disease management factors (13). In contrast, numerous studies have suggested moderate-intensity exercise to prevent common pregnancy complications such as maternal obesity, gestational diabetes, vascular disorders, and heart problems in mice. It is reported that exercise (40 to 65% of VO2max for 8 weeks) during pregnancy is beneficial for both mother and fetus to combat obesity in mice (4). In obese pregnant mice, daily exercise on a treadmill reduces weight gain, lowers serum glucose and fat, and increases insulin sensitivity (4, 14). In humans, exercising (40 to 60% of VO2max) during pregnancy reduces the risk of gestational hypertension and preeclampsia and increases energy consumption (15, 16). The fetal and maternal cardiac response to aerobic, anaerobic, and circular exercise activities has been reported to be safe and beneficial during pregnancy in humans and animals (17–19). In general, exercise in obese mothers not only affects the function of fat tissue, but also affects muscle metabolism, glucose homeostasis, preeclampsia, and heart function (4, 14–19). It seems that one of the reasons for these improvements is due to the placentokines such as apelin, leptin, apela, irisin, and adiponectin, but the placentokine we review in this study is apelin. The release of apelin due to exercise also is more than the other placentokines (15). Apelin also is known as exerkine (exercise + cytokine) and placentokine (placenta + cytokine) (15, 20, 21). Apelin as an exerkine reverses obesity-related placental dysfunction by increasing mitochondrial biogenesis in mice (22). It seems that aerobic training (4 times a week with an intensity of 60-70% of the maximum heart rate for 45-60 minutes) affects improving insulin sensitivity through the regulation of apelin (23). A relationship in studies has been reported between swimming (6 days per week for 9 weeks) and apelin expression in rat heart and vessels to reduce blood pressure (24). Hence, exercise (60 to 70% of VO2max, 55 min, 3 days/week, for 8 weeks) may be associated with reduced arterial stiffness by increasing plasma apelin levels in middle-aged adults (25). In addition, exercise (56 to 61% of VO2max, 5 days/week for 3 weeks) may improve myocardial tolerance to doxorubicin-induced cardiotoxicity through apelin by inhibiting oxidative stress and up-regulating antioxidants in rats (26). On the other hand, Apelin precursors and its receptor (APJ) in cattle, humans, rats, and mice show 76 to 95% homology (1). High homology apelin in mammals appears to play similar roles in different species. In pregnant women, the apelin-APJ system changes may promote oxidation of fatty acids, glucose uptake, angiogenesis, vasodilation, hypotension, myocardial contraction, diuresis, and stress response (1). Therefore, in this study, the direct role of apelin through maternal exercise on common pregnancy complications such as obesity, gestational diabetes, gestational hypertension, and cardiac hypertrophy is investigated. This study was conducted to investigate the role of exercise and apelin during pregnancy and also to find effective mechanisms for pregnancy

Abbreviations: BMI, body mass index; APJ, apelin receptor; AT1, angiotensin II receptor; WAT, white adipose tissue; BAT, brown adipose tissue; TG, triglycerides; FFA, free fatty acids; C/EBPβ, CCAAT-enhancer-binding protein; PPARγ, peroxisome-proliferator-activated receptor-γ; PRDM16, PR domain-containing protein 16; PGC1α, peroxisome proliferator-activated receptor-gamma coactivator 1-alpha; UCP1, uncoupling protein 1; AKT, protein kinase B; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; SNS, central nervous system; HSL, hormone-sensitive lipase; N, norepinephrine; PKC, protein kinase C; RyR1, ryanodine receptor; PLP, phospholamban; SLN, sarcolipin; UCP3, uncoupling protein 3; GLUT4, glucose transporter 4; IRE1α, inositol-requiring enzyme 1α; JNK, Jun N-terminal Kinase; PI3K, phosphatidylinositol 3-kinase; AMPK, AMP-activated protein kinases; ACC, acetyl CoA carboxylase; CoA, malonyl-coenzyme A; mtDNA, mitochondrial DNA; CVD, cardiovascular disease; RAS, renin-aldosterone-angiotensin; sFIt-1, soluble fms-like tyrosine kinase 1; VEGFR, vascular endothelial growth factor receptor; HIF-1, hypoxia-inducible factor 1; RUPP, uteroplacental perfusion pressure; VSMCs, vascular smooth muscle cells; eNOS, nitric oxide synthase; NO, nitric oxide; MDA, malondialdehyde; VEGF, vascular endothelial growth factor; ACE, Angiotensin-converting enzyme; Ang II angiotensin II; ACE2, enzyme converter angiotensin 2; FGF-2, fibroblast growth factor 2.

complications such as obesity, gestational diabetes, preeclampsia, and cardiac hypertrophy.

Apelin and its receptor (APJ)

Apelin is available in various isoforms, including apelin-36, apelin-17, apelin-13, apelin-12, and pyroglutamated [pyr1] apelin-13. Apelin was initially proposed as an endogenous ligand for the angiotensin II protein J (APJ) receptor (27–29). APJ has about 54% homology to angiotensin II receptor (AT1), but angiotensin II cannot bind to it. All apelin isoforms with different biological strengths can bind to APJ (30). Apelin-13 is a high affinity for APJ receptors and apelin-17 and [pyr1] apelin-13 is predominant in plasma (29, 31). Apelin and APJ are present in the muscle, adipocyte, heart, kidneys, and placenta syncytiotrophoblasts (32). Apelin and APJ binding to G protein are expressed in syncytiotrophoblasts for the transport of human placental nutrients (33). Apelin mRNA expression in the placenta is about 10 times that of adipose tissue (34). While apelin in placental villi is 20 times adipose tissue (33). The placenta releases significant levels of apelin, and apelin levels in the fetal circulation are higher than in mothers (1). Decreased levels of apelin in the infant's circulatory system occur rapidly after birth because the placenta is the source of apelin for the fetus (1). Apelin is highly expressed in the mammary gland during lactation and is positively regulated by the mother's high-fat diet. Apelin expression in the mammary gland is positively correlated with breast milk apelin and maternal insulin. Apelin levels of breast milk increase with BMI (35). It is reported that apelin and APJ play a role in metabolic diseases, glucose metabolism, atherosclerosis, cardiovascular diseases, oxidative stress, obesity, and pregnancy (32, 36, 37). For example, a negative correlation has been reported between apelin and ox-LDL during pregnancy. In addition, the level of apelin is shown lower in pregnant women, while BMI and serum lipids are significantly high (36). In mice, apelin also increases the level of brown fat proteins, mitochondrial biogenesis, and placental vasculature (22). There is evidence that apelin has a positive effect on fetal BAT development and offspring metabolic health in mice (5). Therefore, apelin appears to be involved in lipid metabolism during pregnancy (38). Apelin has been also reported to be a good candidate for improving insulin sensitivity (31). Studies showed that apelin significantly ameliorates preeclampsia symptoms, impaired endothelial nitric oxide synthase/nitric oxide signaling, and reduces oxidative stress activation in mice (39). In addition, it is reported that apelin is very important for the formation of the fetal cardiovascular system and early placental development, increasing fetal angiogenesis and energy homeostasis in mice (1). Hence, considering the role of apelin in preventing pregnancy complications, in the continuation of the study, we will look at the effects of apelin on markers of pregnancy obesity, gestational diabetes, pregnancy blood pressure, and pregnancy cardiac hypertrophy.

Association of gestational obesity with white and brown adipose tissue and apelin

Obesity has become an epidemic worldwide due to lack of exercise and overeating, rising from 21.5% to 33.3% in recent years (5, 40). The prevalence of maternal obesity during pregnancy has been reported to be 7-25% and is associated with high birth weight and increases the risk of being overweight in the future (2, 41). Overweight and obesity in pregnant mice predispose their children to obesity and metabolic diseases (42). In obese people, adipose tissue is composed of white adipose tissue (WAT) and brown adipose tissue (BAT). BAT is located in certain places and is the main site of non-shivering thermogenesis in mammals, while WAT collects triglycerides and is responsible for storing metabolic energy (41, 43). WAT reduces metabolic activity due to the lack of thermogenic genes such as PGC-1α and uncoupling protein 1 (UCP1) (44–46), increases lipogenesis during extra calories, and ensures the breakdown of triglycerides (TG) during energy restriction to fuel other organs (46). Increased volume and number of fat cells in WAT is the basis of obesity (47, 48), while brown fat cells lead to decreased due to decreased cellular energy sensor AMP-activated protein kinases (AMPK) and increased inflammatory factors TNF-α (40, 41, 49–51). Hence, maternal obesity in mice impairs the growth of fetal BAT by impairing the thermogenic function of brown fat (5). However, WAT turns into beige or brite cells in response to various stimuli (such as cold and exercise). Beige cells are characterized as thermogenic and brown fat due to high mitochondrial and UCP1 content. While the expression of UCP1 in human WAT is much lower than BAT (52). Therefore, beige cells are found in WAT and burn energy to create heating (53). Browning of beige cells occurs in response to various stimuli such as exposure to chronic cold, caloric restriction, exercise, maternal lifestyle, nutrition, and apelin administration (41, 52). Brown and beige fat cells use free fatty acids (FFA) for mitochondrial beta-oxidation (46, 53). Enhancing the thermogenic function of BAT and beige fat cells eliminates excess energy and leads to the prevention of obesity and metabolic diseases (53). In obesity, apelin appears to act to inhibit adipogenesis through negative feedback at the autocrine level, as obesity increases apelin and APJ secretion. While the inhibition of apelin leads to obesity and obesity increases with the lack of apelin signaling (54). In other words, in obesity, the formation of brown fat is suppressed and the reduction of excess energy consumption in WAT is intensified. On the other hand, apelin expression is increased in WAT and its plasma level is increased in obesity. Apelin activates PI3K/Akt and AMPK signaling in brown preadipocytes (52). Hence, apelin creates a

positive feedback loop to increase the differentiation of brown fat cells and neutralize the adipogenesis of brown disrupted by inflammatory agents. It has been reported that the weight of WAT is significantly reduced in apelin-treated rats, while the weight of BAT is similar to that of the control group. However, the level of brown fat marker proteins in WAT (PRDM16, COX1, and UCP1) increases in mice treated with apelin (52). Hence, apelin is known as a regulator of energy metabolism and has anti-obesity and anti-diabetic properties (52).

The possible effect of apelin on adipose tissue differentiation in obese pregnant women

In rats, apelin mRNA in WAT increases 2.2-fold on day 7 of gestation and returned to baseline on day 14, and remained at baseline during pregnancy (55). Apelin mRNA expression in BAT during pregnancy and lactation is similar to WAT. The reason for the increase in early pregnancy may be related to fat accumulation (55). Hence, appears apelin mRNA levels and apelin expression are increased during the differentiation of fat cells (56, 57). On the other hand, plasma apelin levels are significantly higher in obese patients than in normal individuals (56). In addition, plasma apelin, placental apelin, and APJ expression all increase in obese pregnant rodents (33, 58, 59). However, it is reported that plasma apelin is no different in obese women (BMI = 35.8) with heavy infants than in normal women (BMI = 23.1) with normal-weight infants (33). It has also been reported that in obese mice, the expression of apelin and APJ in the placenta increases, and the concentration of apelin in the mammary glands is higher in obese women and insulin-resistant obese women than in the control group. Also, more APJ mRNA expression was reported in obese women than in normal women. While maternal and neonatal apelin secretion is reduced in obesity and insulin-resistant obesity compared with controls (59). These differences in the level of the apelin are due to the time, dose, and place of measurement during pregnancy. But to answer these differences, it is better to focus on the function of the apelin on adipose tissue.

Apelin stimulates transcription factors such as CCAAT-enhancer-binding protein (C/EBPβ) and peroxisome-proliferator-activated receptor-γ (PPARγ) in the early stages of adipocytes differentiation. While apelin increases transcription factors such as PR domain-containing protein 16 (PRDM16) and peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC1α) for mitochondrial biogenesis and thermogenesis (40, 52, 54). Brown cell adipogenesis is stimulated by the binding of PRDM16 to PPAR-γ and activation of the transcriptional function (60). Apelin then counteracts PPARγ to increase brown fat in the next stage of differentiation. Hence, C/EBPβ and PPARγ are critical for the initiation of WAT and BAT production, while PRDM16 and PGC1α are essential for the determination of BAT. Hence, it seems that increasing apelin is to increase BAT activity and counteracts WAT (40, 52, 54) (Figure 1). Apelin increases the expression of PRDM16 in WAT and leads to the browning of WAT (52). In addition, lack of PRDM16 in brown fat precursors leads to loss of brown fat characteristics, and overexpression of PRDM16 in myoblasts causes them to differentiate into brown fat cells (60). Expression of UCP1, PRDM16, and COX1 also significantly increase in human WAT treated with apelin on days 5-9 (52). Apelin-APJ signaling also differentiates BAT through the PI3K/Akt and AMPK/mTOR signaling pathways and increases the expression of brown fat proteins for mitochondrial biogenesis and thermogenesis (UCP1, CIDE-A, and COX1) (52) (Figure 1). Therefore, PRDM16, UCP1, CIDE-A, and COX1 gene expression can be a key strategy to reduce metabolic disorders caused by obesity because brown fat can increase energy consumption and protect against obesity through a specialized energy-burning program (5, 52, 60). Hence, in childhood and adolescence, BAT plays an important role in metabolic processes such as glucose and lipid metabolism (41). Apelin also has been reported to reduce levels of WAT, serum triglycerides, FFA and glycerol release, abdominal obesity, fat weight, and fat production in obese rats. While mice with apelin deficiency increase serum free fatty acid levels, serum FFA and glycerol levels, abdominal obesity, and body fat (52, 61). In rats, apelin can also significantly reduce TG content, glycerol concentration, the average diameter and adipocytes, and expression of PPARγ and perilipin mRNA in WAT. Apelin can inhibit fat cell differentiation, enhance lipolysis and improve obesity. Possible mechanisms may be to regulate PPARγ expression to inhibit WAT differentiation and to regulate perilipin expression to promote lipolysis (40). Hence the increase in apelin in obesity seems natural because autocrine apelin signaling may serve as a new therapeutic target for obesity and other metabolic disorders (54).

It is reported that daily apelin supplementation during pregnancy results in an increase in BAT marker proteins such as UCP1, PRDM16, and PGC-1α in mice fetal. Apelin administration also induces the expression of UCP1, Ppargc1a, PRDM16, Cidea (BAT-specific marker), and Elovl3 mRNAs in male and female fetuses (5) (Figure 1). In addition, Increased expression of UCP1 regulates many of the genes involved in lipid metabolism in BAT (41). In BAT, UCP1 is activated by FFA, causing oxidative phosphorylation to separate from ATP production as a proton carrier. UCP1 as a proton channel also disperses the electrochemical gradient without ATP production. Therefore, to adapt to the inefficient production of ATP, metabolism must be increased and heat produced. Hence, lipid catabolism in BAT does not lead to the production of ATP but also thermogenic reactions. Fatty acids released from fat droplets are the primary energy source for UCP1-mediated thermogenesis in BAT (46). Glucose metabolism is very important for BAT activity for lipogenesis or filling of fat

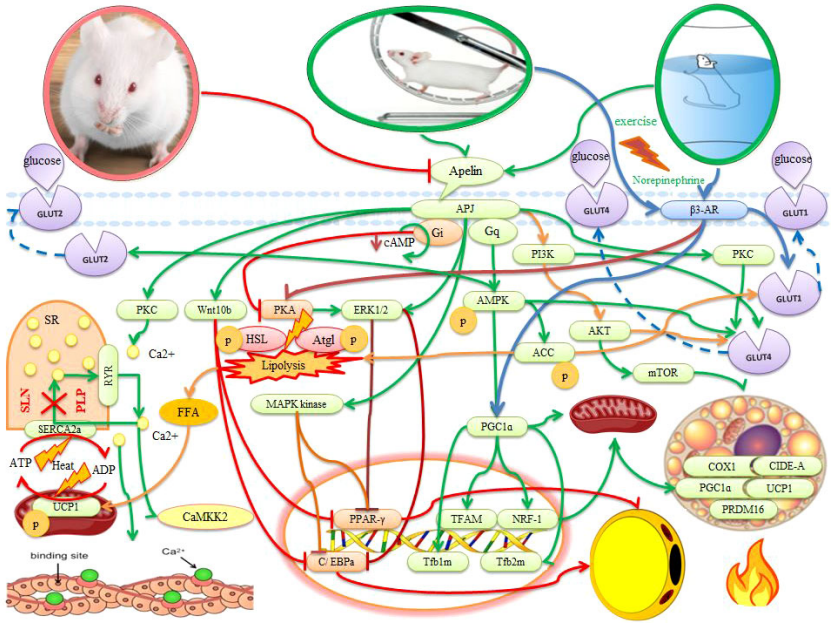

FIGURE 1
The effects of apelin and exercise on adipose tissue, muscle, and placenta in animals. The description is available in the text. Abbreviations are given at the beginning of the study.

droplets and can be used as an alternative fuel (46). These effects of apelin on WAT and BAT indicate the use of energy reserves and the reduction of adipose tissue (2). In addition, apelin increases the basal activity of BAT by increasing the expression of PGC1α, UCP1, mitochondrial biogenesis, and oxygen consumption (52). Thus, positive regulation of thermogenic genes in fetal BAT may be a strategy for the use of fat storages that can be used immediately by infants for UCP1-mediated thermogenic after birth (62). Apelin also has the potential to increase oxygen consumption, and reduce oxidative stress in fat cells, leading to anti-apoptotic effects. The anti-apoptotic effect of apelin in different cells is exerted by mitogen-activated protein kinase (ERK1/2, MAP3/1) and protein kinase B (AKT) (5, 52, 63–65). Therefore, the administration of apelin during pregnancy both increases brown lipid production, oxidative phosphorylation, and mitochondrial activity and inhibits the process of lipid synthesis and differentiation of white fat cells in fetal BAT (5). Thus, apelin can be a new therapeutic target for obesity and metabolic diseases in children of obese mothers (2, 52).

Possible mechanisms of apelin in the adipose tissue

It is reported that apelin administration stimulates AMPK activation and increases DNA demethylation of the PRDM16 promoter, leading to the initiation and maintenance of BAT production and thermogenesis (5). There is evidence also that apelin in pre-adipocytes and adipocytes leads to phosphorylation of MAPK kinase/ERK1/2 and inhibition of expression of adipogenic transcription factors (PPARγ and C/EBPa) (54). In addition, apelin through Wnt (Wnt10b) stimulation may inhibit the expression of PPARγ and C/EBPa in the early stages of differentiation. As a result, this event inhibits adipogenesis (54) (Figure 1). In adipocytes, apelin binds to the APJ and activates G protein subunits that include the Gαq and Gai proteins. Gαq and Gai then activate AMPK and inhibit protein kinase A (PKA) and reduce cyclic adenosine monophosphate (cAMP) synthesis. Hence, apelin inhibits lipolysis through Gq-AMPK phosphorylation and Gi-PKA dephosphorylation (61) (Figure 1). Apelin also performs ACC phosphorylation through AMPK activation An increase in apelin-induced lipogenic enzymes such as ACC leads to an increase in FFA synthesis in BAT, while FFA synthesis decrease in WAT. However, the FA content decreases significantly in BAT because the rate of decomposition of FFA (e.g., β-oxidation) also increases. This represents a collaboration between WAT and BAT for thermogenesis (66). Apelin also inhibits lipolysis by preventing the fragmentation of lipid droplets, increasing the expression of AMP-dependent perilipin, and decreasing the phosphorylation of perilipin. Therefore, the decrease in the release of free fatty acids caused by apelin can be attributed to the dual function of apelin in lipogenesis inhibition and lipolysis inhibition. Hence, apelin and APJ have been shown to inhibit adipogenesis of pre-adipocytes and

lipolysis in mature adipocytes (54). Daily injections of apelin have also been reported to reduce triglyceride content, fat deposition in adipose tissue, and the expression of a variety of genes involved in adipogenesis while increasing thermogenesis and oxygen consumption (40, 52). Since most of these findings are based on animal models, more attention should be paid to human models. The study of these key variables and important pathways in different time and place conditions in pregnant women should be considered.

Possible roles of exercise and apelin on adipose tissue of pregnant women

Among the increase in hormones caused by maternal exercise, apelin has a higher level because it is present in large amounts in the circulation of the mother, fetus, and placenta (41). Exercise-induced placental hypoxia stimulates vascular proliferation and expression of apelin in the placenta, increased apelin in fetal circulation, increased apelin in maternal circulation, and increases fetal brown fat (5). In mice, exercise (45 to 65% VO2max for 8 weeks) positively regulate apelin and is maintained in their offspring after a high-fat diet. In fetal brown adipose tissue, protein levels of UCP1, PRDM16, and PGC-1α are higher in athlete mothers than in controls. In the offspring of athlete mothers *via* apelin, the mRNA expression of BAT markers including UCP1, Ppargc1a, and PRDM16 increases (5) (Figure 2). In addition to the above factors, exercise increases the protein content of BAT markers such as Cidea and Elovl3 in male and female fetuses (5, 22). On the other hand, sedentary, overweight adults increase UCP1 by 12 weeks of exercise (3 times per week, HRmax intensity 70-80%). As a result, it appears that 12 weeks of exercise with apelin changes leads to the expression of brown/beige fat genes in abdominal fat (67). However, after swimming the pregnant mother is not reported any difference in the expression of UCP1 in BAT, which is related to the type of exercise because swimming puts less pressure on the athlete's mother due to carrying part of the body weight in water (2). In addition, pregnancy obesity decreases PRDM16 expression, oxidative metabolism, and mitochondrial biogenesis in the placenta. while maternal exercise-induced administration of apelin and apelin significantly increases PRDM16 expression and is associated with increased oxidative metabolism and mitochondrial biogenesis and expression of nutrient transporter in the placenta (22). Exercise increases BAT markers such as UCP1 and PRDM16 in the offspring of athlete mothers and is associated with oxygen consumption (Figure 2). In mice following a high-fat diet, VO2 and VCO2 levels are higher in the offspring of athlete mothers than in the control group. Thus, maternal exercise increases carbohydrate oxidation in the offspring of athlete mothers who are challenged with a high-fat diet. Increased oxygen consumption and

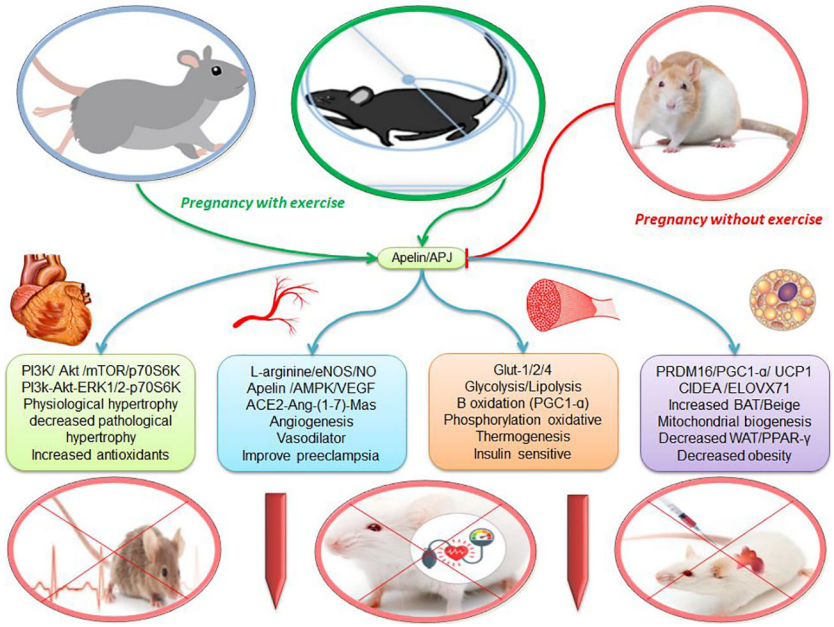

FIGURE 2
The effects of apelin and exercise on adipose tissue, muscle, vascular and heart. escription is available in the text. Abbreviations are given at the beginning of the study.

carbohydrate oxidation are associated with increased weight and BAT temperature in the offspring of athlete mothers (5). Then, WAT weight is reduced for the offspring of athletic mothers and they show better glucose tolerance than the offspring of the control group, which is associated with lower levels of fasting blood sugar and insulin levels in the offspring of athlete mothers (5). There is a negative correlation between UCP1 protein levels and insulin resistance in children of mothers of athletes exposed to high-fat diets, indicating that maternal exercise protects against metabolic disorders from children with high-fat diets. Exercise of the pregnant mother seems to increase the growth of beige/BAT fat in the fetus and children against obesity and metabolic syndromes (5, 41). Maternal exercise (45 to 65% of VO2max for 8 weeks) via apelin reduces pregnancy weight gain and increases the relative weight of BAT by 37.8%, while WAT decreases by about 37.1% compared to control mice. During weaning, body weight and WAT mass are lower in the offspring of athlete mothers, but WAT mass is higher than in the offspring of sedentary mice. Children of athletic mothers consume more food than children in the control group after exposure to a high-fat diet. However, the offspring of athlete mothers show less weight gain than the control group (2, 5). Therefore, exercise and consumption of apelin by the pregnant mother regulates glucose and fat metabolism, oxidative phosphorylation, thermogenesis, mitochondrial biogenesis, and growth of fetal brown fat and prevents the differentiation of white fat cells in fetal brown fat (5, 68).

In rodents, sympathetic stimulation using external stimuli (such as exercise, norepinephrine, prolonged cold exposure, and the PPARγ agonist) activates the beta-adrenergic receptor 3 (β3-AR) (69). In rats, exercise (60% of VO2max for 6 to 9 weeks) also directly activates the central nervous system (SNS) and, releasing norepinephrine and through β3-AR, stimulates BAT activity and WAT browning (70–72). In other words, the SNS innervates adipose tissue and releases the hormone norepinephrine. Norepinephrine stimulates β3-AR in fat cells and leads to an increase in cAMP. The cAMP-dependent PKA is then activated. PKA regulates phosphorylation of lipolytic enzymes and increases access to substrates for thermogenic and UCP1 activation (70) (Figure 1). Increased PKA activation can then phosphorylate hormone-sensitive lipase (HSL), adipose triglyceride lipase (Atgl), and perilipin, thereby activating lipolysis. Activation of PKA causes the excretion of the substrate (FFA) for thermogenesis (69, 72) (Figure 1). The primary source of thermogenic energy in BAT is also FA because it is essential for activating UCP1. BAT thermogenesis appears to be strongly associated with TG hydrolysis and FA oxidation (73). In addition, glucose appears to be used to regenerate ATP in mitochondria *via* UCP1 because lactic acid (an indicator of anaerobic glycolysis) increases significantly with thermogenesis. On the other hand, UCP1-induced thermogenesis impairs BAT thermogenesis by inhibiting glycolysis because increased glycolysis is also necessary to provide sufficient oxaloacetate for the oxidation of FA and acetyl CoA in the citric acid cycle (73–75). Therefore, carbohydrates and fats are consumed in BAT, and heat production prevents obesity. While people with low body temperature or low thermogenesis are more prone to obesity (76). On the other hand, norepinephrine also activates the expression of PGC1α (a key regulator of thermogenic gene expression and mitochondrial biogenesis), COX1, and UCP1 *via* β3-AR receptors. It then increases mitochondrial biogenesis, oxygen uptake and respiratory activity, and metabolic rate in BAT. It seems that increased apelin with exercise can be used as a new therapeutic target for obesity and metabolic diseases (52). Because healthy obese pregnant women have the aerobic capacity for physical activity, it is recommended that obese pregnant women exercise three to four times a week (repetition) and walk 10,000 steps daily (77).

Possible roles of exercise and apelin for skeletal muscle thermogenesis in pregnant women

In rats, the concentration of apelin on the 21st day of pregnancy rises significantly and causes uterine contractions. These contractions do not occur with the protein kinase C (PKC) inhibitor in a calcium-free environment, suggesting that the PKC pathway may be involved in the apelin mechanism (78). This indicates the importance of calcium in muscle contractions and thermogenesis. In myocytes, SERCA is located in the sarcoplasmic reticulum (SR) membrane and transports calcium from the cytosol to the SR using ATP hydrolysis. The gradient of calcium produced by SERCA is dispersed by the ryanodine receptor (RyR1). SERCA transport activity is inhibited by the two peptides phospholamban (PLP) or sarcolipin (SLN), but its ATPase activity remains. Thus, to match Ca2 + transport, mitochondrial ATP synthesis is increased and heat is generated (46) (Figure 1). Under conditions of hyperthermia, there is uncontrollable Ca2+ leakage with stimulation of the calcium cycle without muscle contraction and heat production (73, 79). It is reported that exercise-induced apelin in mothers activates sarcolipin and uncoupling protein 3 (UCP3) in the muscles of children and protects them from obesity (2) because Sarcolipin and UCP3 regulate thermogenesis in muscles to improve metabolic homeostasis (42). In myogenic cells with apelin/APJ deficiency, a decrease in thermogenic genes (Sarcolipin and UCP3) indicates the effect of apelin/APJ on fetal muscle thermogenesis and lower muscle temperature (2). After exercise, apelin activates Gαi and Gαq. Apelin activates CaMKK2 (calcium/calmodulin-dependent protein kinase kinase 2) as a downstream Gαq molecule in the fetal muscle of obese mothers and stimulates fetal muscle thermogenesis (42). In the fetal muscle of obese mothers, CaMKK2 is inactivated

while it is regenerated by apelin administration. Apelin mimics the effects of exercise and increases the thermogenic capacity of fetal muscles (2). Apelin also activates AMPK as a mechanical mediator in the regulation of metabolic pathways. AMPK ablation reduces the beneficial effect of exercise-induced apelin on fetal muscle thermogenic and mitochondrial biogenesis genes (2, 80). Hence, the heat generated by apelin may be due to the activation of AMPK (5, 61, 80). Activation of AMPK through exercise increases the PGC1-α level and its downstream (NRF1 and TFAM) for proliferation and transcription of mitochondrial DNA. PGC-1α stimulates mitochondrial biogenesis and regenerates mitochondrial-rich oxidative muscle tissue to regulate carbohydrate and fat metabolism. Hence, exercise-induced PGC-1α may play a role in improving obesity and type 2 diabetes (80, 81). There is evidence that a high-fat diet of parents through hypermethylation of the PGC-1α promoter leads to impaired metabolic homeostasis in boys at 9 months of age (82). In addition, maternal obesity lower fetal muscle temperature and thermogenic markers while being restored by maternal exercise (2). Thus, exercise prevents child obesity due to maternal obesity, which currently accounts for approximately 35% of pregnancies (42). In summary, maternal obesity is inhibited by exercise through activation of AMPK, PGC-1α, UCP3, and sarcolipin in fetal muscle. It is noteworthy that these changes are maintained in the muscles of children and show intergenerational effects (42). In general, it appears that exercise and apelin can increase thermogenesis in WAT, BAT, beige, and muscle, and these processes can lead to reduced obesity and metabolic disorders.

Possible effects of apelin on insulin sensitivity in pregnancy

Gestational diabetes is a glucose intolerance during pregnancy and its lack of control leads to side effects such as birth defects (31). Gestational diabetes has several short-term and long-term complications in both mother and fetus, including preeclampsia, fetal macrosomia or type 2 diabetes, and cardiovascular disease in the future (1). Nevertheless, the level of apelin in pregnancy with and without diabetes is controversial. For example, no association has been reported between plasma apelin concentrations in gestational diabetes and controls (83). The concentration of apelin also in cord blood has not different reported in diabetics and normal people (31). In addition, some studies have reported no significant difference between women with gestational diabetes and women with normal glucose tolerance at plasma apelin mRNA levels as well as apelin and APJ mRNA expression in adipose tissue, visceral fat, and placental tissue (34). But other studies about the apelin effect in other tissue have reported differently. For example, it has been reported that circulating levels of apelin, an insulin-sensitizing hormone, in the placenta increase in mothers of obese and insulin-resistant pregnant mice (1, 33). It also is reported that in obese patients, plasma apelin and insulin levels are significantly higher and the expression of apelin in fat cells is strongly inhibited by fasting and improves after re-feeding like insulin (56). This means that insulin deficiency increases the concentration of apelin and apelin can act like insulin (31). There is also evidence that the level of apelin in the group of fasting pregnant women is significantly higher than non-fasting pregnant women, which is may be related to insulin resistance at they (84). While severe maternal food restriction during pregnancy reduces fetal apelin, indicating that maternal nutritional and glucose status affects fetal apelin levels (1). Also, maternal plasma apelin levels have been reported to be significantly higher in patients with gestational diabetes compared to pregnant women without diabetes, which is positively correlated with their umbilical cord blood levels (83). In the second trimester, apelin levels also are reported to be higher in patients with gestational diabetes than in controls, with a negative correlation with triglycerides (TG) and total cholesterol (38). In addition, it is reported in pregnant women (24 to 28 weeks of gestation) insulin levels are significantly higher than in non-pregnant women, while serum apelin and glucose levels are lower. Nevertheless, there was a negative correlation between apelin with ox-LDL and HDL-cholesterol in the pregnancy group (36, 85). Thus, apelin appears to be involved in glucose and lipid metabolism during pregnancy. One of the reasons for these differences is mostly due to measurement methods that have used real-time PCR, Western blot, and ELISA methods. On the other hand, feeding conditions, time, and place of measurement can affect these differences. In the following, we will discuss the effects of apelin in gestational diabetes.

Apelin increases pancreatic islet cell mass and beta-cell insulin content in mice (86). Apelin has also a glucose-lowering effect along with increased glucose utilization in skeletal muscle and adipose tissue of normal and obese insulin-resistant mice (34). Administration of low-dose apelin to the fetus on day 21 of the fetus leads to the uptake of glucose into the lungs and muscles of the fetus in an insulin-independent manner. Administration of apelin at higher concentrations decreases fetal insulin and increases fetal glycemia, indicating the inhibitory role of high plasma apelin levels in insulin secretion in the fetus (1). In addition, short- and long-term treatment with apelin improves insulin sensitivity in obese and insulin-resistant rodents by increasing glucose uptake into skeletal muscle (86). Apelin increases glucose uptake *via* insulin and the PI3K/Akt pathway in adipocytes and can increase the transport of glucose transporter 4 (GLUT4) from the cytoplasm to the plasma membrane (87) (Figure 1). Endoplasmic reticulum stress-induced diabetes is ameliorated by oral apelin-13 administration by inhibition of inositol-requiring enzyme 1α (IRE1α) and inhibition of c-Jun N-terminal Kinase (JNK) (31). Direct regulation of apelin expression by insulin in both human and mouse adipocytes is

associated with the stimulation of phosphatidylinositol 3-kinase (PI3K), PKC, and MAPK. Thus, insulin has direct control over the expression of the apelin gene in adipocytes (56). In diabetes or obesity with hyperinsulinemia, apelin levels increase. Hyperapelinemia is a compensatory mechanism that not only inhibits pancreatic secretion but also leads to insulin sensitivity and glucose uptake into insulin-independent muscle tissue in mice (31). In conclusion, the apelin/APJ axis appears to be involved in the regulation of glucose homeostasis in adults by stimulating glucose uptake and insulin sensitivity (1). Increased apelin is believed to be a compensatory mechanism for inhibiting pancreatic secretion, insulin sensitivity, and glucose uptake into insulin-independent muscle tissue (31). Increased plasma apelin in type 2 and type 1 diabetic patients confirms the existence of this compensatory mechanism, which first reduces insulin resistance and then leads to a decrease in apelin levels. These low serum apelin levels in healthy lean individuals may be the result of natural insulin sensitivity (31, 88). Thus, apelin also has anti-diabetic properties and can be used as a therapeutic agent for type I and II diabetes (89). To understand these issues, we need to pay more attention to these apelin mechanisms in pregnancy in future research.

Apelin stimulates the phosphorylation of AMPK and acetyl CoA carboxylase (ACC) in muscle (68, 90). Chronic apelin therapy increases GLUT2 by activating AMPK and enhances glucose uptake and utilization of glucose in skeletal muscle in obese and insulin-resistant mice (31, 91) (Figure 1). On the other hand, the abundance of apelin and APJ in the placenta is strongly related to placental AMPK signaling and causes the transfer of maternal glucose to the fetus because all effects in mice treated with apelin are eliminated by inactivating AMPK (33, 92). In addition, during AMPK activation, ACC is inhibited, then the concentration of malonyl-coenzyme A (CoA) decreases, and beta-oxidation of fatty acids increases in muscle (15, 93, 94). In the human placenta, ACC is phosphorylated *via* p-AMPK to enhance fatty acid oxidation and reduce fatty acid synthesis (95). While ACC phosphorylation in the diabetic macrosomic group is significantly inhibited and leads to macrosomic induction through an excessive synthesis of fatty acids in the placenta (95). In the macrosomic diabetic placenta group, there is a significant negative correlation between neonatal birth weight and p-ACC and p-AMPK protein expression (95) (Figure 1). This means that the levels of the GLUT1 and ACC proteins increase and the p-AMPKα and p-ACC protein levels decrease in the macrosomia of gestational diabetes. As a result, GLUT1 has a potential role in the transfer of placental glucose to infant weight in gestational diabetes, which is the reason for the significant increase in macrosomia due to gestational diabetes in late pregnancy (95). Obese and insulin-resistant mice make better use of lipids, oxidation of free fatty acids, oxidative capacity, and mitochondrial biogenesis in muscle by administering apelin. Hence, injections of apelin for 4 weeks reduce fat mass, glycemic levels, and plasma triglycerides (92). Therefore, apelin can improve insulin sensitivity by increasing glucose uptake, mitochondrial biogenesis, improving oxidative capacity, and full use of lipids in insulin-resistant mice (68, 92).

The effect of exercise on insulin sensitivity in pregnancy

Exercise results on gestational diabetes are challenging. For example, it was reported that there was no difference between a standard 12-week exercise program or standard prenatal care (control group) in the second half of pregnancy to prevent insulin resistance. This study has several drawbacks in implementation. These drawbacks include determining the intensity of exercise (Borg scale), low adherence of participants, exercising at home without supervision, and doing exercise in the control group. These flaws make the results of the study questionable (96, 97). Some other findings support these findings that exercise has no significant effect on gestational diabetes (98, 99). In contrast, other studies report different results, for example, exercise reduces the risk of gestational diabetes. This study states that to achieve at least a 25% reduction in gestational diabetes, pregnant women should exercise at least 600 MET-minutes per week (e.g., 140 minutes of brisk walking, water aerobics, stationary cycling, or resistance training) with moderate intensity (100). There is evidence that obese women with 30 minutes of cycling three times a week (from the 25th week of pregnancy to the end of pregnancy) gain significantly less weight and lower levels of insulin resistance (101). There is evidence that women with gestational diabetes with exercise intervention have the lowest BMI increase in late and mid-pregnancy compared to women with gestational diabetes without exercise intervention. In addition, athletic diabetic women experience a lower risk of preterm delivery, high birth weight, and macrosomia than non-athletic diabetic women (97, 98, 102–104). Studies also show that for some women, exercise may help reduce the risk of gestational diabetes. Therefore, exercise seems to be associated with a reduction in gestational diabetes in obese women (105, 106). Therefore, moderate-intensity exercise in the second and third trimesters of pregnancy can be used to reduce the significant adverse outcomes of gestational diabetes (107–109). The possible mechanisms of these important effects of exercise in gestational diabetes can also be important. Some of these mechanisms are mentioned below.

Possible mechanisms of exercise on gestational diabetes

After exercise, increased expression of UCP1 protein in BAT and WAT in the offspring of exercise mothers compared to control rats leads to improved glucose tolerance, insulin

sensitivity, decreased fasting glucose, and insulin levels (5). After exercise, BAT also clears and absorbs glucose through two different pathways: SNS stimulation and insulin signaling. These two pathways have a synergistic effect on the transfer of glucose transporter to the plasma membrane. In BAT, stimulation of β3-adrenoceptors by epinephrine and norepinephrine increases the expression of GLUT1 and GLUT4 to the plasma membrane. Glucose uptake after adrenergic stimulation is independent of GLUT4 and is dependent on GLUT1 displacement. In BAT, however, insulin enhances GLUT4 transport to the plasma membrane *via* the PI3K-PDK-Akt pathway (Figure 1). Activation of BAT by adrenergic stimulation can reduce high cholesterol and atherosclerosis and regulate lipid metabolism in humans. Hence, brown fat volume is associated with increased lipolysis, free fatty acid oxidation, and insulin sensitivity (41, 110). Therefore, BAT is a major organ for burning fatty acids and glucose for thermogenesis (111).

Exercise-induced apelin secretion by activation of AMPK stimulates the expression of the GLUT4 gene in muscle (93). Hence, there is a significant positive correlation between apelin after exercise and decreased insulin resistance (112). In diabetic mothers, exercise-induced apelin has preventive effects on gestational diabetes by reducing insulin and glucose in maternal circulation, reducing weight, reducing insulin resistance, and improving glucose tolerance in skeletal muscles (2, 5). Apelin from exercise (40 to 60% of VO2max for 1 week) in fetal skeletal muscle activates AMPK for mitochondrial biogenesis by binding to a G protein-coupled receptor. The number of mitochondrial DNA (mtDNA) copies in the muscle of male and female fetuses increase (15, 91). Maternal exercise and consumption of apelin during pregnancy cause DNA demethylation of the peroxisome proliferator-activated receptor γ coactivator-1α (Ppargc1a) promoter and increase mitochondrial biogenesis in fetal muscle (15, 91). After exercise, APJ severely induces mitochondrial biogenesis markers such as Ppargc1a, Tfam, Tfb1m, Tfb2m, Nrf1, and Cox7a1 in males and females myogenic cells (15, 91) (Figure 1). These changes are maintained in the offspring and the offspring muscle in athlete mothers express higher levels of PGC-1α1/4 isoforms. Taken together, these effects have a positive effect on children's endurance capacity and protect children's muscles against metabolic disorders (15, 91). Treatment of mice with insulin resistance by apelin not only improves insulin sensitivity, but also increases fatty acid oxidation, oxidative phosphorylation, and mitochondrial biogenesis (31). Exercise of the pregnant mother also increases oxidative muscle fibers in the offspring by increasing mitochondrial biogenesis in oxidative fibers compared to glycolytic fibers. Oxidative muscle fibers are very efficient at using glucose and fatty acids (91). Hence, the useless energy consumption in the muscle increases while the thermogenic function is disrupted due to obesity caused by high energy nutrition. Therefore, exercise along with thermogenic function in muscle prevents metabolic disorders such as obesity and insulin sensitivity (2, 42). In addition, the sons and daughters of athletic mothers show better glucose tolerance than the children of the control group, which is associated with lower fasting blood sugar and insulin levels in the offspring of athletic mothers (5). These data show the long-term beneficial effects of maternal exercise on the metabolic health of children and support the preventive role of maternal exercise in glucose tolerance and metabolism of athlete children.

Preeclampsia and pregnancy

Hypertension is a very common risk factor for cardiovascular disease (CVD) (113). Women are more likely than men to die from cardiovascular disease, as well as deaths from cardiovascular disease are on the rise for women under 55 years (114). Around the world, about 7 million women are diagnosed with pregnancy hypertension or preeclampsia every year, and it usually appears in the third trimester of pregnancy (after 20 weeks of pregnancy) and is one of the main causes of maternal, fetal, and neonatal mortality (1, 115, 116). Premature birth between 32 and 34 weeks of gestation is very high due to the risk of discomfort to the mother and fetus due to preeclampsia (1). In addition, low birth weight is a recurrent consequence of preeclampsia and is independently associated with increased blood pressure and ischemic heart disease in adulthood (115, 116). This process is because embryonic development is based on the formation of the placenta because it creates conditions for the exchange of nutrients and oxygen between mother and fetus (1). In addition, the initial events of preeclampsia appear to be still placental ischemia/hypoxia. One of the main causes of placental ischemia is insufficient trophoblast invasion, which results in incomplete regeneration of the uterine spiral arteries and endothelial vasospasm (32). Hence, symptoms of preeclampsia include endothelial dysfunction such as vasoconstriction and ischemia (1). Invasion of embryonic trophoblast cells into spiral arteries reduces the resistance of placental arteries and increases blood flow to the embryo-placenta unit (117). However, women and children who survive preeclampsia are at greater risk for future cardiovascular adverse events (115, 116). That is why it is vital to address this issue.

Preeclampsia and the possible roles of apelin and exercise

Several mechanisms are associated with endothelial dysfunction in preeclampsia. These include hypoxia, excessive oxidative stress, renin-aldosterone-angiotensin (RAS) axis, and

imbalance of placental angiogenic factors. This means that the soluble fms-like tyrosine kinase 1 (sFIt-1), as an anti-angiogenic factor, binds to the vascular endothelial growth factor receptor (VEGFR) and neutralizes vascular endothelial growth factor (VEGF) and placental growth factor (114). In addition, preeclampsia is associated with an increase in placental anti-angiogenic agents (sFlt1 or sVEGFR-1) and endoglin because the administration of these agents to pregnant mice causes preeclampsia (1). Recent studies have also identified decreased apelin and APJ as important factors in preeclampsia at the placentas (32, 39, 118, 119). Apelin mRNA is in mature fat cells and stroma vascular fraction (SVF) at approximately equal levels (56). Inefficient Apelin and APJ appear to help initiate preeclampsia by reducing angiogenic activity in the placenta (32, 119). Apelin is important for the formation of the fetal cardiovascular system and the early growth of the placenta and acts in mid or late pregnancy to modulate fetal angiogenesis (1, 39). The role of apelin and APJ in early pregnancy is likely because stronger APJ signals are seen in trophoblast cell membranes (119).

On the other hand, apelin is known to be a potent mediator for placental arteries, and the content of apelin decreases as a result of high-fat diets but is positively regulated after exercise. Exercise for the pregnant mother diverts blood to the muscles and skin, creating a short-term hypoxic environment. Hypoxia in the placenta also activates HIF-1 and VEGF and enhances angiogenesis (4, 120). Consistent with these results, other regulatory factors such as VEGF, VEGFR, and hypoxia-inducible factor 1 (HIF-1) increase significantly with exercise in mice (121). It has also been reported that exercise in pregnant women may increase angiogenesis by increasing placental growth factor (PlGF) (32). As mentioned before, another cause of hypertension associated with reduced uteroplacental perfusion pressure (RUPP) is an imbalance between proangiogenic and anti-angiogenic (soluble fms-like tyrosine kinase 1 (sFIt-1)) factors (121). While exercise reduces RUPP-induced blood pressure by lowering sFIt-1 and increasing the VEGF factor and VEGF/sFIt-1 ratio. Hence, the positive effects of exercise on angiogenic balance in RUPP mice are confirmed by endothelial tube formation. Thus, exercise before and during pregnancy reduces hypertension, angiogenic imbalance, and oxidative stress induced by placental ischemia in RUPP mice (122). In addition, muscles stimulate AMPK and VEGF during high-volume exercise and lead to amelioration of preeclampsia during pregnancy. Therefore, exercise during pregnancy improves placental function (i.e., the ratio of fetal weight to placental weight) in both normal and RUPP pregnancies (32, 123). Although AMPK phosphorylation is suppressed as a result of maternal obesity, increased levels of AMPK phosphorylation and apelin protein in the placenta after exercise indicate a possible role of apelin angiogenesis mediated by AMPK in the placenta. Thus increase in AMPK phosphorylation in trained mice indicates nutrient/oxygen exchange in the placenta (4).

Possible roles of apelin and exercise to control vascular tone

Apelin and APJ are found in many placental cells such as endothelial cells and embryonic artery smooth muscle cells. Apelin appears to affect placental vascular tone and the exchange of oxygen and nutrients between mother and fetus (1). In addition, in the placenta, an organ without autonomic nerves, local control of vascular tone is critical to maintaining fetal growth because endothelial dysfunction is common due to an imbalance between the synthesis of vasodilator and vasoconstrictor molecules in the systemic circulation and placenta (124). Apelin, through the L-arginine/eNOS/NO pathway, increases vasodilation, heart contraction, angiogenesis, and suppression of aortic inflammation (39, 89, 115, 125) (Figure 2). eNOS converts L-arginine to NO molecule (39, 115). The NO released from endothelial cells is transported to adjacent vascular smooth muscle cells (VSMCs), where it causes cGMP production and Ca2+ uptake into intracellular calcium stores. As a result, Ca2+ is reduced, causing VSMC relaxation and vasodilation (126). NO is a strong vasodilator, a blood pressure regulator, and an anti-atherogenic, and prevents cell adhesion and platelet aggregation. In the placenta, eNOS is highly expressed in syncytiotrophoblast, villi endothelium, and macrophages (39, 115). Studies have shown that decreasing levels of apelin, endothelial nitric oxide synthase (eNOS), and nitric oxide (NO) contribute to the pathogenesis of preeclampsia. In preeclampsia, oxidative markers such as malondialdehyde (MDA) increase significantly, and levels of NO, apelin, and eNOS decrease significantly. Mean arterial pressure is negatively correlated with apelin and NO, and MDA is positively correlated with mean arterial pressure (115). Therefore, the decrease in mean serum levels of apelin and eNOS is normal in preeclamptic women, and it is negatively correlated with mean arterial pressure (39, 115, 125, 127, 128). In contrast, rats treated with apelin improve the symptoms of preeclampsia because it significantly increases the expression of eNOS in the placenta and the levels of NO and eNOS in the serum, all of which decrease preeclampsia (1, 39, 129). There is evidence that after the occurrence of preeclampsia, the level of maternal apelin to dilate blood vessels to cope with maternal hypertension increases (1). In addition, intravenous injection of apelin-13 into pregnant women increases the transfer of glucose from mother to fetus through the placenta. Due to the lack of change in the expression of major placental glucose transporters such as GLUT1 and GLUT3, apelin seems to exert its effect by increasing the dilation of placental arteries. Apelin probably absorbs glucose in the muscle through NO-induced vasodilation (1). Shear stress due to moderate exercise is one of the most important mechanisms to improve vascular function in the placenta through the synthesis of NO, which is caused by eNOS and VEGF (124). Exercise during pregnancy in the human placenta leads to a 2-fold increase in eNOS and a 4-

fold increase in NO, as well as a 6% decrease in O2 levels and a 26% decrease in H2O2 (130). On the other hand, chronic endurance training improves blood pressure by increasing mRNA expression and eNOS phosphorylation and reducing oxidative stress (126). Exercise-related mechanisms appear to involve the high expression of eNOS and NO because L-arginine and NO increase after 12 weeks of exercise and contribute to the beneficial effects of exercise on high blood pressure in humans (131). Exercise has been reported to be effective in preventing the onset of preeclampsia because 6 weeks of exercise through small heat shock proteins is involved in oxidative stress and apoptosis and facilitates eNOS-mediated NO synthesis through the larger HSPs (122). Since recent studies have been on animal models, future research with scientific guidelines on humans seems necessary.

The apelin system is associated with other effective cardiovascular systems such as Angiotensin (ACE) (32). Angiotensin-converting enzyme (ACE), angiotensin II (Ang II), and angiotensin 1 receptor (AT1) act as vasoconstrictors, cell proliferation, limb hypertrophy, sodium retention, and aldosterone release. While the enzyme converter angiotensin 2 (ACE2), angiotensin- (1–7), and Mas receptor are involved in vasodilation, anti-proliferation, anti-hypertrophy, cardiac protection, and protective measures (132, 133). Activation of AT1 receptors stimulates apelin secretion through Ca2+-dependent pathways, protein kinase C, and MAPK kinase, while activation of AT2 receptors inhibits apelin secretion through cAMP-and-cGMP-dependent pathways (134). Apelin is also detected by ACE2 (135) and the placenta can participate in apelin clearance by increasing ACE2 expression and helping to reduce maternal apelin levels at term (1). In preeclampsia, a decrease in apelin/APJ is associated with an increase in ACE2 expression because apelin is metabolized by ACE2 (27, 32, 136). Angiotensin II reduces the release of apelin from the human placenta (1). Ang II and apelin have opposite effects on the regulation of blood pressure, vascular tone, inflammation, and fluid homeostasis (27). Apelin inhibits the increase in cytosolic calcium and vasoconstriction induced by Ang II, which in turn contributes to vasodilation (27, 32). After treatment with apelin, the incidence of preterm delivery, cesarean section, and asphyxia are reduced (137). While positive regulation of Ang II and Ang II type 1 receptor (AT1R) is known as a vasoconstrictor and pro-inflammatory peptides in the placenta in women with preeclampsia (27). Although angiotensin II receptors increase at preeclampsia but decrease in trained mice (138). In rats, decreased inflammatory cytokines and angiotensin II receptor type 1 with high-volume exercise confirm this finding (139). Hence, exercise can stimulate the ACE2-Ang- (1–7)-Mas axis in parallel with inhibiting the ACE-Ang II-AT1 pathway. Activation of the ACE2-Ang- (1–7)-MAS receptor axis may play a role in the beneficial effects of physical exercise (133, 140) (Figure 2). Modulation of RAS appears to be a key mechanism in the development of preeclampsia, which can be altered by exercise to prevent preeclampsia (138, 141). In addition, pregnant women without physical activity have the same risks as obesity for preeclampsia (14). Epidemiological studies on exercise during pregnancy show a reduction in the incidence of obesity complications such as gestational diabetes, gestational hypertension, fetal growth retardation, and preeclampsia (32, 142, 143). Finally, current treatment guidelines emphasize the role of physical activity in treating high blood pressure because exercise lowers blood pressure in 75% of people with high blood pressure. Low to moderate-intensity exercise seems to help lower blood pressure in people with high blood pressure (113).

Possible role of apelin in cardiac hypertrophy in pregnancy

Pregnancy is a unique period in a woman's life with anatomical and physiological changes to increase the fetal metabolic demand. Hence, the cardiovascular system must be adapted to meet the needs of the fetus and the enlarged uterus (117). In pregnancy, physiological changes in the heart are proportional to an increase in factors such as PGC-1α, VEGF, angiogpietin1, and fibroblast growth factor 2 (FGF-2). Thus, cardiac adaptations due to pregnancy are more like exercise-induced physiological hypertrophy than pathological hypertrophy (117, 144). Under physiological hypertrophy conditions, heart function is either normal or increases due to a proportional increase in chamber size and wall thickness (11). Pathological cardiac hypertrophy is associated with decreased heart function and heart failure. Volumetric and pressure overload initially change the morphometry of the chamber. This change can be concentric hypertrophy (further increase in wall thickness with small cavities) or eccentric hypertrophy (large ventricular cavities with thin walls) (11). The apelin/APJ system plays an important role in cardiac hypertrophy and cardiovascular disease (89). Early studies show that APJs have apelin-independent functions for heart development. For example, fetuses carrying APJ mutations do not have a heart, and APJ knockout mice show fetal death due to vascular abnormalities, hearts with weak rings, and ventricular abnormalities. Most of the live fetuses also show abnormal vasculature, abnormal myocardium formation, and ventricular wall growth in adulthood (1). There is APJ in the early stages of gastrulation and during the later stages of development, while apelin expression begins only at the end of gastrulation. In general, APJ is very important for the early growth of the heart and apelin is vital for the early growth of the placenta and the formation of the fetal cardiovascular system (1).

Cardiac apelin is significantly reduced in heart failure and is regulated directly by the Ang II-AT1R system. Ang II-induced hypertension gradually puts extra pressure on the heart, leading to pathological hypertrophy. Ang II-treated mice show an

increase in left ventricular mass (LV), cardiomyocyte cross-section, and atrial natriuretic factor (ANF) expression. Transforming growth factor beta1 (TGF-β1) may contribute to Ang II-induced cardiac pathological hypertrophy. In contrast, the treatment of hypotension with an angiotensin-converting enzyme inhibitor or angiotensin receptor antagonist significantly reduces cardiac hypertrophy. Inhibition of the renin-angiotensin system may have beneficial effects, at least in part, through the repair of the cardiac apelin system. Apelin/APJ overexpression reduces the increased cardiac hypertrophy induced by Ang II, TGF-β, and oxidative stress (145). Apelin, on the other hand, can increase ACE2 levels in defective hearts and metabolize Ang II to produce the beneficial heptapeptide Ang- (1–7) as an anti-cardiac hypertrophy agent. Intraperitoneal apelin injection lowers blood pressure in hypertensive mice by inhibiting the renin-angiotensin system. As a result, apelin may activate the ACE2/Ang- (1–7) axis to reduce cardiac hypertrophy. Overexpression of apelin eliminates Ang II-induced cardiac hypertrophy by reducing atrial natriuretic peptide (ANP) protein content in cardiomyocytes by reducing cell size. However, apelin administration has been reported to cause non-pathological cardiac hypertrophy, and apelin/APJ could be a promising therapeutic target for cardiac hypertrophy (146). While specific inhibitors of PI3k, Akt, and ERK1/2 factors reverse the effects of apelin on the diameter, volume, and protein content of cardiomyocytes. Hence the PI3k-Akt-ERK1/2-p70S6K pathway is involved in rat myocardial hypertrophy caused by apelin (147). Serum apelin is significantly lower in patients with left ventricular hypertension than in normal individuals. In mice and humans, there is a direct correlation between plasma apelin and left ventricular mass index and between apelin expression and APJ in the myocardium. However, in mice with diabetic hypertension, an inverse correlation was observed between plasma apelin and myocardial expression. This means that apelin/APJ expression decreases in the myocardium, while plasma apelin increases in left ventricular hypertrophy. Due to the inotropic properties and vasodilation induced by apelin, plasma apelin enhancement appears to be a compensatory mechanism for maintaining cardiac output in rats with hypertensive overload or diabetic cardiomyopathy (148). There is evidence that a high-fat diet leads to increased ER stress (increased Bip and CHOP levels), and increased intracellular Ca2+ in the heart. The calcineurin-NFAT3 signaling pathway is then activated, leading to cardiac hypertrophy. While with the administration of apelin significantly reduces this pathway (89). Oxidative stress is an important factor in the development of cardiac hypertrophy by the overproduction of ROS and decreased cardiac antioxidant capacity. ROS is involved in cardiac pathological hypertrophy through inflammatory agents such as Ang II, TNF-α, leptin, and endothelin-1. In contrast, apelin significantly inhibits ROS production and increases antioxidant capacity by catalase activity in neonatal cardiomyocytes (149). Thus, apelin/APJ can inhibit pathological cardiac hypertrophy associated with oxidative stress.

On the other hand, exercise can improve myocardial morphology and heart function, leading to hypertrophy of the athlete's heart. Moderate-intensity exercise (60 to 70% VO2max for 8 weeks) significantly increases PI3K/Akt, mTOR, and p70S6K, but does not show pathological damage to the myocardium. While long-term high-intensity exercise (80 to 85% VO2max) causes cardiac hypertrophy with heart damage, which carries the risk of pathological changes (150). Akt as an axial regulator increases physiological hypertrophy as opposed to pathological hypertrophy through exercise. Swimming exercise in mice lacking Akt disrupts the cardiac growth response (151, 152). It is reported that IGF-1, PI3K, Akt, and mTOR signaling are also involved in exercise-induced cardiac hypertrophy (153). Thus, these results suggest that apelin/APJ and exercise may prevent pathological cardiac hypertrophy by activating the PI3K/Akt/mTOR/p70S6K and PI3k-Akt-ERK1/2-p70S6K pathways. Pathological hypertrophy is induced by prolonged stimulation of pro-inflammatory cytokines such as interleukin IL-1β, IL-6, TNF-α, TGF-β1, And NF-kB. These factors are strongly associated with increased fibrosis. In contrast, the hearts of athlete animals do not increase pro-inflammatory cytokines, and instead, significantly increase the anti-inflammatory cytokine IL-10 (11). These results show the difference between pathological and physiological hypertrophy. However, hypertrophy caused by pregnancy and exercise is both physiological hypertrophies. In mid-pregnancy, Akt phosphorylation and its downstream targets, such as GSK3β, mTOR, and p70S6, increase significantly. As a result, cardiac adaptation during mid-pregnancy is similar to the response to exercise (117). As a result, it seems that exercise through physiological hypertrophy and reduction of inflammatory factors can be useful to protect the heart of pregnant women. Hence, the American Congress of Obstetrics and Gynecology, the centers for disease control, and the American College of Sports Medicine recommend physical activity during pregnancy. These guidelines state that women should exercise at a moderate intensity for at least 30 minutes most days of the week if not all days of the week (117).

Conclusion

The prevalence of maternal obesity during pregnancy is associated with the risk of metabolic diseases and high birth weight. Overweight and obesity in pregnant women predispose their children to obesity and metabolic diseases in the future (2, 5, 40–42). Obesity is associated with WAT hypertrophy and BAT apoptosis (41, 49). Environmental factors such as active

lifestyle, and type of nutrition such as apelin supplementation can lead to BAT activity and thermogenesis (41). Apelin supplementation and exercise (45 to 65% VO2max) during pregnancy increase BAT proteins such as Cidea, Elovl3, UCP1, PRDM16, and PGC-1α in males and female fetuses (5, 22). UCP1 dissipates energy stored in the mitochondrial electrochemical gradient in the form of heat, and PRDM16 positively regulates many of the genes involved in BAT production (41). Released fatty acids are the primary energy source for UCP1-mediated thermogenic (62). Elevated UCP1 and PRDM16 in the offspring of athletic mothers are associated with oxygen consumption (5). Increased levels of VO2 and VCO2 in the offspring of athlete mothers indicate an increase in carbohydrate oxidation and are associated with an increase in weight and BAT temperature. While WAT weight is reduced and indicates an improvement in fetal growth and metabolism in the offspring of athlete mothers (5). Thus, exercise and consumption of apelin by the pregnant mother regulates glucose and fat metabolism, oxidative phosphorylation, thermogenesis, mitochondrial biogenesis, and growth of fetal brown fat and prevents the differentiation of BAT into WAT (5, 68).

Apelin also increases pancreatic islet cell mass and beta-cell insulin content (86). Apelin has a glucose-lowering effect with increased glucose utilization in skeletal muscle and adipose tissue of normal and obese insulin-resistant mice (34). Apelin increases glucose uptake through the PI3K, Akt, PKC, and MAPK factors into adipocytes and can increase the transport of GLUT4 from the cytoplasm to the plasma membrane (56, 87). In diabetes or obesity with hyperinsulinemia, increased apelin is a compensatory mechanism in which it not only inhibits pancreatic secretion but also leads to insulin sensitivity and glucose uptake into insulin-independent muscle tissue and then leads to a decrease in apelin levels (31, 88). Exercise-induced apelin secretion by activating AMPK stimulates the expression of GLUT4 gene in muscle (93), and then activates mitochondrial biogenesis. The offspring muscle in athlete mothers expresses higher levels of the PGC-1α1/4 isoform and increases oxidative muscle fibers in children. Oxidative muscle fibers are very efficient in the use of glucose and fatty acids (15, 91). Hence the useless energy consumption in the muscle increases (2, 42). In addition, boys and girls of athlete mothers show better glucose tolerance than children in the control group, which is associated with lower fasting blood sugar and insulin levels in the children of athlete mothers (5). Hence, exercise and apelin are known as regulators of energy metabolism and have anti-obesity and anti-diabetic properties (52).

To prevent preeclampsia, exercise in the pregnant mother directs blood to the muscles and skin, creating a short-term hypoxic environment. Hypoxia activates HIF-1, VEGF, and VEGFR and increases angiogenesis (121). Exercise and apelin, through the L-arginine/eNOS/NO pathway, increase vasodilation, angiogenesis, and suppression of inflammation (39, 89, 115, 125). Exercise can stimulate the ACE2-Ang- (1–7)-Mas axis in parallel with inhibiting the ACE-Ang II-AT1 pathway (133). The activity of RAS appears to be a key mechanism in the development of preeclampsia that can be altered by exercise to prevent preeclampsia (138, 141). Moderate-intensity exercise (60 to 70% VO2max) and apelin/APJ may prevent pathological hypertrophy by activating the PI3K/Akt/mTOR/p70S6K, PI3k-Akt-ERK1/2-p70S6K pathways and the anti-inflammatory cytokine IL-10. While pathological hypertrophy is induced by long-term stimulation of pro-inflammatory cytokines such as interleukins IL-1β, IL-6, TNF-α, TGF-β1, and NF-κB (11, 150). As a result, it seems that exercise through these pathways can be beneficial in protecting the heart of pregnant women.

Future prospects

Since the present study studies are more than animal samples, future research should pay more attention to human samples. The use of scientific guidelines for maternal and fetal health should be considered. Future research should pay special attention to the variables of duration and intensity of training. Due to physiological changes during the first, second, and third trimesters of pregnancy, research should consider these time variables.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Follicular fluid lipidomic profiling reveals potential biomarkers of polycystic ovary syndrome: A pilot study

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Background: Polycystic ovary syndrome (PCOS) is a heterogeneous endocrine disorder associated with multiple metabolic conditions including obesity, insulin resistance, and dyslipidemia. PCOS is the most common cause of anovulatory infertility; however, the molecular diversity of the ovarian follicle microenvironment is not fully understood. This study aimed to investigate the follicular fluid (FF) lipidomic profiles in different phenotypes of PCOS and to explore novel lipid biomarkers.

Methods: A total of 25 women with PCOS and 12 women without PCOS who underwent *in vitro* fertilization and embryo transfer were recruited, and their FF samples were collected for the lipidomic study. Liquid chromatography-tandem mass spectrometry was used to compare the differential abundance of FF lipids between patients with different PCOS phenotypes and controls. Subsequently, correlations between specific lipid concentrations in FF and high-quality embryo rate (HQER) were analyzed to further evaluate the potential interferences of lipid levels with oocyte quality in PCOS. Candidate biomarkers were then compared *via* receiver operating characteristic (ROC) curve analysis.

Results: In total, 19 lipids were identified in ovarian FF. Of these, the concentrations of ceramide (Cer) and free fatty acids (FFA) in FF were significantly increased, whereas those of lysophosphatidylglycerol (LPG) were reduced in women with PCOS compared to controls, especially in obese and insulin-resistant groups. In addition, six subclasses of ceramide, FFA, and LPG were correlated with oocyte quality. Twenty-three lipid subclasses were identified as potential biomarkers of PCOS, and ROC analysis indicated the prognostic value of Cer,36:1;2, FFA C14:1, and LPG,18:0 on HQER in patients with PCOS.

Conclusions: Our study showed the unique lipidomic profiles in FF from women with PCOS. Moreover, it provided metabolic signatures as well as candidate biomarkers that help to better understand the pathogenesis of PCOS.

KEYWORDS

polycystic ovary syndrome, follicular fluid, lipidomics, oocyte quality, liquid chromatography-tandem mass spectrometry

Introduction

Polycystic ovary syndrome (PCOS) is a common female endocrine disorder affecting both reproductive and metabolic functions, with a prevalence rate of 5%–10% among women of reproductive age (1–3). PCOS is characterized by the association between clinical and/or biochemical hyperandrogenism and chronic oligo-anovulation and/or ultrasonographic evidence of polycystic ovaries (4). Women with PCOS have a high prevalence of infertility, oligomenorrhoea, androgen excess, obesity, insulin resistance, and dyslipidemia (5–7). Moreover, there is a growing interest in the complications associated with metabolic disturbances in PCOS, which affects the quality of life of numerous women worldwide. However, despite extensive research efforts, understanding the genetic, molecular, and cellular mechanisms underlying the pathophysiology of PCOS is still challenging (8, 9). This highlights the need to further assess the pathogenesis of PCOS and to identify potential biomarkers for prevention of long-term complications *via* appropriate screening, early and accurate diagnosis, and effective intervention.

Previous studies have shown that changes in the follicular microenvironment are correlated with PCOS (10). The search for biomarkers in biologic fluids, including follicular fluid (FF), has emerged as an alternative to invasive methods (11). FF is derived from ovarian antral follicles, which contains important metabolites and serves as a complex microenvironment for follicular development, oocyte maturation, and germ-somatic cell interactions (12). Impaired oocyte quality and outcomes of *in vitro* fertilization (IVF) are related to changes in FF components among patients with PCOS, but the mechanisms have not been fully elucidated yet (13). The metabolomic profiling of FF may have an important impact on oocyte developmental potential and embryo viability. However, whether there are characteristic differences in FF lipid levels between PCOS patients and healthy controls has not been explored. Since intrafollicular communication is critical for normal oocyte development and reproduction, the identification of FF components may provide a better understanding and reveal potential lipid biomarkers of PCOS.

Lipids are essential metabolites that have many crucial cellular functions and can enable a direct readout of cellular metabolic status. Lipidomics is the study of lipidomes using the principles and techniques of analytical chemistry (14, 15). It provides a powerful tool for developing lipid biomarkers to diagnose different diseases. In addition, the discovery of lipid-based biomarkers can be an alternative method for exploring disease states (16). This indicates the potential benefit of clinical lipidomics in the identification and development of disease biomarkers, assessment of signals and metabolic processes, and provision of insights into molecular mechanisms and drug targets. A comprehensive analysis of lipid classes and molecular species has been performed using the separation power of liquid chromatography-mass spectrometry (LC-MS) (17). This post-genomic technology has been applied to rapidly detect global metabolic profiling in biological systems and is a useful strategy for discovering biomarkers, identifying perturbed pathways, diagnosing diseases, and measuring responses to medical treatment (18).

Therefore, the current study aimed to investigate FF lipidomic changes underlying the different phenotypes of PCOS. Here we show that women with PCOS had elevated FF ceramide (Cer) and free fatty acids (FFA) concentrations and decreased FF levels of lysophosphatidylglycerol (LPG), which might affect high-quality embryo rate. Moreover, we demonstrate that Cer,36:1;2, FFA C14:1, and LPG,18:0 could represent candidate lipid biomarkers of embryo quality in PCOS patients. Our findings suggest that altering ovarian lipid metabolism may help improve oocyte quality and early embryo development, which provides a potential intervention on pregnancy outcomes in patients with PCOS.

Materials and methods

Participants

Participants were recruited from the Center for Reproductive Medicine, Renji Hospital, Shanghai Jiao Tong University School of Medicine. In total, 25 patients with PCOS and 12 women of

similar age without PCOS were included in the study. Patients with PCOS were diagnosed based on the Rotterdam criteria, which exclude other endocrine disorders and require the presence of two or more of the following three signs: oligo- or amenorrhea, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovarian morphology on ultrasound (19). PCOS patients were further divided as follows (1): the obese PCOS (Obese) group (n = 9) with body mass index (BMI) ≥ 25 kg/m^2 and the lean PCOS (Lean) group (n = 16) with BMI < 25 kg/m^2; (2) the insulin-resistant PCOS (IR) group (n = 13) with homeostatic model assessment of insulin resistance (HOMA-IR) ≥ 2.5 and the non-insulin-resistant PCOS (NIR) group (n = 12) with HOMA-IR < 2.5 (20); (3) the hyperandrogenism PCOS (HA) group (n = 14) with free androgen index (FAI) ≥ 5 and the non-hyperandrogenism PCOS (NHA) group (n = 11) with FAI < 5 (21). The control group included women with normal ovarian reserve (regular menstrual cycles and AMH concentration of ≥ 2 ng/mL) and normal BMI who presented with infertility caused by fallopian tube disorders or male factors (azoospermia or severe oligo −/asthenoteratozoospermia). All controls had regular menstruation and normal androgen levels, glucose tolerance, and ovarian appearances on ultrasound. Women who received hormonal treatment, insulin sensitizer, anti-hypertensive drugs, or other medications affecting lipid or glucose metabolism within the last 3 months were excluded from the study. This study was approved by the Ethics Committee of Renji Hospital (approval number: 2015030308) and was performed in accordance with the Declaration of Helsinki. A written informed consent was obtained from all participants.

Clinical measurements and biochemical analyses

BMI was calculated as weight in kilograms divided by the square of height in meters. Fasting blood glucose (FBG) concentration was quantified using the glucose oxidase method (22). The concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), E2, total T, fasting serum insulin (FINS), and sex hormone-binding globulin (SHBG) were assessed using electrochemiluminescence (cobas e 601 module; Roche Diagnostics, Indianapolis, IN, USA). The levels of AMH were evaluated using an enzyme-linked immunosorbent assay kit (Kangrun Biotech, Guangzhou, China) according to the manufacturer's protocol. Insulin resistance index, as determined by HOMA-IR, was calculated using the following formula: (FBG [in millimoles per milliliter] × FINS [in milliunits per milliliter])/22.5 (23). FAI was calculated as (TT × 100)/SHBG. The participants underwent ultrasound examination using the Voluson E8 System (GE Healthcare, Chicago, IL, USA) between the second and fifth days after menstruation. After the IVF procedure, data on the number of oocytes and oocytes in meiosis II stage (MII), oocyte fertilization rate (defined as the number of oocytes fertilized divided by the number of oocytes inseminated), and pregnancy test results were collected from the participant's medical record. According to the modified Peter's scoring system for cleavage-stage embryos (24), the embryos on day 3 after fertilization were divided into the following four grades: grade I - embryos with blastomeres of equal size and fragmentation of < 10%; grade II - embryos with blastomeres of slightly uneven or unequal size with 10%–20% cytoplasmic fragmentation; grade III - embryos with blastomeres of distinctly unequal size with 21%–50% fragmentation; and grade IV - embryos with blastomeres of severe fragmentation with the amount of debris > 50%. Based on these criteria, 6–10 cell and grade I–II embryos on day 3 are considered high-quality embryos, and the rest are regarded as low-quality embryos.

Sample collection and preparation

Regarding IVF, all patients received controlled ovarian hyperstimulation and medical management according to our established protocols (25). The GnRH antagonist protocol was applied for ovarian stimulation. Each patient received individual doses of gonadotropins, FSH, and/or human menopausal gonadotropin, starting on day 2 or 3 of the menstrual cycle. Recombinant human chorionic gonadotropin (hCG) was administered to the patient to induce final follicular maturation when the dominant follicles reached a diameter of ≥ 18 mm (26, 27). With respect to the standard clinical practice of oocyte retrieval at the Center for Reproductive Medicine, Renji Hospital, all accessible follicles were punctured and aspirated using a 16-gauge needle under ultrasound guidance 35–37 h after the administration of recombinant hCG. Preovulatory ovarian FF samples were collected during oocyte retrieval, and only FF samples with no macroscopic blood contamination were included for further analyses. FF obtained from three dominant follicles with diameters of 18–22 mm was pooled and centrifuged at 1,500 × g for 10 min to remove cellular debris and insoluble particles. The supernatant was then collected and stored at −80°C for further use.

Lipidomics

Solvents for sample preparation and mass spectrometry (MS) analysis, including methanol, chloroform, and water, were purchased from Burdick and Jackson (Muskegon, MI, USA). Other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

FF lipidomic data were acquired using liquid chromatography with electrospray ionization mass spectrometry (LC-ESI-MS) (28). Details of the experimental protocols including sample preparation and spectroscopy have been described previously

(29). Briefly, FF lipids were extracted using a modified methyl tert-butyl ether protocol. Samples were separated on a UPLC HSS T3 column C18 column at 400 µL/min using a gradient comprising mobile phases A and B. Mobile phase A is a mixture of acetonitrile, water, and ammonium acetate. Mobile phase B is a mixture of acetonitrile, isopropanol, and ammonium acetate. MS analysis was performed using a QTRAP 5500 mass spectrometer (SCIEX, Framingham, MA, USA) (30). The mass spectrometer was operated in the negative ion mode *via* multiple reaction monitoring. Analyst 1.6.3 software (SCIEX) was used for data acquisition and processing. FF samples were analyzed in random order, and quality control samples were inserted every eight samples to ensure repeatability. Finally, a total of 581 lipids were quantified.

Statistical analysis

All data were analyzed using the SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA). Data distribution was assessed using the normal Quintal plot. Continuous data with a normal distribution were presented as means ± standard deviation (SD). Data with non-normal distributions were analyzed after logarithmic transformation. Comparative analysis of quantitative data was performed using the independent samples *t*-test between the PCOS and control groups. Intergroup comparisons of the measured metabolite intensities were performed by one-way analysis of variance with Bonferroni correction. Categorical variables were expressed as frequency (composition ratio) and were analyzed using the $\chi 2$ test. The statistical relationship between the variables of interest was evaluated using Pearson or Spearman correlation. Receiver operating characteristic (ROC) analysis was performed, and the area under the ROC curve (AUC), 95% confidence interval (CI), and corresponding P values were calculated to establish the predictive value of each parameter in PCOS. A P value of < 0.05 was considered statistically significant.

Results

Demographic and clinical characteristics of the study participants

A total of 37 patients were recruited in this study. Twenty-five patients had PCOS based on the Rotterdam criteria. Of the PCOS patients, 16 (64%) had a normal BMI, whereas nine (36%) had a BMI of > 25 kg/m^2; 13 (52%) patients had insulin resistance classified according to HOMA-IR, and 12 (48%) did not; 11 (44%) patients had no clinical or biochemical evidence of androgen excess, and 14 (56%) patients presented with hyperandrogenism. Table 1 shows the baseline characteristics and clinical outcomes of patients with different phenotypes of PCOS and controls. Women with and without PCOS did not significantly differ in terms of age (27.92 ± 2.43 vs. 28.16 ± 2.34 years, P = 0.772). A broad spectrum of metabolic and endocrine changes was observed in PCOS patients, including significantly high BMI (23.77 ± 5.18 vs. 20.58 ± 0.91 kg/m^2, P = 0.006), remarkably elevated serum levels of FINS (12.22 ± 6.59 vs. 5.67 ± 1.18 mIU/L, P < 0.001), basal LH (8.98 ± 4.83 vs. 5.58 ± 2.21 mIU/mL, P = 0.013), AMH (15.14 ± 7.71 vs. 5.81 ± 3.62 ng/mL, P < 0.001), TT (1.69 ± 0.71 vs. 0.69 ± 0.28 nmol/L, P < 0.001), and low levels of basal serum FSH (5.59 ± 1.00 vs. 7.08 ± 1.48 mIU/mL, P = 0.001). As expected, women with PCOS had significantly higher values for HOMA-IR (2.67 ± 1.45 vs. 1.23 ± 0.26, P < 0.001) and FAI (4.78 ± 4.93 vs. 1.29 ± 0.56, P = 0.021) than those without PCOS. There were no statistically significant differences in the FBG and basal E2 levels between the PCOS group and controls (P = 0.914 and 0.112, respectively). Furthermore, subgroup analyses were conducted to further detect the impact of obesity, insulin resistance, and hyperandrogenism in PCOS. Among PCOS patients, obese women showed significantly higher FINS levels (18.00 ± 4.98 vs. 8.97 ± 5.00 mIU/L, P < 0.001), HOMA-IR values (3.95 ± 1.21 vs. 1.95 ± 1.02, P < 0.001), and FAI (10.10 ± 8.12 vs. 3.33 ± 2.77, P < 0.001) than those with a normal BMI; patients with insulin resistance had a significantly higher BMI compared with patients without insulin resistance (26.61 ± 5.60 vs. 20.68 ± 2.08 kg/m^2, P = 0.001); women with hyperandrogenism had higher BMI (25.69 ± 5.67 vs. 21.32 ± 3.29 kg/m^2, P = 0.029) and HOMA-IR values (3.18 ± 1.57 vs. 2.03 ± 1.02, P = 0.048) than non-hyperandrogenic women. As compared with controls, the numbers of oocytes retrieved (14.67 ± 6.88 vs. 20.28 ± 8.91, P = 0.036) and MII oocytes (8.67 ± 4.33 vs. 15.08 ± 7.61, P = 0.002) during IVF were significantly higher in PCOS patients. No statistical differences in fertilization rate, high-quality embryo rate (HQER), clinical pregnancy rate, and live birth rate were observed between the groups of patients with PCOS and controls. However, the PCOS IR group had a significantly lower HQER than the control group (0.42 ± 0.27 vs. 0.69 ± 0.23, P = 0.031). Overall, these changes were clinically associated with the phenotypes of PCOS and complemented the metabolomic analysis.

Lipidomic changes between healthy controls and patients with PCOS

Lipids play important roles both as basic substrates and as regulators in many metabolic pathways. To compare global variation in the lipidomic profiles of FF between the PCOS and control groups, the lipid content of pooled FF samples was assessed by LC-MS/MS analysis. All samples were analyzed blindly, and nineteen specific lipids were identified in FF (Supplementary Table 1). Three lipids were differentially highlighted, and the quantification information are given in Table 2. We observed that concentrations of total ceramide and

TABLE 1 Demographics and clinical outcomes of women with PCOS and controls.

Parameter	Control (n=12)	PCOS							
		All PCOS (n=25)	P	Classified according to BMI		Classified according to HOMA-IR		Classified according to FAI	
				Lean (n=16)	Obese (n=9)	NIR (n=12)	IR (n=13)	NHA (n=11)	HA (n=14)
Age, years	27.92 ± 2.43	28.16 ± 2.34	0.772	28.25 ± 2.30	28.00 ± 2.55	28.17 ± 2.04	28.15 ± 2.67	27.36 ± 2.54	28.79 ± 2.05
BMI, kg/m^2	20.58 ± 0.91	23.77 ± 5.18[a]	0.006	20.57 ± 1.90	29.44 ± 4.12[a,b]	20.68 ± 2.08	26.61 ± 5.60[a,c]	21.32 ± 3.29	25.69 ± 5.67[a,d]
FBG, mmol/L	4.92 ± 0.55	4.94 ± 0.56	0.914	4.93 ± 0.40	4.97 ± 0.79	5.03 ± 0.47	4.87 ± 0.68	4.90 ± 0.49	4.98 ± 0.62
FINS, mIU/L	5.67 ± 1.18	12.22 ± 6.59[a]	< 0.001	8.97 ± 5.00	18.00 ± 4.98[a,b]	6.62 ± 2.16	17.40 ± 4.71[a,c]	9.38 ± 4.98	14.46 ± 7.00[a]
HOMA-IR	1.23 ± 0.26	2.67 ± 1.45[a]	< 0.001	1.95 ± 1.02	3.95 ± 1.21[a,b]	1.49 ± 0.46	3.76 ± 1.15[a,c]	2.03 ± 1.02	3.18 ± 1.57[a,d]
Basal FSH, mIU/mL	7.08 ± 1.48	5.59 ± 1.00[a]	0.001	5.65 ± 1.05[a]	5.47 ± 0.94[a]	5.93 ± 1.00	5.27 ± 0.92[a]	5.81 ± 0.99[a]	5.42 ± 1.01[a]
Basal LH, mIU/mL	5.58 ± 2.21	8.98 ± 4.83[a]	0.013	8.45 ± 4.92	9.39 ± 4.95	9.10 ± 5.64	8.87 ± 4.19	7.69 ± 3.21	9.99 ± 5.72
Basal E2, nmol/L	32.15 ± 6.25	41.78 ± 17.22	0.112	38.37 ± 13.04	47.83 ± 22.50	39.17 ± 12.28	44.19 ± 21.01	40.34 ± 16.24	42.91 ± 18.47
AMH, ng/mL	5.81 ± 3.62	15.14 ± 7.71[a]	< 0.001	16.67 ± 9.26[a]	12.41 ± 2.05[a]	17.13 ± 10.49[a]	13.30 ± 3.20[a]	14.61 ± 8.56[a]	15.56 ± 7.27[a]
TT, nmol/L	0.69 ± 0.28	1.69 ± 0.71[a]	< 0.001	1.54 ± 0.63[a]	1.96 ± 0.81[a]	1.59 ± 0.59[a]	1.78 ± 0.83[a]	1.15 ± 0.62	2.12 ± 0.45[a,d]
SHBG, nmol/L	64.88 ± 37.55	51.22 ± 38.63	0.510	59.51 ± 39.32	20.81 ± 14.33	63.68 ± 41.52	28.79 ± 20.61	64.54 ± 38.09	17.91 ± 6.62[a,d]
FAI	1.29 ± 0.56	4.78 ± 4.93[a]	0.021	3.33 ± 2.77	10.10 ± 8.12[a,b]	3.55 ± 2.97	6.99 ± 7.21	2.19 ± 1.11	11.25 ± 4.87[a,d]
No. of oocytes retrieved	14.67 ± 6.88	20.28 ± 8.91[a]	0.036	21.75 ± 9.15	17.67 ± 8.34	22.17 ± 10.61	18.54 ± 7.00	20.18 ± 6.93	20.36 ± 10.48
No. of MII oocyte	8.67 ± 4.33	15.08 ± 7.61[a]	0.002	16.87[a] ± 7.72	12.00 ± 6.73	16.58 ± 8.74[a]	13.69 ± 6.43	16.73 ± 7.31[a]	13.79 ± 7.86
Nuclear maturation (MII) rate	0.63 ± 0.25	0.75 ± 0.17	0.104	0.78 ± 0.18	0.69 ± 0.15	0.76 ± 0.19	0.74 ± 0.16	0.82 ± 0.16	0.69 ± 0.16
Fertilization rate	0.55 ± 0.16	0.62 ± 0.16	0.194	0.66 ± 0.11	0.55 ± 0.21	0.65 ± 0.12	0.59 ± 0.19	0.63 ± 0.13	0.61 ± 0.18
High-quality embryo rate	0.69 ± 0.23	0.51 ± 0.27	0.054	0.56 ± 0.27	0.42 ± 0.27	0.60 ± 0.25	0.42 ± 0.27[a]	0.55 ± 0.24	0.47 ± 0.29
Clinical pregnancy rate	0.79 (11/14)	0.71 (25/35)	0.843	0.74 (17/23)	0.67 (8/12)	0.71 (12/17)	0.72 (13/18)	0.65 (11/17)	0.78 (14/18)
Live birth rate	0.71 (10/14)	0.63 (22/35)	0.477	0.61 (14/23)	0.67 (8/12)	0.59 (10/17)	0.67 (12/18)	0.53 (9/17)	0.72 (13/18)

Data are presented as mean ± SD. [a]Compared with the control group, P < 0.05; [b]Compared with the Lean group, P < 0.05; [c]Compared with the NIR group, P < 0.05; [d]Compared with the NHA group, P < 0.05.

AMH, anti-Müllerian hormone; BMI, body mass index; E2, estradiol; FAI, free androgen index; FBG, fasting blood glucose; FINS, fasting serum insulin; FSH, follicle-stimulating hormone; HOMA-IR, homeostatic model assessment of insulin resistance; MII, metaphase II; LH: luteinizing hormone; SHBG, sex hormone-binding globulin; TT, total testosterone.

total FFA were elevated in statistically significant manner (P = 0.029 and 0.008, respectively), whereas the levels of LPG were significantly decreased in FF from the patients with PCOS compared with the controls (P < 0.001), thereby indicating the aberrance of lipid metabolism in PCOS.

To further understand the potential interaction between biochemical states and lipidomic abnormalities in women with PCOS, we investigated the concentrations of ceramide and LPG in each PCOS subgroup. The concentrations of total ceramide and total FFA showed an increasing trend (P < 0.05) in women with PCOS, while the concentrations of LPG had a decreasing trend (P < 0.001). In particular, ceramide levels in FF were considerably higher in both obese PCOS patients and PCOS patients with insulin resistance than those of controls (P = 0.027 and 0.026, respectively), whereas they did not exhibit statistically significant differences in PCOS women with hyperandrogenism (P = 0.073; Figure 1A). As shown in Figure 1B, PCOS patients with obesity the had significantly higher FFA levels in FF than controls (P = 0.005). The total FFA levels in PCOS patients with insulin resistance or hyperandrogenism were significantly elevated compared with controls (P < 0.001) and PCOS patients without insulin resistance (P = 0.027) or hyperandrogenism (P = 0.015). Moreover, the concentrations of LPG in each PCOS phenotype were obviously lower than the control group independently of obesity, insulin resistance, and hyperandrogenism (P < 0.001; Figure 1C). It is noteworthy that total LPG identified in FF includes LPG with default acyl group and alkyl-LPG. Since no significant difference was found in the alkyl-LPG (LPG O,18:1) level between patients with PCOS and controls, we only analyzed LPG, which represents the default acyl-LPG in this manuscript. Therefore, women with PCOS had an altered FF lipidomic profile in terms of total ceramide, total FFA, and LPG levels.

TABLE 2 Changes in the relative levels of candidate lipids in the FF for discrimination between women with PCOS and without PCOS.

Parameter	Control (n = 12)	PCOS (n = 25)	*P* value	PCOS *vs.* Control
Total Cer	416.24 ± 116.52	520.53 ± 136.39	0.029	↑
Total FFA	75435.12 ± 16657.29	92830.67 ± 17811.83	0.008	↑
LPG	66.27 ± 22.36	19.13 ± 6.87	< 0.001	↓

Data are presented as mean ± SD.
Cer, ceramide; FFA, free fatty acid; LPG, lysophosphatidylglycerol.

Analysis of lipid subclasses and selection of potential biomarkers in the FF of patients with PCOS

To investigate lipidomic differences between the PCOS and control groups and to further identify potential lipid biomarkers, we examined changes in the subclasses of ceramide and LPG in the FF samples. The lipids associated with differences in characteristics among the various groups were identified. As presented in Table 3, the FF levels of nine ceramide subclasses (Cer,34:1;2, Cer,36:1;2, Cer,36:2;2, Cer,38:1;2, Cer,38:2;2, Cer,40:0;2, Cer,40:1;2, Cer,40:2;2, and Cer,42:1;2), 11 FFA subclasses (C14:0, C14:1, C16:0, C16:1, C18:1, C18:3, C20:1, C20:4, C20:5, C22:0, and C22:6), and three LPG subclasses (LPG,18:0, LPG,18:1, and LPG,18:2) differed between patients with PCOS and control subjects (P < 0.05). Among the differential lipid subclasses in FF, concentrations of Cer,36:1;2, Cer,36:2;2, Cer,38:1;2, Cer,38:2;2, Cer,40:0;2, FFAs (C14:0, C14:1, C16:0, C16:1, C18:1, C18:3, C20:4, and C20:5) were statistically significantly higher in all of the Obese, IR, and HA groups than in the control group (P < 0.05). In addition, obese PCOS patients had higher FFA C16:0 levels than lean PCOS patients (P = 0.030); the IR group had higher FFAs (C16:0, C18:1, C18:3, and C20:4) concentrations than the NIR group (P < 0.05); the HA group also had significantly higher FF concentrations of FFAs (C16:0, C18:1, and C18:3) than the NHA group (P < 0.05). Furthermore, LPG,18:0 and LPG,18:1 levels were observed to be lower in obese, insulin-resistant, and

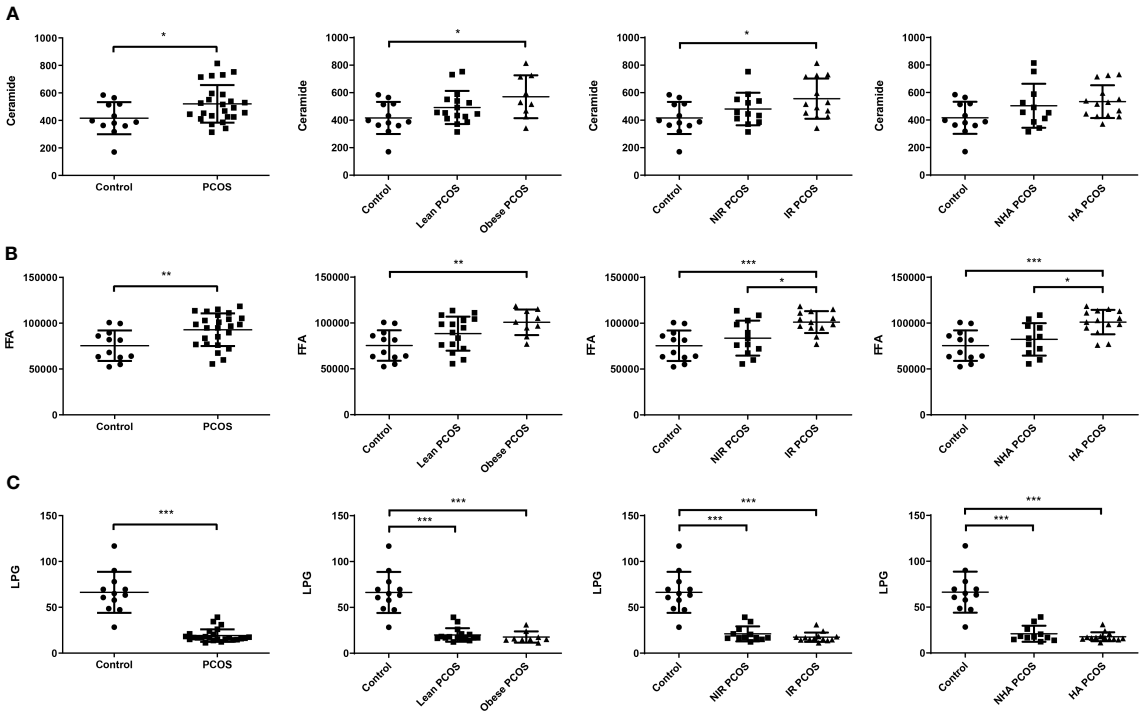

FIGURE 1
Changes in FF lipids in patients with PCOS (*n* = 25) and healthy controls (*n* = 12). FF ceramide **(A)**, FFA **(B)**, and LPG **(C)** levels in PCOS patients with different phenotypes and controls. Data are expressed as mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

TABLE 3 FF lipid subclasses in controls and women with PCOS.

Lipid Subclass	Control(n=12)	PCOS							
		All PCOS(n=25)	P	Classified according to BMI		Classified according to HOMA-IR		Classified according to FAI	
				Lean (n=16)	Obese (n=9)	NIR (n=12)	IR (n=13)	NHA (n=11)	HA (n=14)
Cer,34:1;2	54.31 ± 17.49	68.38 ± 20.14[a]	0.046	68.50 ± 21.88	68.16 ± 17.85	67.70 ± 24.47	69.00 ± 16.16	66.58 ± 16.56	69.79 ± 23.08
Cer,36:1;2	7.72 ± 1.58	10.95 ± 3.08[a]	< 0.001	10.39 ± 2.89[a]	11.95 ± 3.31[a]	9.98 ± 2.90[a]	11.85 ± 3.07[a]	9.81 ± 2.84	11.84 ± 3.06[a]
Cer,36:2;2	11.14 ± 4.09	17.89 ± 7.85[a]	0.008	16.14 ± 8.89	21.00 ± 4.45[a]	15.49 ± 10.19	20.10 ± 4.12[a]	15.06 ± 5.76	20.12 ± 8.73[a]
Cer,38:1;2	4.54 ± 1.13	6.27 ± 2.03[a]	0.002	5.98 ± 1.79	6.79 ± 2.42[a]	5.59 ± 1.51	6.90 ± 2.29[a]	5.67 ± 1.95	6.74 ± 2.03[a]
Cer,38:2;2	2.21 ± 0.72	3.04 ± 1.04[a]	0.018	2.78 ± 1.12	3.51 ± 0.73[a]	2.61 ± 1.24	3.44 ± 0.64[a]	2.76 ± 0.93	3.26 ± 1.11[a]
Cer,40:0;2	14.43 ± 5.29	19.92 ± 6.04[a]	0.011	18.43 ± 5.70	22.56 ± 6.02[a]	18.02 ± 5.61	21.67 ± 6.09[a]	18.18 ± 6.01	21.28 ± 5.92[a]
Cer,40:1;2	29.86 ± 9.21	39.03 ± 14.44[a]	0.032	36.06 ± 11.00	44.30 ± 18.70[a]	33.66 ± 8.51	43.98 ± 17.18[a]	37.05 ± 17.83	40.58 ± 11.59
Cer,40:2;2	9.17 ± 3.04	12.35 ± 4.87[a]	0.028	11.33 ± 3.81	14.16 ± 6.18[a]	10.60 ± 3.21	13.97 ± 5.67[a]	11.83 ± 5.23	12.76 ± 4.73
Cer,42:1;2	101.94 ± 37.75	143.00 ± 61.71[a]	0.031	129.90 ± 56.80	166.29 ± 66.51[a]	121.25 ± 42.60	163.08 ± 71.01[a]	131.60 ± 59.70	151.96 ± 63.98
FFA C14:0	225.46 ± 41.17	270.26 ± 46.20[a]	0.007	265.99 ± 51.51	277.85 ± 36.43[a]	250.87 ± 45.70	288.16 ± 40.39[a]	270.00 ± 53.03	270.46 ± 42.16[a]
FFA C14:1	18.52 ± 4.73	29.53 ± 6.33[a]	< 0.001	28.73 ± 7.02[a]	30.96 ± 4.93[a]	27.62 ± 6.45[a]	31.30 ± 5.91[a]	28.19 ± 5.77[a]	30.59 ± 6.75[a]
FFA C16:0	7843.83 ± 1521.67	9715.17 ± 1770.00[a]	0.003	9076.43 ± 1487.33	10850.72 ± 1727.49[a,b]	8657.06 ± 1464.87	10691.89 ± 1464.82[a,c]	8722.47 ± 1484.99	10495.16 ± 1611.98[a,d]
FFA C16:1	894.72 ± 233.15	1265.87 ± 331.47[a]	0.001	1229.92 ± 346.54[a]	1329.77 ± 311.89[a]	1178.28 ± 356.48	1346.73 ± 297.49[a]	1152.35 ± 309.92	1355.06 ± 330.89[a]
FFA C18:1	23446.51 ± 6480.94	30192.11 ± 6002.36[a]	0.004	28138.12 ± 5922.91	33843.65 ± 4343.97[a]	26924.01 ± 6066.24	33208.82 ± 4217.17[a,c]	25444.40 ± 4910.58	33922.46 ± 3729.05[a,d]
FFA C18:3	638.61 ± 211.96	900.21 ± 321.02[a]	0.015	809.68 ± 300.40	1061.14 ± 307.29[a]	721.88 ± 255.10	1064.82 ± 292.05[a,c]	728.31 ± 208.43	1035.27 ± 334.93[a,d]
FFA C20:1	731.96 ± 224.56	907.02 ± 233.85[a]	0.038	888.34 ± 271.64	940.24 ± 154.18	869.15 ± 299.21	941.98 ± 156.69	852.25 ± 300.46	950.06 ± 164.28
FFA C20:4	2542.89 ± 673.50	3731.62 ± 1233.04[a]	0.004	3516.01 ± 1234.55	4114.93 ± 1202.17[a]	3129.24 ± 1033.11	4287.67 ± 1168.16[a,c]	3243.78 ± 1216.73	4114.93 ± 1143.89[a]
FFA C20:5	69.32 ± 30.24	137.82 ± 64.95[a]	0.001	130.50 ± 61.31[a]	150.84 ± 72.89[a]	128.74 ± 65.89[a]	146.20 ± 65.57[a]	115.94 ± 61.33	155.01 ± 64.59[a]
FFA C22:0	33.75 ± 9.48	38.60 ± 6.67[a]	0.040	38.07 ± 6.53	39.53 ± 7.21	37.60 ± 6.87	39.51 ± 6.63	36.90 ± 6.28	39.93 ± 6.89
FFA C22:6	3531.41 ± 1114.99	5216.42 ± 1848.96[a]	0.006	5319.68 ± 2184.56[a]	5032.86 ± 1117.44	5020.88 ± 2471.28	5396.93 ± 1079.58[a]	4876.29 ± 2269.82	5483.67 ± 1474.26[a]
LPG,18:0	55.82 ± 20.68	14.52 ± 5.70[a]	< 0.001	15.42 ± 5.91[a]	12.92 ± 5.24[a]	16.54 ± 6.41[a]	12.65 ± 4.41[a]	15.98 ± 7.06[a]	13.37 ± 4.28[a]
LPG,18:1	5.38 ± 2.91	1.00 ± 0.65[a]	< 0.001	1.04 ± 0.55[a]	0.92 ± 0.83[a]	1.01 ± 0.55[a]	0.99 ± 0.75[a]	1.10 ± 0.63[a]	0.92 ± 0.67[a]
LPG,18:2	5.07 ± 1.49	3.62 ± 1.29[a]	0.004	3.48 ± 1.45[a]	3.86 ± 0.98	3.48 ± 1.68[a]	3.75 ± 0.85	3.85 ± 1.55	3.44 ± 1.07[a]

Data are presented as mean ± SD. [a]Compared with the control group, P < 0.05; [b]Compared with the Lean group, P < 0.05; [c]Compared with the NIR group, P < 0.05; [d]Compared with the NHA group, P < 0.05.
Cer, ceramide; FFA, free fatty acid; LPG, lysophosphatidylglycerol.

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hyperandrogenic women in the PCOS subgroup than the healthy controls (P < 0.001). The findings suggest that the levels of 23 lipid subclasses were significantly changed in the FF of women with PCOS, and they could potentially serve as the indicator of PCOS.

Correlations between FF lipids and oocyte quality

To further evaluate the potential interferences between FF lipid levels and oocyte quality in PCOS, the correlations between the levels of specific lipids and HQER were assessed. Six lipid subclasses were found to be highly correlated with oocyte quality using the Pearson or Spearman correlation tests in women with and without PCOS (Figure 2). Further linear regression analysis confirmed that the FF concentrations of Cer,36:1;2 (rs = –0.486, P = 0.002), Cer,38:1;2 (rs = –0.426, P = 0.009), Cer,38:2;2 (rs = –0.331, P = 0.045), Cer,40:0;2 (rs = –0.333, P = 0.044), and FFA C12:0 (rs = –0.385, P = 0.019) were significantly negatively associated with HQER (Figures 2A–E). Conversely, the levels of LPG,18:0 in FF showed a significantly positive correlation with HQER (rs = 0.355, P = 0.031; Figure 2F). We also found that the FF levels of differential lipids were closely correlated with the endocrine-metabolic parameters (Supplementary Table 2).

Identification of potential lipids responsible for distinguishing between women with and without PCOS

In total, 23 potential lipidomic biomarkers were identified by MS analysis. When evaluated alone Cer,36:1;2 yielded an AUC of 0.837, which was stronger than for total ceramide concentrations (0.713; Figure 3A). The AUC value of FFA C14:1 was higher than that of total FFA concentrations (0.913 vs. 0.763; Figure 3B). Among LPG, LPG,18:0 had the highest AUC (0.993; Figure 3C), indicating a strong impact of LPG activity.

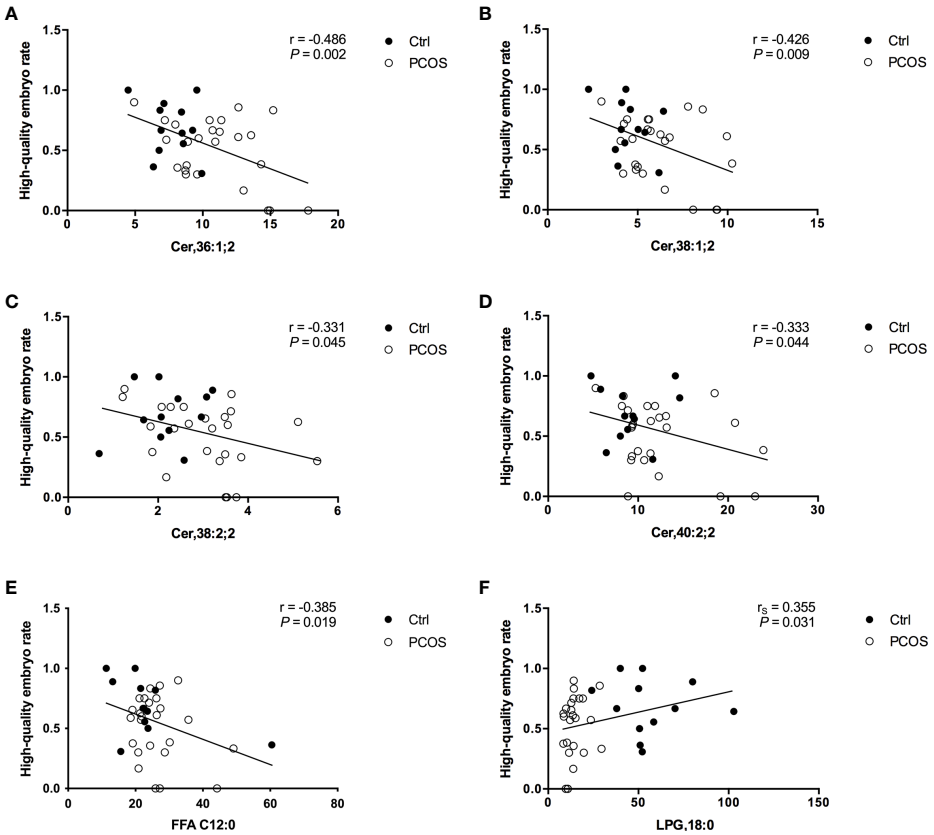

FIGURE 2
Correlation between specific FF lipids and embryo quality in patients with (white dots; *n* = 25) and without (black dots; *n* = 12) PCOS. Regression analysis between HQER and Cer,36:1;2 **(A)**, Cer,38:1;2 **(B)**, Cer,38:2;2 **(C)**, Cer,40:0;2 **(D)**, FFA C12:0 **(E)**, and LPG,18:0 **(F)** respectively was performed. The lines indicate the fitted regression curves.

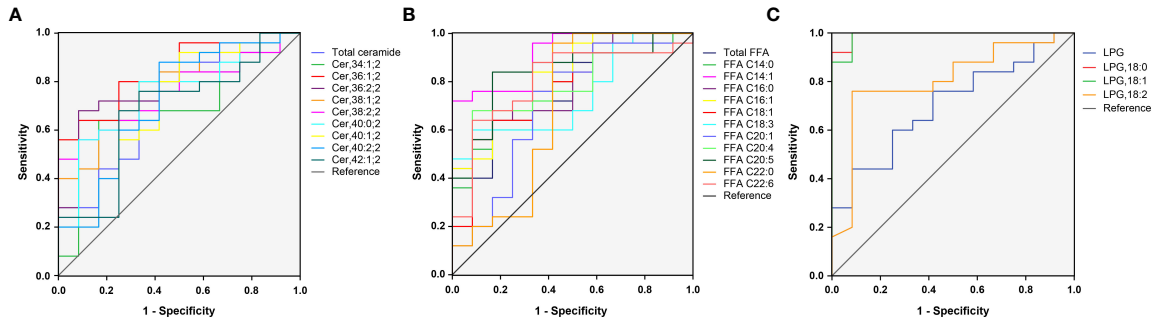

FIGURE 3
ROC curves for 23 selected lipid subclasses for differentiating women with PCOS from normal healthy individuals. The AUC value of Cer,36:1;2, FFA C14:1, and LPG,18:0 was larger than that of the total ceramide **(A)**, total FFA **(B)**, and LPG **(C)**, respectively.

Since some lipids have significant changes between PCOS subgroups, such as NIR/IR and NHA/HA, we selected four candidate lipid subclasses according to Table 3, and then ROC curve analysis was performed for PCOS subgroups. The AUC value of FFA C16:0 (0.827) was higher than that of the other lipid subclasses for distinguishing between PCOS women with and without insulin resistance (Supplementary Table 3), and FFA C18:1 yielded the highest AUC (0.903) for distinguishing between NHA and HA subgroups (Supplementary Table 4), indicating that these lipids could be used as subtype indicators of PCOS.

Discussion

Ovarian FF constitutes the microenvironment for follicle development and oocyte maturation. The FF comprises secretions from the granulosa cells (GCs), theca cells, and oocytes within or surrounding the follicle. The composition of FF has attracted considerable interest, and several metabolomic studies, including those on carbohydrate, amino acid, and lipid metabolism, have been conducted (31–35). However, research on sphingolipid metabolism, especially ceramide and its subclass in the FF of PCOS patients with different phenotypes is limited. In the previous study, we found that the content of ceramide in the serum of patients with PCOS was significantly higher than that of BMI-matched controls, which is similar to those reported by Li et al. (36, 37). We also found that the two subclasses Cer (OH_N16:0/N18:0) and Cer (N22:0) may act as lipid biomarkers for predicting PCOS and may play an important role in the pathogenesis of PCOS (36). Therefore, the current study aimed to investigate the lipidomic profiles of FF in PCOS patients with different phenotypes and their association with IVF outcomes.

Ceramide is an important lipid signaling molecule regulated by tumor necrosis factor-α, interferon-γ, Fas ligands, interleukin-1, and nerve growth factor. Ceramide is closely correlated with insulin resistance, metabolic inflammation, and non-alcoholic fatty liver disease (38–40). For example, elevated ceramide levels in the adipose tissue have been observed in obese patients and exacerbated metabolic disorders by inducing adipose tissue inflammation. Ceramide inhibits the protein phosphatase 2A (PP2A) or protein kinase C ζ/Akt signaling pathway, leading to insulin resistance (41, 42). We observed that patients with PCOS had a higher concentration of FF ceramides than controls. Further analysis showed that the ceramide levels in the Obese and IR groups were significantly higher than that of the control group, whereas no difference was seen between the HA and control groups, indicating that obesity or insulin resistance is significantly associated with high ceramide concentrations, rather than testosterone concentrations.

Moreover, ceramide and its metabolites are also involved in the regulation of cell differentiation, proliferation, and apoptosis (43). Eliyahu et al. (44) revealed that acid ceramidase (AC), a sphingolipid hydrolase that hydrolyzes ceramide into sphingosine, is highly expressed in FF, cumulus cells, and oocytes. Recombinant AC could enhance the survival rates and quality of oocytes and embryos grown *in vitro*, as well as embryo development *in vivo* after implantation, while conditional knockout of AC in mice showed elevated ceramide level in the ovary with a correspondingly significant decrease in fertility. These data indicate that high ceramide levels could impair fertility (45). Consistently, we also observed that Cer,36:1;2, Cer,38:1;2, Cer,38:2;2, and Cer,40:2;2 were negatively associated with HQER.

Oocyte maturation is dependent on the activation of the PI3K/Akt signaling pathway (46). Elevated ceramide concentration inhibits the Akt signaling pathway (40, 47), which may impair oocyte maturation. In addition, accumulating evidence suggests that intracellular free Ca^{2+} levels play an important role in the regulation of oocyte maturation and early embryonic development (48–50). The endoplasmic reticulum (ER) may be a vital mediator of Ca^{2+}

homeostasis *via* the inositol (1, 4, 5)-trisphosphate receptor (IP3-R) and sarco/ER Ca^{2+}-ATPase (SERCA) channels (51, 52). Ginzburg et al. (53) found that the inhibition of SERCA by saccharides alone is extremely weak, and the inhibitory effects increase significantly when the saccharides was attached to a ceramide backbone. Ceramide increases the concentration of IP3 in a dose-dependent manner *via* the activation of the Gq/11 and phospholipase C (PLC) signal pathway. IP3 triggers calcium release from the ER by activating IP3-R in the oocyte of *Xenopus laevis* (54). Calcium oscillations triggered by ceramide *via* the Gq/11-PLC-IP3 pathway may be involved in the regulation of calcium homeostasis during oocyte maturation and follicle development.

Patients with PCOS had higher FF FFA levels than controls. The total FFA levels were significantly increased in the Obese, IR, and HA groups compared with the control group. Furthermore, the IR and HA groups had significantly higher total FFA levels than the NIR and NHA groups respectively, thus suggesting that insulin resistance and hyperandrogenemia increase lipolysis and release more FFA into the circulation (55, 56). Further analysis revealed that the levels of 11 FFA subclasses (C14:0, C14:1, C16:0, C16:1, C18:1, C18:3, C20:1, C20:4, C20:5, C22:0, and C22:6) were increased in PCOS group. In addition, the IR and HA groups had higher C16:0, C18:1, and C18:3 levels than the NIR and NHA groups, while only the IR group had a higher C20:4 level than the NIR group. Palmitic acid (PA, C16:0), the most abundant saturated fatty acid in the circulation, is the main fuel for β oxidation and produces energy. It also causes lipid toxicity, which in turn leads to metabolic inflammation and insulin resistance. PA remarkedly suppresses GC survival in a time- and dose-dependent manner leading to reproductive abnormalities (57). Arachidonic acid (AA, C20:4) is one of the most abundant omega-6 polyunsaturated fatty acids in the blood and is a precursor to inflammatory mediators such as prostaglandins (PGE2, PGD2, PGI2, PGF2a, and PGJ2). Prostaglandins produced by the cyclooxygenase metabolic pathway are involved in different reproductive processes, such as follicle development, oocyte maturation, ovulation, and embryo implantation. Elevated AA levels in FF may produce excessive prostaglandins, leading to local inflammation, which in turn affects the follicle development and ovulation (58–60).

Herein we report that levels of three LPG subclasses (LPG,18:0, LPG,18:1, and LPG,18:2) were significantly lower in PCOS patients than in control subjects. Phosphatidylglycerol (PG) was first discovered in *Lactobacillus arabinoses* by Benson and Maruo in 1958, and it is widely found in animal plants and almost all bacteria. In animals, PG is mainly stored in the plasma membrane. With very few researches on the physiological and pathological role of PG and LPG, neither clinical nor basic studies on the relation between LPG and PCOS have been performed. We found a significant decline in FF LPG of patients with PCOS and no difference between the IR and NIR, HA, and NHA groups, suggesting that PCOS patients have decreased levels of LPG in FF with a unique mechanism. Additionally, the level of LPG O,18:1 was not associated with HQER in women with and without PCOS, and its ROC AUC was only 0.540. The effect of LPG on follicle development and oocyte maturation needs to be further assessed.

This study has several limitations. First, the relatively small sample size affected statistical efficiency. Second, FF samples were collected from patients who received gonadotropin stimulation and hence may not represent their natural state. Third, the composition of FF was highly variable and was associated with the developmental stage of follicles. Therefore, our findings could only reflect changes in the lipid composition of FF just before ovulation.

Conclusions

The present study found that patients with PCOS had elevated FF ceramide and FFA concentrations and decreased FF levels of LPG, especially in Obese and IR groups. High ceramide and FFA levels and low LPG levels might impair follicle development and oocyte maturation, which then affect HQER. Moreover, we showed that Cer,36:1;2, FFA C14:1, and LPG,18:0 could represent useful lipid biomarkers of embryo quality in PCOS patients. Thus, altering ovarian ceramide, FFA, and LPG synthesis or metabolism may help improve oocyte function and early embryo development, which provides a potential intervention for the treatment of PCOS.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Renji Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YD, SL, and YS designed the study. YL collected clinical information. QZ and YH performed biochemical analysis. YW and JQ collected patient samples. MZ and HY performed MS analysis. YJ, YF, and RH analyzed the data. YD and YJ wrote the

manuscript. SL and YS revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.960274/full#supplementary-material

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S100P promotes trophoblast syncytialization during early placenta development by regulating YAP1

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Recurrent pregnancy loss (RPL) is a severe complication of pregnancy that is caused by genetic abnormalities, immune dysfunction, aberrant cell biology, and tissue structure destruction. Among which, placental dysfunction is crucial in the pathogenetic progression of RPL. Although some regulatory factors associated with RPL have been reported, the placental changes correlated with RPL still need to be elucidated. Here, we found that a portion of RPL patients presented with low serum and placental S100P expression. Using a human trophoblast stem cell model, we demonstrated that S100P was exclusively expressed in syncytiotrophoblast (ST)-like syncytia (ST(2D)-TS^{CT}) and that loss of S100P expression in ST(2D)-TS^{CT} cells impaired β-hCG secretion, leading to syncytialization failure during early placental development. Moreover, we found that S100P is involved in regulating trophoblast syncytialization by downregulating the protein level of Yes-associated protein 1 (YAP1), which plays a pivotal role in maintaining trophoblast stemness. Together, our findings suggest that S100P plays an essential role in regulating trophoblast syncytialization during early placental development in humans *via* YAP1. Additionally, lower serum S100P levels may predict poor pregnancy outcomes and represent a potentially useful marker for evaluating placental biological function during early pregnancy.

KEYWORDS

trophoblast, syncytialization, S100P, YAP1, RPL

Introduction

Recurrent pregnancy loss (RPL) is defined as two or more consecutive spontaneous abortions of a clinically established intrauterine pregnancies before 20 weeks with the same spouse (1). Treatment of infertility caused by RPL has been a global challenge for decades, and RPL affects 2-5% of all pregnancies (2, 3). RPL is an intractable complication of pregnancy that is caused by a variety of factors, such as abnormal genetic development, immune dysfunction, cervical insufficiency, maternal endocrine abnormalities, infectious diseases, and male factors (4–7), leading to increased risks of affective disturbance, obstetric complications and longer-term health problems (8). The placenta is a crucial organ involved in the pathogenetic progression of RPL.

The syncytial layer, which is located at the outermost surface of the placenta and has a continuous surface measuring 12 to 14 m^2 at term, is directly bathed in maternal blood and is involved in nutrient exchange and hormone communication between the mother and fetus (9). The development of the syncytial layer initially includes the formation of multinucleated cells by lateral fusion of cytotrophoblast cells (CTBs). This process is completed *via* the fusion of new CTBs for the establishment of the multinucleated cell layer - the syncytiotrophoblast (STB); this process is called syncytialization (10). Unlike other epithelial differentiation processes, trophoblast syncytialization is both an endocrinological event and a cell fusion event (10, 11), and is regulated by various molecular and signaling pathways at the right temporal and spatial point (12). On the one hand, syncytialization results in the secretion of chorionic gonadotropin (CG) subunit proteins encoded by chorionic gonadotropin alpha (*CGA*) and chorionic gonadotropin beta (*CGB*), which maintain ovarian corpus luteum function (13). In addition, syncytialization results in the expression of 11β-HSD2, which is a microsomal enzyme complex that is responsible for the interconversion of cortisol and cortisone and thus protects the embryo from the potentially detrimental effects of cortisol (14). On the other hand, zonula occludens-1 (ZO-1) is located at intercellular boundaries between CTBs and between CTBs and the STB, forms tight junctions, and its expression is substantially decreased during cell fusion (15). Another transmembrane protein, E-cadherin, which is involved in cell-cell adhesion by promoting the formation of adherent junctions, is decreased during syncytialization due to alterations in both F-actin structure and expression (16, 17). However, the underlying mechanisms that are critical to regulating syncytialization are poorly defined.

In 2018, human trophoblast stem (TS) cells were successfully established from human blastocysts (TS^{blast}) and proliferative cytotrophoblast (TS^{CT}) cells which have the ability to differentiate into extravillous cytotrophoblast (EVT)- and syncytiotrophoblast (ST)-like cells (18, 19). Human TS cells have been demonstrated to be a valuable *in vitro* model for molecular and functional characterization analysis of human trophoblast cells because their transcriptomes and methylome profiles are similar to those of primary trophoblast cells (18, 20). When induced by exogenous agents, TS^{CT} cells cease proliferation and commence differentiation into STB and EVTs. The gene expression profiles of TS^{CT}-derived ST-like cells were similar to those of primary ST cells. Therefore, human TS cells are useful for studying placental development and understanding the pathogenesis of placental disorders (18).

S100P is a member of the S100 family. As a 95-amino acid calcium-binding protein involved in calcium signaling transduction, S100P was first purified and characterized from placentas (21–23). The structure of S100P is altered after binding to calcium, resulting in protein activation and the acquisition of a conformation enabling binding of other proteins (24). S100P regulates many intracellular and extracellular activities, including gene transcription, protein phosphorylation, enzyme activities, cytoskeleton component dynamics, cell proliferation and differentiation (25). S100P contains two EF-hand domains. Each domain consists of two helices, and a calcium-binding loop exists between them. S100P plays a key role in the progression of a variety of cancers (26). In lung cancers, S100P increases cell migration, invasion and metastasis by interacting with integrin α7, which activates focal adhesion kinase (FAK) and AKT (27). S100P is overexpressed in colorectal cancer tissues and participates in regulating cell invasion and metastasis by binding to the SLC2A5 promoter, thereby reducing its methylation and activating its transcription (28). Although most studies have focused on the function of S100P in malignant tumors, a relationship between S100P and calcium signaling in mammalian embryo and placental development has also been reported given that S100P promotes cell proliferation, survival, invasion and vascularization (29–32). Although there is a lack of evidence showing that S100P is directly involved in regulating mouse placenta development, calcium signaling is required for trophoblast proliferation, invasion, differentiation, and fusion during mouse placentation (23). In humans, recent studies have indicated that S100P is highly expressed in the human endometrium during the "implantation window" (33–35). As gestation progresses, S100P is strongly expressed on the syncytial layer of the human placenta, and this expression lasts throughout gestation (36). In addition, the transient receptor potential (TRP) channel protein TRPV6, which plays a vital role in calcium transport, is expressed in human CTBs, and this expression increased during syncytialization (37). Furthermore, S100P enhanced the motility and invasion of human trophoblast cell lines (38) and was able to regulate trophoblast-like cell proliferation *via* the P38 mitogen-activated protein kinase (MAPK) pathway (39).

The Hippo signaling pathway is a highly conserved signaling pathway that participates in a variety of physiological processes,

including organ size control, tissue regeneration, cell stemness and differentiation (40–43). The Hippo signaling pathway is activated by numerous triggers, such as high cell density, G protein-coupled receptor signaling, and cell polarity, provoking phosphorylation of macrophage-stimulating protein kinases 1/2 (MST1/2) and large tumor suppressor kinases 1/2 (LATS1/2) (44). Yes-associated protein 1 (YAP1), a key component of the Hippo signaling pathway, interacts with the PDZ-binding motif (TAZ), resulting in its inactivation by either degradation or cytoplasmic retention. Conversely, when the Hippo signaling pathway is "off", YAP1 is dephosphorylated and translocates to the nucleus, where it maintains stemness and promotes proliferation by acting as a coactivator of TEA domain (TEAD) transcription factor families. The Hippo signaling pathway could be a main driver of placental development (45). Intriguingly, YAP/TEAD4 transcriptional complexes play vital roles in murine trophectoderm (TE) development in preimplantation embryos by activating *Cdx2* (46, 47). Regarding the importance of YAP/TEAD4 complexes for trophoblast development in postimplantation embryos, recent studies have demonstrated that both YAP1 and TEAD4, which are specifically conserved in the CTB progenitors of human first-trimester placentas, are crucial for the self-renewal of TS cells and the proliferation of progenitors (48).

S100P expression is regulated by a variety of transcription factors. It has been reported that the SMAD and STAT families are promoters of S100P that can upregulate S100P expression (49). On the one hand, inhibition of the kinase Warts (Wts), an upstream factor of the Hippo signaling pathway, or activation of the downstream transcriptional cofactor Yorkie (Yki) reduces the expression level of STATs (50). On the other hand, SMADs have also been confirmed to be negatively regulated by YAP/TAZ, transcriptional cofactors downstream of the Hippo pathway (51, 52). Research has shown that the YAP/TEAD complexes suppress lung and breast cancer metastasis by sequestering S100P *via* the lncRNA *NORAD* (31). Furthermore, it has been demonstrated that the induction of S100A7, a member of the S100 family, can be significantly inhibited by nuclear YAP, and TEAD1 is required for YAP transcriptional repression of S100A7 (53, 54).

Although a variety of studies have shown that S100P is regulated by YAP1 of the Hippo signaling pathway in different tissues and organs, the relationship between S100P and YAP1 in the human placenta and their roles in regulating trophoblast syncytialization remain unclear. Therefore, in this study, we aimed to investigate the vital role of S100P in trophoblast syncytialization during early gestation. We used human trophoblast stem cells and gain- and loss-of-function methods to evaluate S100P-mediated molecular mechanisms involved in trophoblast syncytialization. Furthermore, using patient-derived villi and serum, we tested whether downregulation of S100P is associated with idiopathic RPL. Our analyses demonstrated an underlying mechanism in which S100P regulates trophoblast syncytialization during early gestation by inhibiting YAP1 to induce placentation and ensure progression of pregnancy.

Materials and methods

Human samples

The first-trimester human placenta tissues of RPL patients and healthy controls (HCs) were collected from women undergoing dilatation and curettage at the sixth to eighth week of gestation in the Department of Obstetrics and Gynecology of the Sir Run Run Shaw Hospital affiliated to the School of Medicine, Zhejiang University. The study was reviewed and approved by the Ethics Committee of Sir Run Run Shaw Hospital affiliated to the School of Medicine, Zhejiang University. All participants were provided with written informed consent to participate in this study.

The HCs were included women who already had a healthy pregnancy at term and opted for an elective abortion at 6-8 weeks in a subsequent apparently healthy pregnancy. Patients suffering from RPL were included if they had an empty gestational sac or embryo stoppage confirmed by type-B ultrasound scans at least twice, containing the current pregnancy. The exclusion criteria for RPL were (i) genital malformation; (ii) abnormal karyotype of parents or abortuses; (iii) endocrine or metabolic disorders; (iv) autoimmune diseases; (v) other major diseases such as hypertension and cancers; and (vi) improper drug treatment, exposure to chemicals or radiation (55). A total of 16 HC and 16 RPL first-trimester placentas were collected. The clinical characteristics of the recruited patients are provided in Table 1.

TABLE 1 Clinical characteristics of the recruited patients.

	Healthy controls	Recurrent pregnancy losses	*p* value
Sample size	16	16	
Maternal age (years)	25.8 ± 2.6	28.4 ± 2.3	ns
Gestational age at D&C (days)	48 ± 3.8	50 ± 3.4	ns
Number of spontaneous abortions	n/a	2.5 ± 0.63	n/a

Data are mean values ± SEM. D&C, dilatation and curettage; ns, not significant; n/a, not applicable.

Isolation, differentiation and identification of human trophoblast cells

The isolation and culture of human TS^{CT} cells were performed following a published protocol (18). Three first-trimester placental villi from healthy mothers were collected. The sex and developmental stage of each human sample used for the derivation of TS^{CT} cells are summarized in Supplementary Table SI. In brief, the first-trimester placental villi were collected and cut into small pieces. All tissues were enzymatically digested thrice in a mixture containing equal amounts of TrypLE (Thermo Fisher Scientific, USA) and Accumax (Innovative Cell Tech, USA) for 20 min at 37°C. Cell suspensions were then filtered through a 70 μm mesh filter (352350, BD Falcon, USA). ITGA6 is a widely used cell lineage marker for CTBs (56). CTBs were immunomagnetically purified using an EasySep phycoerythrin (PE)-positive selection kit (Stemcell Technologies, Canada) and a PE-conjugated anti-ITGA6 antibody (130-097-246, Miltenyi, Germany). The selected cells were seeded in a 6-well plate (Corning, USA) coated with 5 μg/mL collagen IV (354233, Corning, USA) at a density of 0.5-1×10^6 cells per well and cultured in 2 mL of TS medium. CTBs were dissociated with TrypLE for 10-15 min at 37°C. The cells at passages 10-30 were used for analyses and differentiation assays. Information regarding the TS medium is presented in Supplementary Table SII.

For ST(2D)-TS^{CT} cell differentiation, TS^{CT} cells were collected and then seeded into a 6-well plate precoated with 2.5 mg/mL collagen IV at a density of 1×10^5 cells per well. The ST(2D) medium was replaced at day 3, and the culture was continued for 3 days.

To assess ST(3D)-TS^{CT} cell differentiation, TS^{CT} cells were seeded in 6-cm Petri dishes and cultured in 3 mL of ST(3D) medium. An equal amount of fresh ST(3D) medium was added at day 3, and the cells were collected at day 6 after they were passed through a 40-μm mesh filter (352340, BD Falcon, USA) to remove dead cells and debris. The cells remaining on the 40-μm mesh filter were used for further research.

For the induction of EVT-TS^{CT} cells, TS^{CT} cells were seeded in 6-well plates precoated with 1 mg/mL collagen IV at a density of 0.75×10^5 cells per well. Matrigel (354234, Corning, USA) was added to a final concentration of 2% immediately after suspending TS^{CT} cells in the precooled medium. The medium was replaced with EVT medium without NRG1 on day 3, and Matrigel was added to a final concentration of 0.5%. After the cells reached 80% confluence at day 6, they were dissociated with TrypLE for 15-20 min at 37°C and passaged to a new collagen IV precoated 6-well plate at a 1:2 split ratio. The cells were suspended in the EVT medium without NRG1 and KSR, and Matrigel was added to a final concentration of 0.5%. EVT-TS^{CT} cells were collected and analysed after two additional days. Information regarding ST(2D), ST(3D) and EVT media is presented in Supplementary Table SII.

The morphologies of TS^{CT}, ST(2D)-TS^{CT} and EVT-TS^{CT} cells were captured by an inverted microscope Primovert with a 10x objective using bright field microscopy (Zeiss, Germany).

Measurement of β-hCG and S100P levels

The supernatant of TS cells was collected as a control. The ST(2D)-TS^{CT} cell medium was replaced on day 3 and collected on day 3 and 6. All supernatants were centrifuged at 10000 × g for 10 minutes to remove impurities before further research. The levels of secreted β-hCG and secreted S100P were measured using an hCG enzyme-linked immunosorbent assay (ELISA) kit (ABN-KA4005, Abnova, Japan) and a S100P ELISA kit (ABN-KA0093, Abnova, Japan), respectively. Total protein was extracted from cells using Radio Immunoprecipitation Assay (RIPA) buffer (P0013B, Beyotime, China) at 4°C for 30 minutes and measured with a Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (23225, Thermo Scientific, USA). The final concentrations of secreted β-hCG and secreted S100P were normalized to the total protein concentration. Serum secreted S100P levels were measured with a S100P ELISA kit (ABN-KA0093, Abnova, Japan) following the instructions.

Real-time quantitative PCR analysis

Total RNA samples were isolated from TS^{CT} and ST(2D)-TS^{CT} cells using an RNA-Quick Purification Kit (RN001, ES Science, China) in accordance with the manufacturer's protocol. Total RNA samples (1 μg) were reverse-transcribed with HiScript II Reverse Transcriptase (R201-1, Vazyme, China). SYBR Green Supermix (D7260, Beyotime, China) and the CFX96 system (Bio-Rad, USA) were used for real-time PCR. The relative gene expression levels in the experimental samples were compared with those in the control samples, and all experiments were performed in triplicate. The primer sequences are listed in Supplementary Table SIII.

Western blotting

Protein samples were extracted from tissues and cells using ice-cold RIPA lysis buffer containing 1% phenylmethanesulfonyl fluoride (PMSF) (ST506, Beyotime, China). Total prepared protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (PG113 and PG114, Epizyme Biotech, China) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore,

USA). After blocking with 5% skim milk (232100, BD Difco, USA) at room temperature for at least 1 hour, the membranes were incubated with primary antibodies overnight at 4°C. The following primary antibodies were used in the experiments: S100P (ab133554, 1:1000, Abcam, USA), TEAD4 (ab58310, 1:1000, Abcam, USA), β-hCG (sc271062, 1:300, Santa Cruz, USA), YAP1 (sc101199, 1:500, Santa Cruz, USA), 11β-HSD2 (sc365529, 1:500, Santa Cruz, USA), E-cadherin (3195, 1:1000, CST, USA), ZO-1 (13663, 1:1000, CST, USA), and GAPDH (60004-1-Ig, 1:1000, Proteintech, China). After washing in Tris-buffered saline with Tween 20 (TBST) buffer, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (7074, 1:3000, CST, USA) or anti-mouse IgG secondary antibodies (7076, 1:3000, CST, USA), and visualized by enhanced chemiluminescence (WBKLS0500, Millipore, USA). The images were captured using a ChemiDoc Touch Imaging System (Bio-Rad, USA). In addition, other membranes were incubated with preabsorbed goat anti-rabbit IgG H&L IRDye 800 (ab21677, 1:5000, Abcam, USA) or donkey anti-mouse IgG H&L Alexa Fluor 680 (ab175774, 1:5000, Abcam, USA) secondary antibodies and then visualized using an Odyssey Infrared Laser Imaging System (Licor Bioscience, USA). ImageJ software was used to quantify the intensities of the Western blotting bands.

Immunofluorescence staining and fusion index calculation

Cells were fixed with 4% paraformaldehyde (MA0192, Meilunbio, China) for at least 30 min and then permeabilized with 0.3% Triton X-100 (T8787, Sigma-Aldrich, USA) for 5 min at room temperature. The frozen sections were permeabilized with 0.3% Triton X-100 for 10 min at room temperature. After blocking in 2% goat serum for 1 hour at room temperature, cells or sections were incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: S100P (ab133554, 1:100, Abcam, USA), β-hCG (ab9582, 1:50, Abcam, USA), YAP1 (sc101199, 1:50, Santa Cruz, USA), TEAD4 (ab58310, 1:50, Abcam, USA), HLA-G (ab7758, 1:50, Abcam, USA), E-cadherin (3195, 1:50, CST, USA), and ZO-1 (13663, 1:50, CST, USA). After washing with TBST buffer, cells or sections were incubated with Alexa Fluor 488- or Alexa Fluor 568-conjugated goat secondary antibody (A11001; A10037; A11034; A11036, 1:500, Invitrogen, USA) for 1 hour at room temperature. The nuclei were stained with Hoechst 33342 Stain Solution (H3570, 1:1000, Invitrogen, USA) for an additional 10 min, and images were captured with a laser scanning confocal microscope (Zeiss, LSM800, Germany).

The fusion index is calculated as the ratio of the number of nuclei in the syncytia divided by the total number of nuclei, and the number of nuclei in the syncytia, defined as nuclei at least three are surrounded by a cell membrane (11). Cell boundaries were marked by ZO-1 (13663, 1:50, CST, USA). The total number of nuclei and the number of nuclei in the syncytia were counted in each slide. At least ten images from each group were captured and analyzed. All slides were counted by two independent individuals for comparison.

Immunohistochemistry staining of paraffin sections

Paraffin slides were dewaxed with xylene for 20 min and 100%, 95%, and 75% ethanol in sequence for a total of 30 min and boiled in Antigen Retrieval Buffer (citrate buffer, pH 6.0, or Tris-EDTA buffer, pH 9.0) (ab93678 and ab93684, Abcam, USA) at 100°C for 15 minutes. The slides were blocked with 2% goat serum for 1 hour at room temperature and incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: S100P (ab133554, 1:100, Abcam, USA) and β-hCG (ab9582, 1:50, Abcam, USA). After washing with TBST buffer, the slides were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature (GK500710, Gene Tech, China). HRP-conjugated antibodies were detected with diaminobenzidine (DAB) (GK500710, Gene Tech, China) for 10 min. The nuclei were then stained with hematoxylin solution (ab220365, Abcam, USA) for an addition 5 min. Images were captured with an Axio Scope A1 (Zeiss, Germany).

Small interfering RNA transfection

To knockdown *S100P* expression, TS^{CT} and ST(2D)-TS^{CT} cells were cultured in 12-well plates and transfected with small interfering RNAs (siRNAs) targeting *S100P* (siS100P) or negative control siRNA (siNC) with Lipofectamine™ 3000 Transfection Reagent (L3000015, Thermo Fisher Scientific, USA) following the manufacturer's instructions. The siRNAs were purchased from Ribo Bio Co., Ltd. (Guangzhou, China). The siS100P sequence was 5-CAAGGATGCCGTGGATAAA-3, and the negative control sequence was 5-GATCATACGTGCGATCAGA-3.

Overexpression in TS^{CT} cells

Cell lines overexpressing *S100P* were generated by infection with a lentiviral vector (CMV-MCS-3FLAG-Ubi-ZSGreen-IRES-Puro) carrying the *S100P* cDNA sequence; the vector was purchased from Guannan Bio Co., Ltd. (Hangzhou, China). TS^{CT} cells were seeded in six-well plates at 80-90% confluence the day before infection. The next day, TS^{CT} cells were infected with lentivirus in culture medium for 24 hours.

Then, the medium was replaced before treatment. Approximately 80% of the cells were infected with overexpression lentivirus. Successful infection was confirmed by qPCR and Western blotting assessments of S100P levels. For long-term culture, clones were maintained in the presence of 2 μg/mL puromycin.

The recombinant human S100P protein (12635-HNAE, Sino Biological, China) consisted of 95 amino acids and had a calculated molecular mass of 10.4 kDa. The recombinant protein was first diluted with ultrapure water at room temperature and then added to TS^{CT} cell culture medium at concentrations of 5, 10, 15, and 20 μM for 24 hours.

RNA-seq

TS^{CT} cells were seeded into 10-cm cell culture dishes and differentiated into ST(2D)-TS^{CT} cells at a 1:1 ratio. On day 1, the ST(2D)-TS^{CT} cells were treated with siRNA targeting *S100P* or negative control as described above. After 4 days, the cells were harvested and total RNA from each group was extracted with TRIzol reagent (Vazyme, China). Three replicate samples were collected for each group. The samples were then processed for reverse transcription, cDNA purification and library construction by Applied Protein Technology company (Shanghai, China). The results were analyzed on the Illumina NovaSeq 6000/MGISEQ-T7 platform. Differentially expressed genes were analyzed with significance criteria of log2 (fold change) ≥1 and multi-test adjusted $p \leq 0.05$ by Cufflinks (version 2.1.1) and R (version 3.5.1). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using clusterProfiler (version 3.18.1). The processed RNA-seq data with fragments per kilobase per million (FPKM) values of all genes are presented in Supplementary Table SIV.

Statistical analysis

All the experiments in this study were performed on three TS^{CT} cell lines at least in triplicate. GraphPad Prism 6.0 software was used for statistical analysis. Statistical comparisons between two groups were carried out using unpaired Student's t test after confirming the normal distribution of the data by one-sample Kolmogorov-Smirnov test for Gaussian distribution or using Mann-Whitney U test for comparisons of data that did not show Gaussian distribution. The comparison of continuous variables among groups was carried out by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) tests. All the data are shown as the mean values ± standard error of the means (SEMs).

Results

S100P expression is decreased in the serum and placentas of RPL patients during early gestation

We collected villi from HC and RPL women in the sixth to eighth week of gestation and performed the experiments laid out in the flowchart (Figure 1). We found that S100P expression was exclusively localized with β-hCG and 11β-HSD2 expression on the syncytial layer of villi from healthy human placentas (Figure 2A). To further determine S100P expression levels in placenta tissues, we collected villi from healthy mothers and patients suffering from RPL during early gestation. The immunohistochemistry results showed low S100P and β-hCG expression in the villi of RPL patients (Figure 2B), in line with the Western blotting results, which indicated low expression of S100P (Figure 2C). As S100P is a secreted calcium-binding protein that can be detected in both human serum and plasma, we measured the serum levels of S100P in patients with RPL and compared them with those in HCs. Interestingly, the serum S100P levels were significantly decreased in patients with RPL (Figure 2D).

S100P is exclusively expressed during trophoblast cell syncytialization *in vitro*

To detect the role of S100P in syncytialization regulation, we first isolated and identified TS^{CT} cells, which had the ability to differentiate into ST- and EVT- like cells (Figure S1A). TEAD4 is a marker for TS^{CT} cells, β-hCG is a marker for ST(2D)-TS^{CT} cells, and human leucocyte antigen-G (HLA-G) is a marker for EVT-TS^{CT} cells (Figure S1B). After the successful establishment of the TS^{CT} cell model, we treated human TS^{CT} cells with forskolin (FSK) as previously described (18). Under the presence of FSK for 6 days to fully induce TS^{CT} syncytialization, S100P expression was progressively increased at both the mRNA and protein levels in ST(2D)-TS^{CT} cells, as measured at days 3 and 6 (Figures 3A, B). The increased expression of 11β-HSD2 indicated successful syncytialization (Figure 3B). In addition, S100P was expressed both in the cytoplasm and nucleus of ST(2D)-TS^{CT} and ST(3D)-TS^{CT} cells (Figure 3C). Interestingly, the level of secreted S100P was significantly increased during FSK-induced syncytialization *in vitro* (Figure 3D), and the level of secreted β-hCG, which is a marker for syncytialization, was also increased (Figure 3E). These results indicated that S100P was progressively and exclusively expressed intracellularly and secreted outside the cells during TS^{CT} cell syncytialization. In addition, the localization of S100P in ST(2D)-TS^{CT} cells indicated that it

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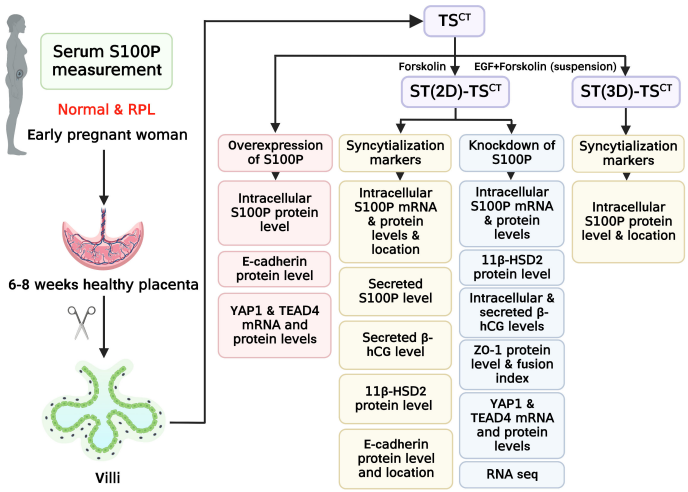

FIGURE 1
Flowchart of the experimental design. Healthy controls and patients with RPL were chosen based on the inclusion criteria. Villi and serum samples from participants were collected to measure tissue and serum S100P levels, respectively. Human TS^{CT} cells were isolated and cultured from human villous cytotrophoblasts. ST(2D)-TS^{CT} and ST(3D)-TS^{CT} cells were used to investigate intracellular S100P expression and location, whereas ST(2D)-TS^{CT} cells were used to detect 11β-HSD2 protein levels, E-cadherin expression and localization, and secreted S100P and β-hCG levels in the culture medium. After knocking down *S100P* in ST(2D)-TS^{CT} cells, intracellular S100P mRNA and protein levels, 11β-HSD2 protein levels, intracellular and secreted β-hCG levels, ZO-1 expression and localization, the fusion index, YAP1 and TEAD4 mRNA and protein levels, and RNA-seq were detected. After overexpressing *S100P* in TS^{CT} cells, intracellular S100P and E-cadherin protein levels, and YAP1 and TEAD4 mRNA and protein levels were detected.

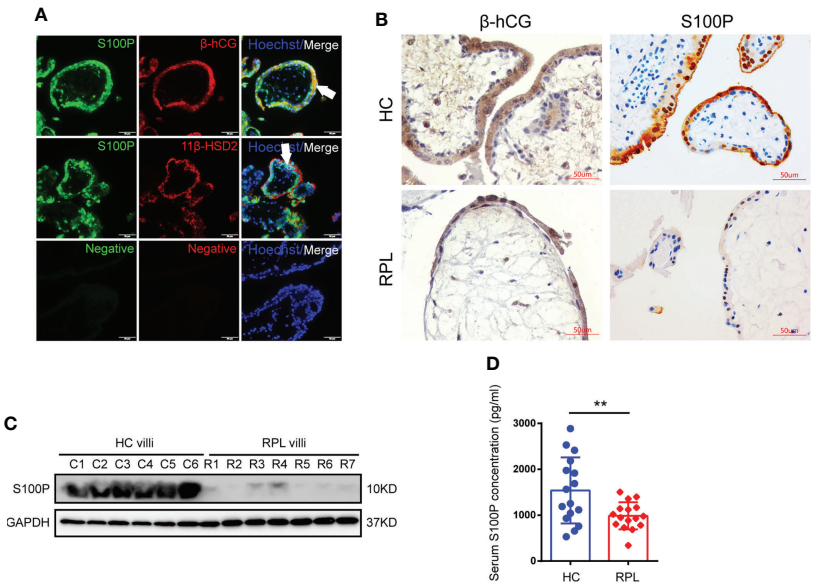

FIGURE 2
Placenta and serum S100P levels in healthy controls and RPL patients. **(A)** Immunofluorescence staining of S100P (green), 11β-HSD2 (red), β-hCG (red) and Hoechst staining (blue) in human healthy placentas (sixth to eighth week of gestation). White arrows indicate STB. Representative images from three independent experiments are shown. Scale bar, 50 μm. **(B)** Immunohistochemistry staining of S100P in human placentas extracted from women at six- to-eight weeks of gestation. β-hCG was used to identify syncytiotrophoblast layers. Scale bars, 50 μm. **(C)** Representative Western blotting of S100P expression in normal and RPL placental tissues (Ctrl n=6 vs. RPL n=7). **(D)** Measurement of serum S100P levels in healthy controls and RPL patients by ELISA (n=16). $^{**}p=0.0079$.

may be involved in different biological processes, such as cellular signaling transduction and gene transcription assistance.

S100P is involved in regulating trophoblast syncytialization

To determine the biological significance of S100P in human trophoblast cells, we used small interfering RNAs to interfere with *S100P* expression and a lentiviral vector to overexpress *S100P* in human trophoblast stem cells. After transfection followed by differentiation for 6 days, we found a substantial decrease in S100P expression at both the mRNA and protein levels in ST(2D)-TS^{CT} cells (Figures 4A, B). Cells transfected with siS100P exhibited a reduction in 11β-HSD2 expression levels by Western blotting (Figure 4B), intracellular β-hCG expression by Western blotting and immunofluorescence (Figures 4C, D), and secreted β-hCG concentration by ELISA (Figure 4E) compared with cells transfected with the control siRNA. ZO-1 expression was significantly increased in ST(2D)-TS^{CT} cells with *S100P* knockdown (Figure 4C, representative images are shown in Figure 4F), while the fusion index was decreased (Figure 4G). Conversely, neither overexpression of the *S100P* gene nor exogenous addition of recombinant human S100P protein triggered syncytialization of TS^{CT} cells, whereas E-cadherin protein expression was decreased (Figures 4H, I). These results demonstrated that S100P was involved in regulating the trophoblast syncytialization process, but failed to directly trigger TS^{CT} cell syncytialization even if the expression of the cell-cell adhesion protein E-cadherin was significantly decreased after *S100P* was overexpressed.

YAP1 maintains the progenitor status of CTB-derived human trophoblast stem cells

YAP1 colocalized with E-cadherin in the cytotrophoblast layer of human healthy placentas (six-to-eight weeks of gestation) (Figure 5A). YAP1 mRNA and protein expression

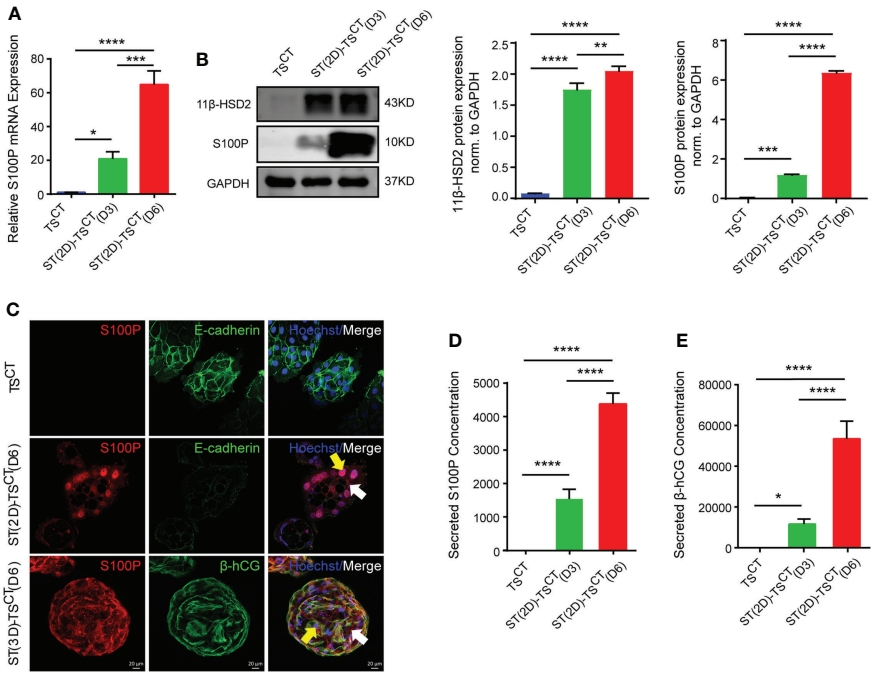

FIGURE 3
Increased S100P expression during FSK-induced syncytialization of TS^{CT} cells. **(A)** Quantification of *S100P* mRNA expression in ST(2D)-TS^{CT} cells measured on D3 and D6. The mean values ± SEMs (normalized to *GAPDH*) are shown. D3: day 3, D6: day 6. *p=0.0110; ***p=0.0002; ****p<0.0001. **(B)** Western blotting analysis of S100P and 11β-HSD2 in ST(2D)-TS^{CT} cells measured on D3 and D6. Semiquantitative analysis of S100P and 11β-HSD2 protein expression normalized to GAPDH (n=3). **p=0.0073; ***p=0.0006; ****p<0.0001. **(C)** Immunofluorescence staining of S100P (red), E-cadherin (green), β-hCG (green) and Hoechst staining (blue) in human TS^{CT}, ST(2D)-TS^{CT} (D6) and ST(3D)-TS^{CT} cells (D6). White arrows indicate cytoplasmic S100P expression, and yellow arrows represent nuclear S100P expression. Representative images from three independent experiments are shown. Scale bar, 20 μm. **(D)** Measurement of the secreted S100P levels in human TS^{CT} and ST(2D)-TS^{CT} cultures (D3 and D6) by ELISA. The levels were measured in three independent experiments. ****p<0.0001. **(E)** Measurement of secreted β-hCG levels in human TS^{CT} and ST(2D)-TS^{CT} cultures (D3 and D6) as assessed by ELISA. The levels were measured in three independent experiments. *p=0.0283; ****p<0.0001.

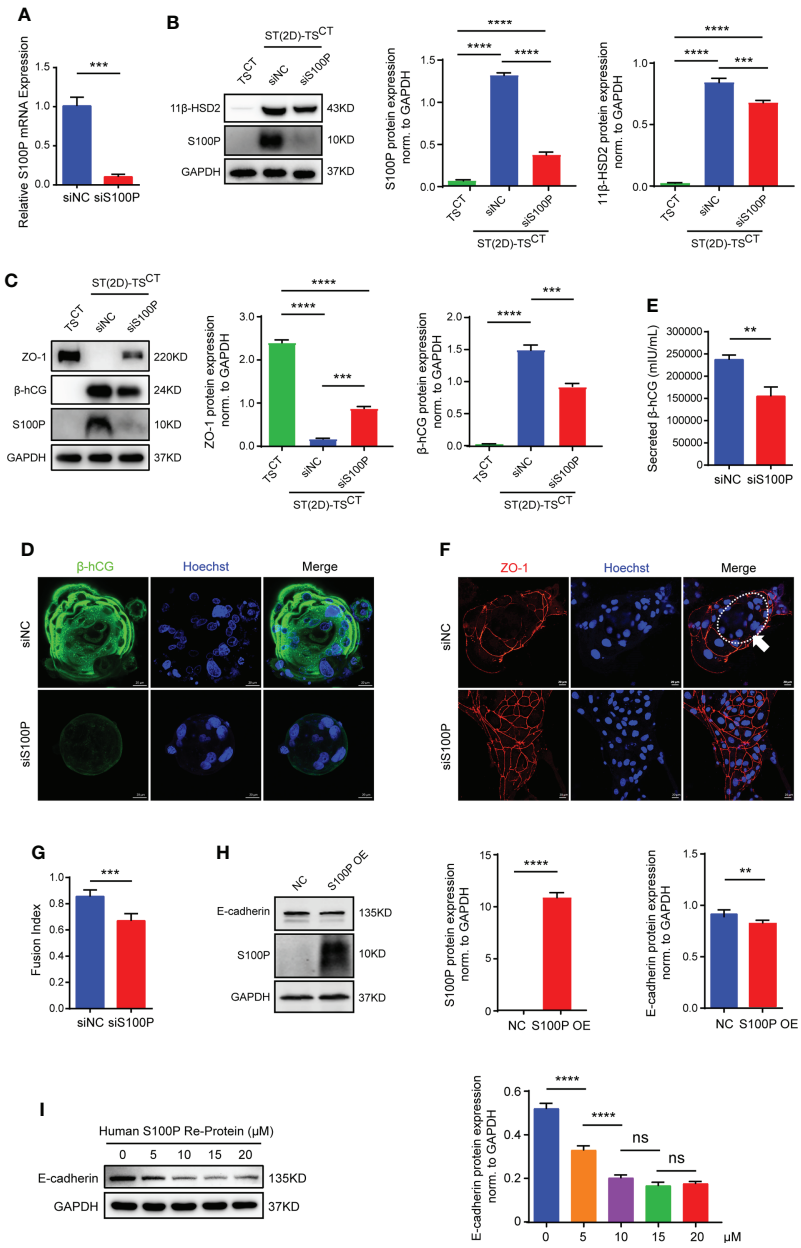

FIGURE 4
S100P is involved in regulating trophoblast syncytialization. **(A)** Quantification of *S100P* mRNA expression. The mean values ± SEMs (normalized to *GAPDH*) of ST(2D)-TS^{CT} cells (D6) are shown. ***p=0.0002. **(B)** Representative Western blotting of S100P and 11β-HSD2 in siS100P-transfected ST (2D)-TS^{CT} cells (D6). Semiquantitative analysis of S100P and 11β-HSD2 protein expression normalized to GAPDH (n=3). ***p=0.0003; ****p<0.0001. **(C)** Representative Western blotting of ZO-1, β-hCG, and S100P in siS100P-transfected ST(2D)-TS^{CT} cells (D6). Semi-quantification of ZO-1 and β-hCG protein expression normalized to GAPDH (n=3). ***p=0.0001, p=0.0003; ****p<0.0001. **(D)** Representative immunofluorescence images stained for the syncytialization marker β-hCG are shown. Hoechst staining was performed to visualize nuclei (n=3, ST(3D)-TS^{CT} cells). Scale bars, 20 μm. **(E)** ELISA measurement of the β-hCG level secreted into the supernatants of the cultures of ST(2D)-TS^{CT} cells (D6) transfected with siS100P. The mean values ± SEMs are shown. **p=0.0038. **(F)** Representative immunofluorescence images of ST(2D)-TS^{CT} cells (D6) transfected with siS100P with ZO-1 (red) and Hoechst staining (blue). The white arrow indicates the fusion area. Representative images of three independent experiments are shown. Scale bar, 20 μm. **(G)** Fusion index of ST(2D)-TS^{CT} cells (D6) transfected with siS100P. The index was assessed in ten images, and the mean values ± SEMs are shown. ***p=0.0002. **(H)** Representative Western blotting of S100P and E-cadherin expression in TS^{CT} cells infected with lentiviral vectors overexpressing *S100P*. Semiquantitative analysis of S100P and E-cadherin protein expression normalized to GAPDH (n=3). **p=0.0084; ****p<0.0001. **(I)** Representative Western blotting of E-cadherin expression in TS^{CT} cells treated with recombinant human S100P protein (5 μM, 10 μM, 15 μM, and 20 μM) (n=3). Semiquantitative analysis of E-cadherin protein expression normalized to GAPDH (n=3). ****p<0.0001; ns: p=0.1208, p=0.9457.

gradually decreased with syncytialization, as measured on day 3 and 6 (Figures 5B, C). The expression of E-cadherin and TEAD4, another key component of the YAP1/TEAD4 transcriptional complex that is crucial for the self-renewal of TS^{CT} cells and proliferation of progenitors, also decreased with syncytialization (Figure 5C). In addition, YAP1 expression showed a significant decrease on day 6 after syncytialization (Figure 5D). These results demonstrated that YAP1 decreased with trophoblast syncytialization.

Suppression of YAP1 and TEAD4 by S100P is involved in regulating trophoblast syncytialization

We reasoned that *S100P* affects syncytialization by repressing CTB progenitor gene expression or inducing the expression of genes that promote STB formation. First, we detected the mRNA expression levels of a few syncytialization-related genes; interestingly, none of these genes were altered after *S100P* knockdown or overexpression (Figures S2A, 2B). Therefore, to identify the potential repressed CTB progenitor gene that mediated *S100P* syncytialization, we examined *YAP1* and *TEAD4* mRNA and protein expression levels after transfection with siRNA targeting *S100P* during TS^{CT} cell syncytialization. Through this analysis, we found that the mRNA levels of *YAP*1 and *TEAD4* were not significantly upregulated (Figure 6A), whereas their protein levels were both increased (Figure 6B). The immunofluorescence results further confirmed the upregulated expression of *YAP1* and ZO-1 in ST(2D)-TS^{CT} cells transfected with siS100P (Figure 6C). Consistently, *YAP1* and *TEAD4* mRNA levels showed no significant change, whereas their protein levels were obviously decreased in *S100P*overexpressing human TS^{CT} cells (Figures 6D, E). Immunofluorescence staining also confirmed the decreased YAP1 protein levels (Figure 6F). Moreover, TS^{CT}

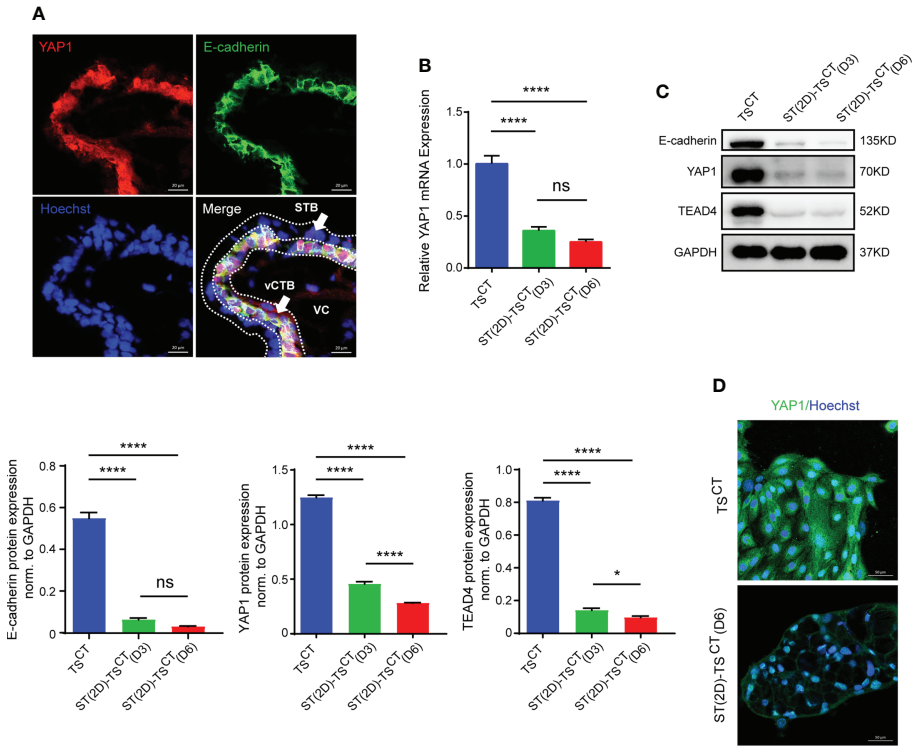

FIGURE 5
YAP1 and TEAD4 expression is decreased during trophoblast cell syncytialization *in vitro*. **(A)** Immunofluorescence staining of YAP1 (red), E-cadherin (green) and Hoechst staining (blue) in human healthy placentas (sixth to eighth week of gestation). White arrows indicate CTBs and STB, respectively. VC, villous core. Representative images from three independent experiments are shown. Scale bar, 20 μm. **(B)** Quantification of *YAP1* mRNA expression. The mean values ± SEMs (normalized to *GAPDH*) of human TS^{CT}, ST(2D)-TS^{CT} (D3) and ST(2D)-TS^{CT} (D6) cell cultures, as measured in duplicate, are shown. ****p<0.0001; ns: p=0.1255. **(C)** Representative Western blotting of E-cadherin, YAP1, and TEAD4 expression in TS^{CT} and ST(2D)-TS^{CT} cells (D3 and D6). Semiquantitative analysis of E-cadherin, YAP1, and TEAD4 protein expression normalized to GAPDH (n=3). *p=0.0185; ****p<0.0001; ns: p=0.1220. **(D)** Immunofluorescence staining of YAP1 (green) and Hoechst staining (blue) in human TS^{CT} and ST(2D)-TS^{CT} cells (D6). Scale bar, 50 μm.

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cells treated with the recombinant human S100P protein showed similar results regarding YAP1 and TEAD4 protein levels compared to those with *S100P* gene overexpression (Figure 6G). Together, these data suggested that S100P may be involved in regulating trophoblast syncytialization *via* YAP1, which is vital for maintaining the self-renewal capacity of TS^{CT} cells.

Gene expression profiling of ST(2D)-TS^{CT} cells with *S100P* knockdown

To further explore the potential mechanism of S100P regulated trophoblast syncytialization, we performed transcriptome sequencing (RNA-seq) analysis to detect the differentially expressed genes in ST(2D)-TS^{CT} cells with *S100P*

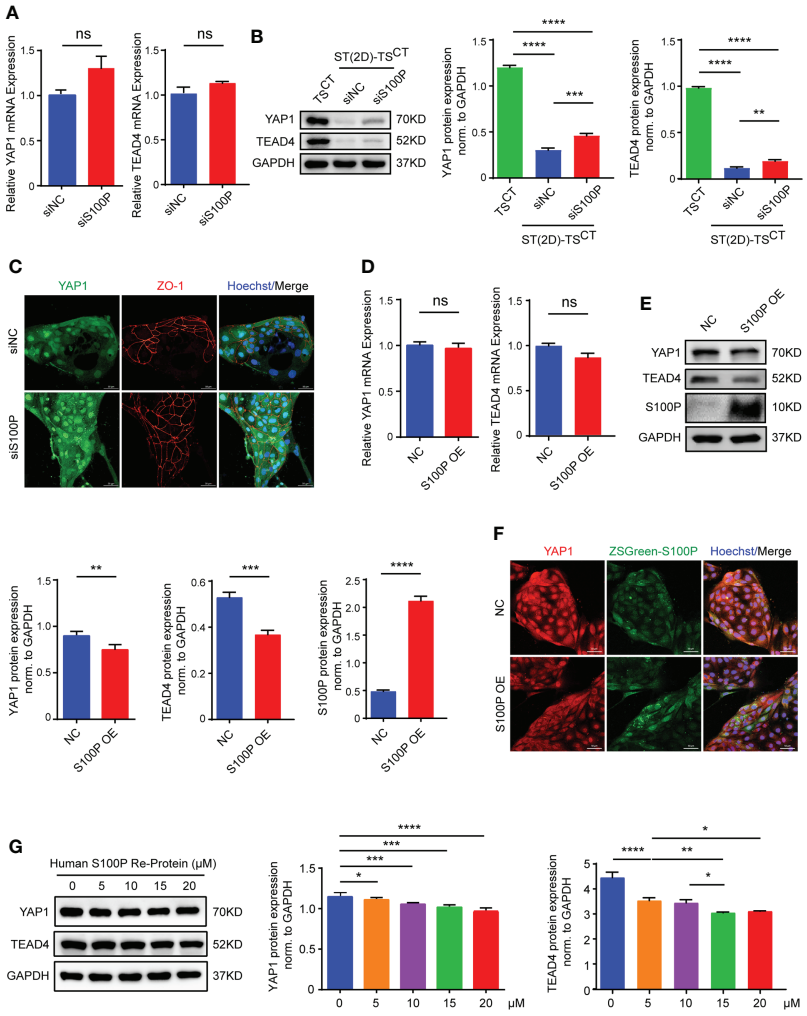

FIGURE 6
YAP1 and TEAD4 expression are increased in *S100P*-knockdown cells and decreased in S100P-overexpressing cells. **(A)** Quantification of *YAP1* and *TEAD4* mRNA expression. ST(2D)-TS^{CT} cells transfected with siRNA underwent syncytialization for 6 days (n=3). All the bar graphs depict the mean values ± SEMs (normalized to *GAPDH*), and the values were measured in duplicate; ns: p=0.0739, p=0.1537. **(B)** Representative Western blotting of YAP1 and TEAD4 protein levels in TS^{CT} and ST(2D)-TS^{CT} cells that were transfected with siS100P and underwent syncytialization for 6 days (n=3). Semi quantification of YAP1 and TEAD4 protein expression normalized to GAPDH (n=3). **p=0.0023; ***p=0.0007; ****p<0.0001. **(C)** Representative immunofluorescence images of staining for YAP1 and the TS^{CT} cell boundary marker ZO-1. Hoechst staining indicates nuclei (n=3). Scale bars, 50 μm. **(D)** Quantification of *YAP1* and *TEAD4* mRNA expression was performed by qPCR. TS^{CT} cells were infected with a lentiviral vector inducing overexpression of *S100P* (n=3). All the bar graphs show the mean values ± SEMs (normalized to *GAPDH*). ns: p=0.1643, p=0.0796. **(E)** Representative Western blotting of YAP1, TEAD4, and S100P expression in *S100P*-overexpressing TS^{CT} cells. Semi quantification of YAP1, TEAD4 and S100P protein expression normalized to GAPDH (n=3). **p=0.0018; ***p=0.0006; ****p<0.0001. **(F)** Representative immunofluorescence images of ZSGreen-S100P and YAP1 staining. Hoechst staining indicates nuclei. Scale bars, 50 μm. **(G)** Representative Western blotting of YAP1 and TEAD4 expression in TS^{CT} cells treated with recombinant human S100P protein (5 μM, 10 μM, 15 μM, and 20 μM) (n=3). Semi quantification of YAP1 and TEAD4 protein expression normalized to GAPDH (n=3). *p=0.0151, p=0.0126, p=0.0203; **p=0.0051; ***p=0.0005, p=0.0001; ****p<0.0001.

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knockdown. Data analysis identified 33 significantly differentially expressed genes, 11 of which were upregulated and 22 of which were downregulated in the *S100P* knockdown group compared to the negative control ($p<0.05$ and log2-fold change>1) (Figure 7A). We validated the expression of some genes that are known to affect syncytialization, migration and invasion of trophoblast cells by RT-qPCR. We found that *COL1A2, LRP1, NOTCH3, IGFBP3* and *LBP1* gene expression was downregulated, which was consistent with the RNA-seq results (Figure 7B). Moreover, the enriched differentially upregulated- and downregulated genes between the two groups of ST(2D)-TS^{CT} cells are shown in the heatmap (Figure 7C). We performed GO analysis of the downregulated genes in the *S100P* siRNA interference group, and the results showed specific enrichment of growth factor binding and extracellular matrix organization (Figure 7D). Overall, these results demonstrated that the syncytialization of ST(2D)-TS^{CT} cells was negatively regulated after *S100P* knockdown, and extracellular matrix components may play a vital role in the syncytialization process.

Discussion

S100P is involved in a variety of signal transduction pathways and exerts its biological effects by interacting with its target proteins; however, the mechanism has not been fully elucidated. To date, a number of target proteins that interact with S100P have been found, such as the Receptor of Advanced Glycation End products (RAGE), Calcyclin-Binding Protein (CacyBP/SIP), Ezrin, and Cathepsin D (57). Recent studies have suggested that intracellular S100P exerts its biological effects through Ezrin, whereas the secreted S100P protein mainly acts by binding to RAGE (58). As a temporally and spatially expressed protein, S100P has been demonstrated to play an important role in human embryo and placenta development (59). After the blastocyst is implanted, S100P is first expressed in the TE

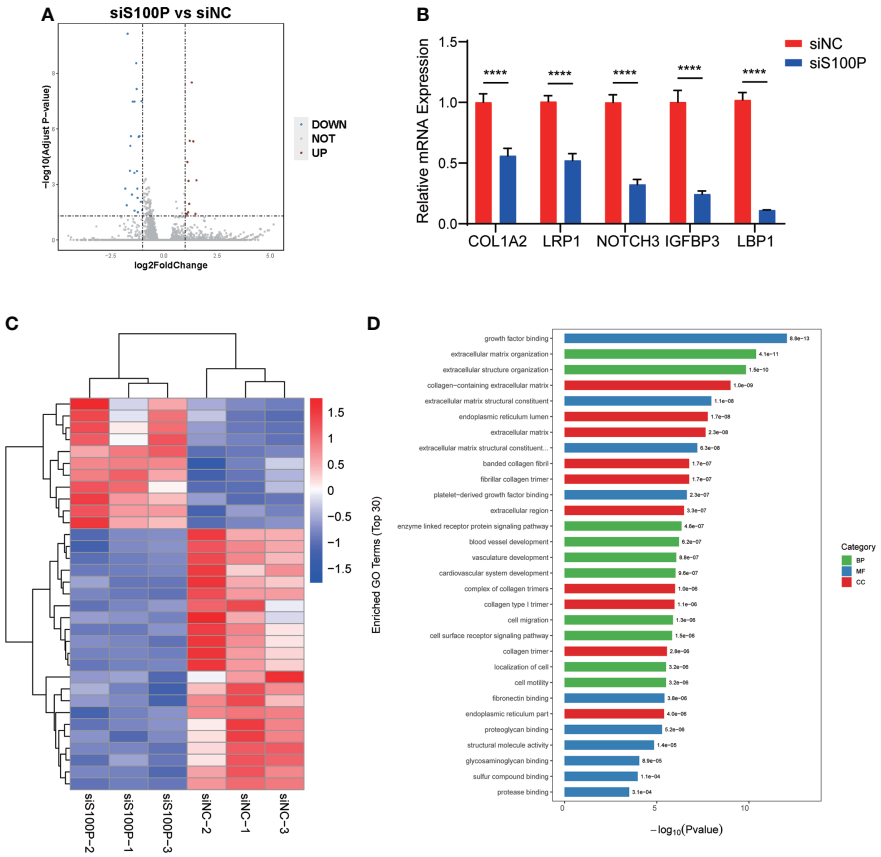

FIGURE 7
Transcriptional profiling of ST(2D)-TS^{CT} cells with *S100P* interference. **(A)** Volcano plots of differentially expressed genes after *S100P* knockdown. The red dots indicate upregulated genes, and the blue dots indicate downregulated genes. **(B)** RT-qPCR analysis of some downregulated and upregulated genes from the RNA-seq data analysis. Data are presented as the mean values ± SEMs. ****$p<0.0001$. **(C)** Heatmap of all the upregulated- and downregulated genes between the two groups. **(D)** Enriched biological process, molecular function, and cellular component terms of the downregulated differentially expressed genes in siS100P ST(2D)-TS^{CT} cells as assessed by GO analysis.

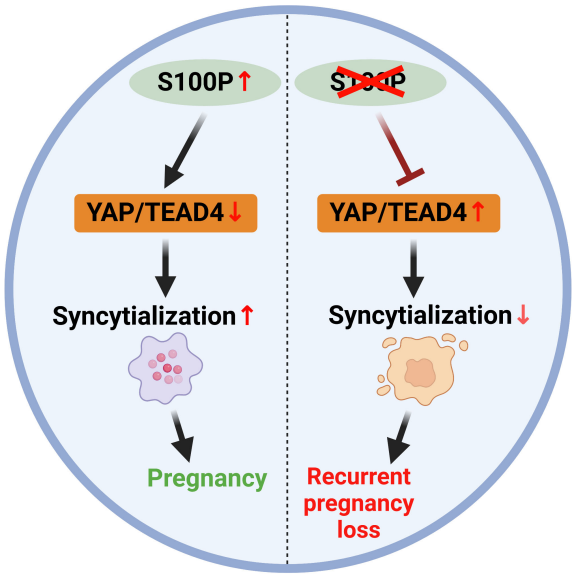

FIGURE 8
Schematic model illustrating that S100P participates in trophoblast syncytialization during early placenta development by downregulating YAP1 and TEAD4.

of the embryo (59). As gestation progresses, S100P becomes highly expressed in placental tissue, and the levels remain elevated throughout the pregnancy cycle (11). S100P affects trophoblast proliferation (60) and enhances the motility and invasion of different trophoblast cell lines (38). In addition, our research found that S100P is also involved in regulating trophoblast syncytialization as knockdown of *S100P* decreased β-hCG secretion and cell boundary stability during the syncytialization process.

S100P expression gradually increased with syncytialization, and reached the maximum level on the sixth day of syncytialization, which was also accompanied by an increase in β-hCG. After knocking down *S100P* for 72 hours from the third day of syncytialization, the levels of intracellular and secreted β-hCG protein and the cell fusion index were significantly decreased on day 6. These results indicated that the deletion of *S100P* affected the trophoblast syncytialization process. Interestingly, the overexpression of the S100P gene and protein in TS^{CT} cells failed to trigger syncytialization; however, the expression of the cell-cell adhesion protein E-cadherin was significantly decreased, which was the same as the decrease in E-cadherin protein expression observed during cell fusion under physiological conditions (61). This result may be attributed to the activation of Ezrin by the S100P protein to reduce the accumulation of E-cadherin in the cell membrane (58, 62).

YAP1 and TEAD4 are key genes involved in maintaining trophoblast cell stemness (45, 48). After knocking down or overexpressing *S100P*, we surprisingly found that YAP1 and TEAD4 were inversely upregulated- or downregulated at the protein level with S100P, whereas no significant alteration in their mRNA levels was observed. In addition, YAP1 and TEAD4 were naturally downregulated with trophoblast syncytialization compared to TS^{CT} cells. *S100P* knockdown partially reversed YAP1 and TEAD4 expression at the protein level in ST(2D)-TS^{CT} cells, but the results did not reach to their levels observed in TS^{CT} cells. This finding indicates that the direct regulation of YAP1 by S100P was not very strong, and S100P may affect YAP1 expression through other pathways. As YAP/TEAD4 complexes repress the expression of STB-associated genes, such as *CGB*, on its promoter regions, it can thereby impair autocrine, hCG-dependent cell fusion and differentiation (45). Thus, we supposed that S100P may indirectly regulate β-hCG expression mediated by YAP1 in a protein-protein interaction manner. Similar results suggesting that S100P participates in cell activities by interacting with its target proteins have been reported in recent years. In hepatocellular carcinoma (HCC), aberrant S100P expression suppresses cell growth and apoptosis by downregulating cyclin D1 and CDK expression at the protein level (63). Moreover, S100P promotes colorectal cancer cell epithelial-to-mesenchymal transition (EMT) as well as migration and invasion by upregulating S100A4 protein levels together with Trx-1 (64). However, it remains unclear how S100P interacts with YAP1 at the protein-interaction level.

Previous studies reported that the YAP/TEAD complexes either sequestered or inhibited S100 family expression, which was contradictory to our findings. In this study, however, the effect of S100P on YAP1/TEAD4 complexes was evident. As all gene expression exhibited strict temporal specificity; *S100P* and *YAP1*

were highly expressed at different times during the TS^{CT} cell syncytialization process. Before TS^{CT} cell syncytialization, YAP1 was highly expressed, *S100P* expression was not altered significantly with *YAP1* knockdown because *YAP1* knockdown did not trigger syncytialization induced by FSK. However, after knocking down the highly- expressed *S100P* gene during syncytialization, YAP1 expression was significantly upregulated at the protein level. In carcinoma cell lines, S100P and YAP1 expression levels were both increased under the same treatment, whereas S100P was apparently up- or downregulated after *YAP1* knockdown or overexpression (53, 54). The above findings may differ from the interpretation of the effect of S100P on YAP/TEAD complexes during the trophoblast syncytialization process.

We performed an RNA-seq assay and found that some genes related to trophoblast syncytialization were downregulated after *S100P* interference. It has been demonstrated that trophoblast cell invasion can be enhanced by IGFBP3 upregulation (65). COL1A2 is a crucial component of the extracellular matrix that plays an important role in trophoblast fusion, invasion, angiogenesis and placental barrier maintenance (66–68).

S100P is a secreted calcium-binding protein and has been detected both in human peripheral serum and placentas *in situ* (36). Serum S100P levels are elevated in patients with diabetic peripheral neuropathy and are considered a significant indicator of peripheral neuropathy in patients with type 2 diabetes (69). Moreover, S100P may be recognized as a novel differential diagnostic marker for HCC and a potential predictor of microvascular invasion (MVI) status in HCC patients before surgery. Downregulating S100P in endometrial tissue induces epithelial cell apoptosis, which suggests that S100P is a potential clinical target to improve the success of *in vitro* fertilization (IVF) (70). Our research found reduced S100P expression levels in placentas and serum in RPLs, suggesting that S100P may represent a potential marker for poor pregnancy outcomes.

In summary, S100P is involved in regulating trophoblast syncytialization by regulating YAP1 at the protein level. Moreover, serum S100P levels are decreased in patients with RPL compared with controls, suggesting that low serum S100P levels are related to poor pregnancy outcomes and may represent a useful marker for evaluating placental biological function during early pregnancy (Figure 8). This study demonstrates that S100P plays a pivotal role in trophoblast syncytialization and gestation maintenance by regulating YAP1 and provides a possible approach for predicting early pregnancy maintenance.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject, PRJNA798551.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang University School of Medicine. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

HJZ and YP performed experiments. HJZ, CZ, BH, XJ, TZ and NL collected the clinical samples. HJZ, WY, XS and HYZ analyzed the data. HJZ made the figures and drafted the article. SZ and HYZ critically reviewed the article. SZ and HYZ conceived and supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.860261/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Establishment of human proliferative CT cells in culture. **(A)** Phase contrast images of TS^{CT} cells at postnatal day 6 (P6), ST(2D)-TS^{CT} cells (measured on day 6), and EVT-TS^{CT} cells (measured on day 8). Similar results were obtained with three independent cell lines. D6: day 6, D8: day 8. Scale bars, 50 µm. **(B)** Representative immunostaining images of TEAD4 (CT marker), CGB (ST marker), and HLA-G (EVT marker) in TS^{CT}, ST(2D)-TS^{CT} (D6), and EVT-TS^{CT} (D8) cells. The nuclei were stained with Hoechst 33342. Scale bars, 50 µm. Similar results were obtained with three independent cell lines.

SUPPLEMENTARY FIGURE 2
Alteration of fusion-associated gene expression with S100P knockdown and overexpression. **(A)** qPCR quantification (duplicates) of *CGA, CGB, HSD11β2, GCM-1, ERVW-1, ERVFRD-1, ERVV-1* and *ERVV-2* mRNA expression in siS100P-transfected ST(2D)-TS^{CT} cells that underwent syncytialization (6 days) (n=3). All the bar graphs present the mean values ± SEMs (ns, not significant). **(B)** qPCR quantification (duplicates) of *CGA, CGB, HSD11β2, GCM-1, ERVW-1, ERVFRD-1, ERVV-1* and *ERVV-2* mRNA expression in TS^{CT} cells infected with lentiviral vectors inducing S100P overexpression. All the bar graphs show the mean values ± SEMs (ns, not significant).

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Maternal lipid profile during early pregnancy and birth weight: A retrospective study

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Introduction: Elevated maternal serum lipid concentrations have been related to an adverse intrauterine environment and lead to abnormal birth weight.

Objective: In this study, we aimed to explore the association between maternal lipid profiles during early pregnancy and birth weight with stratified pre-pregnancy body mass index (BMI).

Methods: This retrospective cohort study was based on a large population from two major maternity centers in Shanghai, China. We included 57,516 women with singleton live birth between January 2018 and October 2020. All of the enrolled women had fasting lipid concentrations measured in early pregnancy. The primary outcomes were birth weight and risks of adverse birth outcomes, including macrosomia, large for gestational age (LGA), low birth weight (LBW), and small for gestational age (SGA).

Results: Higher maternal concentrations of total cholesterol (TC), triglyceride (TG), and low-density cholesterol (LDL-c) in early pregnancy were associated with increased birth weight. Ln transformed TG and levels exhibited a positive association with LGA and macrosomia (OR = 1.33, 95% CI: 1.25, 1.42 and OR = 1.37, 95% CI: 1.24, 1.52) and showed a negative relationship with SGA (OR = 0.73, 95% CI: 0.62, 0.85). High TG (>75th percentile, 1.67 mmol/L) group also showed higher risks of LGA and macrosomia (OR = 1.21, 95% CI: 1.15, 1.28 and OR = 1.20, 95% CI: 1.10, 1.31) and decreased prevalence of SGA (OR = 0.71, 95% CI: 0.61, 0.83). Moreover, significant combined effects of pre-pregnancy BMI and lipid profiles on LGA and macrosomia were identified.

Conclusions: Elevated maternal lipid profiles in early pregnancy are associated with higher birth weight and increased risks of LGA and macrosomia. We

propose that serum lipid profiles in early pregnancy and pre-pregnancy BMI could serve as screening indexes for high-risk women.

KEYWORDS

maternal lipid profiles, body mass index, birth weight, large for gestational age, macrosomia

Introduction

According to Developmental Origins of Health and Disease (DOHAD) theory, maternal metabolism and intrauterine environment could affect fetal development and further impact their health status in adulthood (1, 2). Among the prenatal metabolic factors, maternal lipids play an important role in excess fetal growth. During pregnancy, maternal lipid profiles, including total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol (HDL-c), are taken up by placenta and primarily provide energy for maternal metabolism and fetal development (3, 4). To adapt to maternal-fetal physiology, maternal lipid levels rise progressively throughout gestation, suggesting the importance of these metabolic changes in fetal development (5).

Overweight and obesity in women of reproductive age, which are related to increased maternal lipid levels in pregnancy (6), keep increasing in China (7). However, hyperlipidemia not only occurs in overweight or obese women but also in normal weight women during pregnancy. Maternal hyperlipidemia has a variety of effects on intrauterine fetal growth and could significantly impact perinatal outcomes (8–12). Elevated TG concentrations in plasma may contribute to increased risks of impaired glucose tolerance and gestational diabetes (13, 14). Moreover, several studies have shown that higher TG and oxidized LDL were associated with preeclampsia (15, 16). Besides pregnancy complications, maternal hyperlipidemia may predict adverse birth outcomes as well, including preterm birth, large for gestational age (LGA), and macrosomia (11, 17). Beyond pregnancy, women with gestational hyperlipidemia were prone to metabolic syndrome and cardiovascular disease (18, 19). Thus, it is important to control maternal lipid concentrations in an optimal range for women in all body mass index (BMI) groups. However, there is no consensus on optimal normal ranges for lipids in pregnant women.

Birth weight is an important outcome reflecting intrauterine conditions and predicting short- and long-term morbidities. LGA and macrosomia indicate excess intrauterine weight gain and are related to adverse obstetrical outcomes such as postpartum hemorrhages, traumatic deliveries, and still birth (20, 21). LGA and macrosomia infants were also prone to diabetes and obesity in adulthood and childhood (22, 23). On the other hand, small for gestational age (SGA) and low birth weight (LBW) infants had higher incidence of hypoxic ischemic encephalopathy, seizures, neonatal sepsis (21) and associated with stroke, kidney disease, hypertension, and depression in later life (24, 25). Therefore, understanding the effect of maternal lipid profile on fetal growth is necessary for optimizing birth outcomes and subsequently decreasing the prevalence of numerous diseases beyond infancy.

So far, most studies focused on the impact of maternal lipid profile in the second and third trimesters during pregnancy on birth outcomes (26, 27). However, it would be ideal if high-risk women could be identified as early as possible. So, our study aims to shed more light on the association between maternal lipid profiles in the first trimester and birth weight and adverse birth outcomes. In addition, we attempted to find a reference value of maternal lipid profile considering the prevalence of LGA and macrosomia, which could be potentially applied to screen high-risk pregnant women for prenatal health care.

Materials and methods

Study population

This study recruited data from Obstetrics and Gynecology Hospital of Fudan University (Ob & Gyn Hospital) and International Peace Maternity and Child Health Hospital (IPMCHH), which are two major maternal health hospitals in Shanghai. Pregnant women who underwent prenatal health care since the first trimester and gave birth at the hospital from January 2018 to October 2020 were included in the analysis. Women who had a twin pregnancy or still birth, or with key medical data missing, namely, pre-pregnancy BMI and lipid profiles in early pregnancy, were excluded. All data, including serum TC, TG, LDL-c, HDL-c concentrations in early pregnancy, and birth information were collected. This study has been approved by Ob & Gyn Hospital (No. 2021-90) and IPMCHH Ethical committees (No. GKLW2019-05).

Data collection and measures

All data were collected by in-person interviews during hospital visits and medical records. Maternal sociographic characteristics included residence, occupation, maternal age at birth, marital status, education, insurance status, and consumption of alcohol and cigarettes. Maternal pre-pregnancy weight was self-reported by enrolled women, and pre-pregnancy BMI (kg/m^2) was calculated as pre-pregnancy weight (kilograms) divided by the square of height (meters). According to WHO classification, pre-pregnancy BMI was categorized into underweight (< 18.5), normal weight (18.5–24.9), overweight (25.0–29.9), and obesity (≥30) (28). Information related to pregnancy complications, including gestational diabetes, gestational hypertension disorders, intrahepatic cholestasis of pregnancy, and mode of conception were ascertained from medical records.

Fasting venous blood samples were drawn during 7:00 a.m. and 9:00 a.m. at the first prenatal visit during 8 to 13 gestational weeks. The lipid profiles were tested by the biochemical laboratory of IPMCHH and Ob & Gyn Hospital. Serum TC and TG concentrations were determined by GPO-POD method with a commercial enzymatic colorimetric assay (Beckman Coulter, CA, USA and Fujifilm, Osaka, Japan) and Beckman AU5800 analyzer and HITACHI 7600. LDL-c and HDL-c were examined by the direct method with a commercial reagent (Beckman Coulter, CA, USA and SEKISUI, TX, USA) and Beckman AU5800 analyzer and HITACHI 7600.

Birth outcomes

In this study, birth weight and risks of LGA and macrosomia of singleton live births were the primary outcomes. Birth weight was standardized based on gestational age at birth (29). Data on birth weight, fetal sex, and gestational age were collected in the medical records at delivery. LGA was defined as an infant with birth weight larger than the 10^{th} percentile for his/her gestational age and sex, whereas small for gestational age (SGA) as smaller than the 10^{th} percentile (30). Macrosomia was diagnosed when the newborn weighed more than 4,000 g, whereas LBW was diagnosed with a birth weight of less than 2,500 g (31).

Statistical analysis

Distributions of maternal TG, TC, LDL-c, and HDL-c concentrations were right-skewed. Therefore, the concentrations of lipids were natural log-transformed to improve the normality of their distributions (32). We conducted multiple linear regression models to evaluate the association between maternal lipids and neonatal birth weight. Ln transformed lipid concentrations were divided into quartiles, and $>75^{th}$ percentile was defined as reference points of high lipid groups. Multiple logistic regression models were used to estimate odds ratios (ORs) and 95% confidential intervals (CIs) for the association between TC, TG, HDL-c, and LDL-c with LGA and SGA. Subgroup analyses were performed according to maternal pre-pregnancy BMI ranges. Furthermore, we investigated the combined effects of pre-pregnancy BMI and lipids in early pregnancy on LGA and macrosomia by adding a product interaction term of pre-pregnancy BMI × lipid concentrations (TC, TG, HDL-c, and LDL-c) in the models. Heat maps were constructed to exhibit the differences based on combinations of pre-pregnancy BMI and maternal TC, TG, HDL-c, LDL-c concentrations (red represents high incidence and blue represents low incidence). Confounders were included if they were previously reported in researches or were found correlated with the primary outcome. In our analyses, all the birth outcome models were adjusted for potential confounders, including maternal pre-pregnancy BMI (except in subgroup analyses), age at birth, mode of conception, parity, education attainment, consumption of cigarettes, infant sex, gestational diabetes, and gestational hypertension disorders. For confounders with missing data, multiple imputations were used based on the Markov chain Monte Carlo method. All analyses were performed using R software (version 4.0.4) with the "rms," "mice," and "visreg," packages.

Results

Study population

A total of 57,516 women with live singleton deliveries were included in this study, and their descriptive characteristics were shown in Table 1. The mean age of mothers was 31.16 years, and the mean BMI was 21.29. Gestational diabetes and hypertension disorders affected 13.7 and 4.8% enrolled women. Among the infants, 51.7% were male and 5.1% were preterm birth. The mean value (SD) of birth weight was 3321.52 (453.25) g, with LGA, SGA, macrosomia, and LBW proportions of 18.1, 2.3, 5.7, and 3.3%, respectively.

The mean levels of TC, TG, LDL-c, and HDL-c in early pregnancy were 4.56 (1.37–12.79) mmol/L, 1.42 (0.10–13.11) mmol/L, 2.29 (0.51–9.58) mmol/L, and 1.58 (0.38–3.45) mmol/L, respectively. Their 75^{th} percentiles were 5.02 mmol/L, 1.67 mmol/L, 2.99 mmol/L, and 2.01 mmol/L (Table 2).

Maternal lipid profile and birth weight

The association between maternal lipid concentrations in early pregnancy and birth weight was presented in Figure 1. After adjusting for confounders, the results displayed a

TABLE 1 Descriptive statistics of study population.

Maternal characteristics	(*n* = 57516)
Age, Mean ± SD, years	31.16 ± 3.95
Pre-gestational BMI, Median (95% CI), kg/m^2	20.8 (16.9, 28.3)
Gestational diabetes (%)	7875 (13.7)
Gestational hypertension disorders	
Gestational hypertension (%)	1284 (2.2)
Pre-eclampsia (%)	1070 (1.9)
Eclampsia (%)	404 (0.7)
Intrahepatic cholestasis of pregnancy	447 (0.8)
Parity	
Nullipara (%)	38182 (66.4)
Primi or multipara (%)	19334 (33.6)
Fetal characteristics	
Fetal sex	
Male (%)	29709 (51.7)
Female (%)	27807 (48.3)
Birth weight, Mean ± SD, Kg	3,321.52 ± 453.25
LBW (%)	1907 (3.3)
Macrosomia (%)	3258 (5.7)
SGA (%)	1320 (2.3)
LGA (%)	10390 (18.1)
Preterm birth (%)	
Very preterm	307 (0.5)
Late preterm	2664 (4.6)

SD, standard deviation; LBW, low birth weight; SGA, small for gestational age; LGA, large for gestational age.

significant positive relationship between maternal TC, TG, and LDL-c concentrations and birth weight. Each unit of natural log (ln) increase in TC, TG, and LDL-c was associated with 0.086 (95% CI: 0.036, 0.136) SD, 0.159 (95% CI: 0.136, 0.181) SD, and 0.071 (95% CI: 0.031, 0.111) increase in birth weight. However, we observed no association between ln-transformed HDL-c and birth weight.

Subgroup analyses showed a similar association. Ln-TG showed a significant association with birth weight in all BMI subgroups, whereas there were no visible association between birth weight with ln-TC nor ln-LDL-c in pre-pregnancy overweight women. In addition, in normal weight and obesity groups, we did not find any correlation between ln-LDL-c and birth weight (Table 3). In fetal sex subgroup analysis, a similar relationship was observed, whereas ln-TC showed a significant positive association with birth weight in male fetus but not female fetus.

Maternal lipid profile and birth outcomes

After adjustment for confounders, we observed a positive association between each unit increase of maternal ln-TG in the first trimester and odds of macrosomia (OR = 1.37, 95% CI: 1.24, 1.52) (Figure 2). The increase of ln-TG (OR = 1.33, 95% CI: 1.25, 1.42) and ln-LDL-c (OR = 1.12, 95% CI:1.02, 1.24) in early pregnancy also exhibited a positive association with the risks of LGA (Figure 3). Moreover,while first trimester lipid profiles was not associated with LBW (Figure s1), ln-TC (OR = 0.67, 95% CI: 0.49, 1.00), ln-TG (OR = 0.73, 95% CI: 0.62, 0.85) and HDL-c (OR = 0.83, 95% CI: 0.68, 1.00) were inversely associated with the prevalence of SGA (Figure s2).

In subgroup analyses based on pre-pregnancy BMI ranges, the relationship between ln-TG and LGA was observed in all BMI categories, with ORs ranging from 1.24 to 1.72. However, no significant association between first trimester ln-TG and the incidence of macrosomia was observed in pre-pregnancy overweight and obese women. Similarly, we did not find any correlation between ln-LDL-c and LGA in overweight and obese women, whereas a negative association was observed between ln-HDL-c and odds of LGA in underweight women. In addition, the prevalence of SGA was shown to be inversely related to ln-TG in all subgroups and negatively associated with HDL-c in pre-pregnancy underweight group. However, we did not find any association between lipid levels in early pregnancy with the risk of LBW in none of the groups (Table s1).

Compared with women of normal serum lipid concentrations, women with high-maternal TC (> 5.02 mmol/L) and TG (> 1.67 mmol/L) exhibited significant increase risks of LGA (OR = 1.07, 95% CI: 1.00, 1.14 and OR = 1.21, 95% CI: 1.15, 1.28), and women in high TG and LDL-c (> 2.99 mmol/L) group showed elevated risks of macrosomia (OR = 1.20, 95% CI: 1.10, 1.31 and OR = 1.09, 95% CI: 1.10, 1.19). We also observed decreased prevalence of SGA in high TG group (OR = 0.71, 95% CI: 0.61, 0.83) (Table 4).

TABLE 2 Quartiles of maternal lipid profiles in first trimester (mmol/L).

	Mean ± SD	25th	50th	75th
TC	4.56 ± 0.83	3.99	4.48	5.02
TG	1.42 ± 0.65	0.98	1.29	1.67
LDL-c	2.29 ± 0.63	2.19	2.59	2.99
HDL-c	1.58 ± 0.52	1.21	1.67	2.01

TC, total cholesterol; TG, triglyceride; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol.

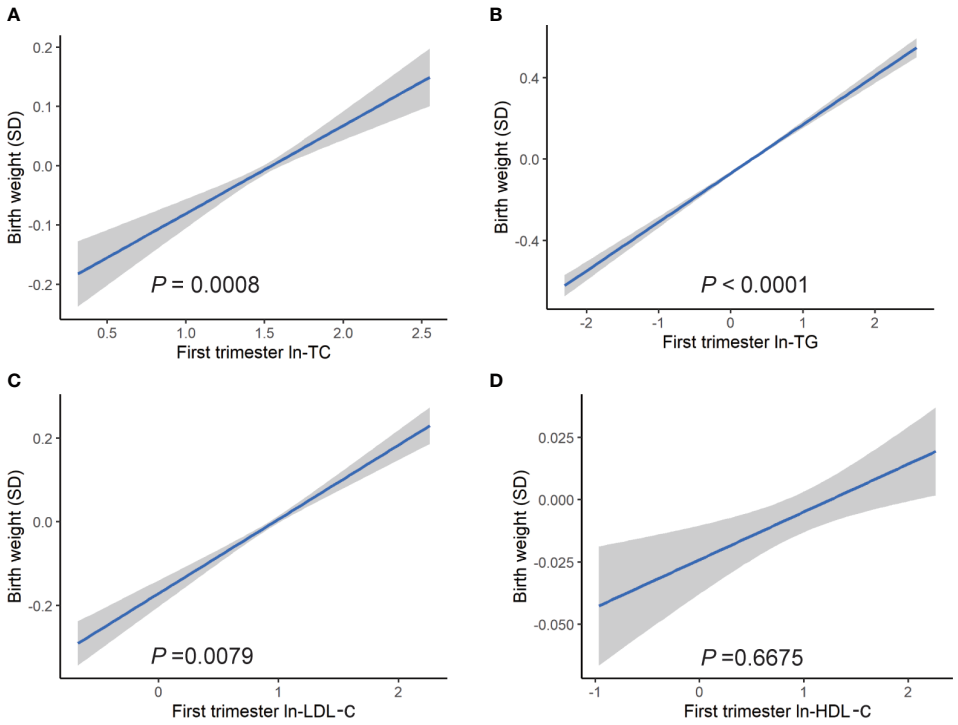

FIGURE 1
Association between ln transformed maternal lipid profiles in early pregnancy and birth weight. Linear regression models for **(A)** ln-TC, **(B)** ln-TG, **(C)** ln-LDL-c, **(D)** ln-HDL-c, and birth weight plotted as predicted mean with 95% CIs. Analyses were adjusted for maternal pre-pregnancy body mass index, age, mode of conception, parity, education, consumption of cigarettes, infant sex, gestational diabetes, and gestational hypertension disorders.

Combined effects of lipid concentrations and pre-pregnancy BMI on the risks of LGA and macrosomia

Increased BMI was believed to be companied by unfavorable lipid levels characterized by high concentrations of TG, TC and LDL-c, and low concentrations of HDL-c (Table s2) (6). Additionally, we observed a positive association between pre-pregnancy BMI and birth weight (Figure s3). Therefore, we investigated the combined effects of first trimester lipid profiles and pre-pregnancy BMI on birth outcomes. Figures 4, 5 displayed heat maps for the combined association of pre-pregnancy BMI (*x*-axis) (ranged from 15.2 to 34.8 kg/m^2) and maternal ln-TC, ln-TG, ln-HDL-c, or ln-LDL-c (*y*-axis) in the first trimester with the incidence of LGA (%) and macrosomia (%) (*z*-axis; red represents higher incidence and blue represents lower incidence).

Considerable differences in the incidence of LGA according to the combination of pre-pregnancy BMI and first trimester ln-TG were identified (*P* for interaction = 0.0224; Figure 4). The interactive effect also showed that, in women with pre-pregnancy BMI less than 35 kg/m^2, increased ln-TG in early pregnancy and pre-pregnancy BMI was associated with higher risks of LGA. A similar effect was observed in the combination of pre-pregnancy BMI and ln-TC levels (*P* for interaction = 0.0131) (Figure 4). The interaction between pre-pregnancy BMI and ln-LDL-c was also significantly identified (*P* for interaction < 0.0001). While ln-LDL-c displayed a positive association with LGA in women with pre-pregnancy BMI less than 25 kg/m^2, a negative relationship was observed in obese women (BMI > 30) (Figure 4). Differently, increasing HDL-c indicated lower risks of LGA in women with pre-pregnancy BMI < 35 kg/m^2 (Figure 4). Similarly, significant combined effects of ln-TG/ln-LDL-c and pre-pregnancy BMI on macrosomia were observed (ln-TG, *P* for interaction = 0.0319; ln-LDL-c, *P* for interaction = 0.0021, Figures 5B). Like the effect on LGA, ln-LDL-c exhibited a negative association with macrosomia in obese women, too. However, we did not observe any significant combined effect on SGA or LBW (Figures s3, s4).

Discussion

In this large retrospective study, we found that elevated maternal lipids in early pregnancy, especially TG, were significantly associated with higher birth weight and increased

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TABLE 3 The association between blood metabolic markers in early pregnancy and fetal birth weight by different BMI groups.

	β (95% CI)[a]	*P*
Normal		
TC	0.06 (0.00, 0.12)	0.0357*
TG	0.15 (0.12, 0.17)	<0.0001*
LDL-c	0.04 (-0.02, 0.10)	0.1980
HDL-c	-0.01 (-0.03, 0.01)	0.4571
Underweight		
TC	0.23 (0.11, 0.36)	0.0003*
TG	0.16 (0.10, 0.22)	<0.0001*
LDL-c	0.21 (0.12, 0.29)	<0.0001*
HDL-c	-0.01 (-0.05, 0.04)	0.8774
Overweight		
TC	-0.01 (-0.19, 0.16)	0.8785
TG	0.20 (0.13, 0.28)	<0.0001*
LDL-c	-0.07 (-0.19, 0.05)	0.2839
HDL-c	0.02 (-0.17, 0.21)	0.82401
Obesity		
TC	0.52 (-0.04, 1.08)	0.0698
TG	0.41 (0.18, 0.65)	0.0007*
LDL-c	0.30 (-0.10, 0.70)	0.1358
HDL-c	0.02 (-0.17, 0.21)	0.82401

[a]Adjusted for maternal age, mode of conception, parity, education attainment, consumption of cigarettes, infant sex, gestational diabetes, and gestational hypertension disorders.
LGA, large for gestational age; TC, total cholesterol; TG, triglyceride; LDL-c, low density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; CI, confidential interval.
* A significant association was found statistically.

risks of LGA and macrosomia. We also proposed 75th percentile as reference points for first trimester lipid concentrations based on a large Chinese population. Our results firstly suggested a considerable combined effect of pre-pregnancy BMI and first trimester TG on birth outcomes, which may provide valuable information on early pregnancy screening and contribute to perinatal health care.

To meet the increasing physiological demands of fetal development, maternal serum lipid concentrations during gestation are generally higher than that in non-pregnant status. Recently, several studies displayed a discernible decrease of lipid levels in the first 6 weeks of pregnancy (33, 34). Later on, the lipid levels keep raising and peak at late third trimester (35). Clinicians usually use the lipid criteria for non-pregnant people to evaluate gestational lipid levels considering that an accurate normal lipid concentration range especially for pregnant women is lacking. However, pregnancy is a vulnerable period during which both mothers and infants are susceptible to adverse lipid environment; thus, a reference range for lipids in pregnant women is urgently needed. As maternal overnutrition is an increasing issue in Shanghai instead of maternal undernutrition, our study aimed to find out lipid reference values to prevent excessive birth weight. Based on a large sample size in two centers in China, we compared the risks of LGA and macrosomia in different percentile groups and we recommended that the 75th percentile of lipid concentrations in the current study could serve as a reference point predicting the prevalence of adverse birth outcomes. In pregnant women whose TG levels were below 75th, the risks of LGA and macrosomia decreased from 18.7 and 5.7% to 15.3 and 5.3%, compared with 16.7 and 5.5% in women whose TG was below 95th percentile. In our study, the reference points of 75th in early pregnancy were slightly lower than the lipid criteria for non-pregnant people (95th percentile) considering the vulnerability of pregnant women. Because maternal serum lipid levels continue increasing in second and third trimesters, the elevated lipids in early pregnancy may forecast a more serious hyperlipidemia in late pregnancy and predict higher risks of large infants. Our reference values could be helpful for screening high-risk pregnant women in early pregnancy.

The influence of TG during pregnancy has gained some attention, and we found a stable association between TG with birth weight and adverse birth outcomes in all of the BMI groups in the current study. Our study is in line with several previous studies that reported a positive association between maternal TG concentrations in early pregnancy and higher birth weight and LGA (36–38). In a study proposed by *Wang et al.* in 2016, maternal TG concentrations in early pregnancy were divided into quartiles, and they did not observe any association between the highest quartile and an increased prevalence of LGA, whereas in the current study, the highest TG quartile showed a significantly higher risk of LGA (39). Because the 75th percentile reported by *Wang et al.* was 1.40 mmol/L, which is lower than that in our study (1.67 mmol/L), the differences of lipid levels may contribute to the discrepancies in results. An obvious increase of first trimester TG concentrations was also noticed in Chinese pregnant women during the last 5 years, which may be related to the changes in diet structure (39). In addition, our study observed that increased TG levels in the first trimester were correlated with elevated risks of macrosomia, which was rarely studied previously. A negative association between TG levels and SGA was also found in the current study, which is consistent with several previous studies (12, 40). In a word, TG exhibited a significant relationship with birth weight and adverse birth outcomes, indicating that TG might play an important role in fetal growth. Additionally, our team previously found that first trimester TG could be a major predictor of gestational diabetes and was associated with preterm birth (17, 41), and others also suggested that high TG might induce gestational hypertension disorders and influence infant post-natal growth (37, 42), emphasizing the important role of TG during pregnancy. Our results supported that first trimester TG could predict adverse birth outcomes, thus the screening in early pregnancy is essential in prenatal health care.

Except for TG, first trimester TC levels exhibited a positive association with birth weight. However, we did not observe any significant association between TC and LGA nor macrosomia in both total population and subgroup analyses,

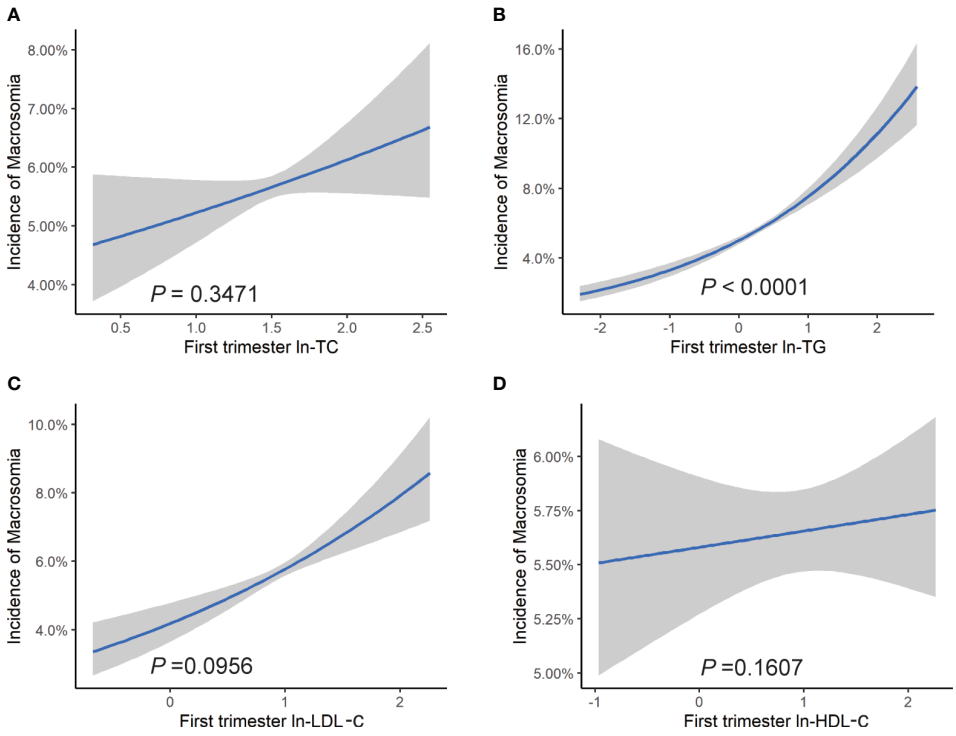

FIGURE 2
Risk of macrosomia associated with maternal lipid profiles in early pregnancy. Logistic regression models for ln transformed **(A)** TC, **(B)** TG, **(C)** LDL-c, **(D)** HDL-c, and macrosomia, expressed as predicted mean with 95% CIs. Analyses were adjusted for maternal pre-pregnancy BMI, age, mode of conception, parity, education, consumption of cigarettes, infant sex, gestational diabetes, and gestational hypertension disorders.

which is consistent with previous researches (37, 43). While previous studies did not propose any association between maternal LDL-c levels and abnormal birth weight (44–46), we observed elevated LDL-c in the first trimester increased odds of LGA, but not macrosomia. In the current study, no detectable association between maternal HDL-c and birth weight or adverse birth outcomes was found as previously suggested in several studies (47, 48) (36, 49). The inconsistent results in different studies could be explained by different gestational weeks when the blood was collected and the variances in the study population.

Unfavorable lipid levels were considered to be related to increased pre-pregnancy BMI. Independent of lipid concentrations, maternal pre-pregnancy overweight, and obesity were reported to increase birth weight and the risks of relevant birth outcomes (50, 51), so we performed a subgroup analysis according to BMI categories. In all subgroups, the association between TG and birth weight and LGA was consistent, suggesting that TG might be a key factor influencing birth weight and predicting LGA. The higher concentrations of TG and smaller subgroup population may explain the inconsistent results in macrosomia. Our results also showed significantly increased risks of excessive birth weight in mothers with pre-pregnancy overweight and obesity (Table 4). Furthermore, the combined effects of pre-pregnancy BMI and lipid profiles on birth outcomes were studied, and significant differences were found according to the combination of pre-pregnancy BMI and first trimester TG levels. Our findings indicated that maternal pre-pregnancy BMI could influence gestational lipid levels and further affect birth weight. Taken together, we hypothesized that pre-pregnancy BMI along with first trimester TG would predict birth outcomes in large measure, and that pregnant women with elevated first trimester lipid concentrations and pre-pregnancy BMI should pay more attention to gestational weight management, including diet control, nutrition education, and moderate exercise. Surprisingly, we observed a negative association between ln-LDL-c with LGA and macrosomia in obese women in the combined effect analysis. As Vahratian et al. reported that the increasement of LDL-c levels during gestation in obese women was discernibly smaller than that in normal pregnancies, we assumed that the metabolic dysregulation and relatively low LDL-c concentrations in late pregnancy may account for the results (52).

Most previous studies focused on the effect of intrauterine lipid exposure in late pregnancy on birth weight, as it is conventionally believed that placental blood flow extensively

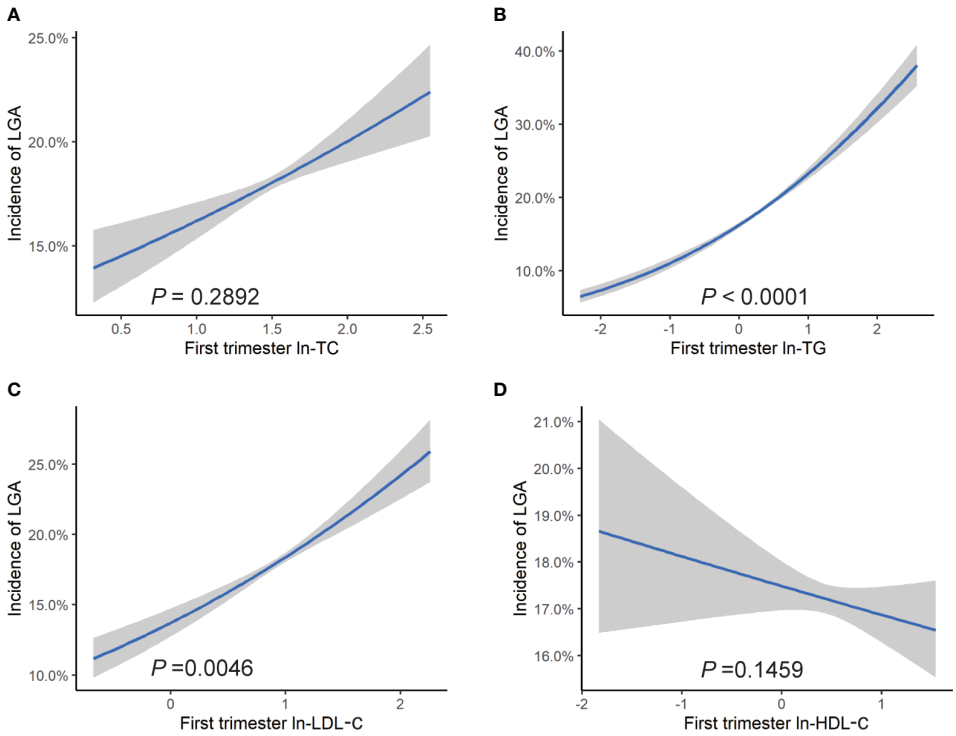

FIGURE 3
Risk of LGA associated with maternal lipid profiles in early pregnancy. Logistic regression models for ln transformed **(A)** TC, **(B)** TG, **(C)** LDL-c, **(D)** HDL-c, and LGA, expressed as predicted mean with 95% CIs. Analyses were adjusted for maternal pre-pregnancy BMI, age, mode of conception, parity, education, consumption of cigarettes, infant sex, gestational diabetes, and gestational hypertension disorders.

TABLE 4 Maternal lipid profiles and risks of LGA, macrosomia, LBW, and SGA by BMI groups.

	All		Normal weight		Underweight		Overweight		Obesity	
	≤75th	> 75th	≤75th	> 75th	≤75th	> 75th	≤75th	> 75th	≤75th	> 75th
TC										
LGA										
n (%)	7131 (16.5)	2543 (17.7)	5400 (16.5)	1916 (17.6)	544 (8.7)	168 (9.5)	1007 (27.4)	381 (25.8)	180 (35.8)	78 (36.3)
OR (95% CI)	1.00 (reference)	1.07 (1.00, 1.14)	1.00 (reference)	1.09 (1.03, 1.17)	1.00 (reference)	1.08 (0.87, 1.33)	1.00 (reference)	0.91 (0.78, 1.05)	1.00 (reference)	0.94 (0.66, 1.36)
Macrosomia										
n (%)	2407 (5.6)	851 (5.9)	1792 (5.5)	621 (5.7)	132 (2.1)	53 (3)	408 (11.1)	148 (10.0)	75 (14.9)	29 (13.5)
OR (95% CI)	1.00 (reference)	1.05 (0.96, 1.16)	1.00 (reference)	1.09 (0.98, 1.22)	1.00 (reference)	1.39 (0.93, 2.07)	1.00 (reference)	0.93 (0.74, 1.16)	1.00 (reference)	0.88 (0.53, 1.48)
LBW										
n (%)	1407 (3.3)	500 (3.5)	1000 (3.1)	348 (3.2)	243 (3.9)	72 (4.1)	146 (4)	64 (4.3)	18 (3.6)	16 (7.4)
OR (95% CI)	1.00 (reference)	0.98 (0.83, 1.16)	1.00 (reference)	0.93 (0.76, 1.14)	1.00 (reference)	1.00 (0.67, 1.48)	1.00 (reference)	1.07 (0.62, 1.84)	1.00 (reference)	3.06 (0.77, 12.08)
SGA										
n (%)	1002 (2.3)	318 (2.2)	708 (2.2)	229 (2.1)	235 (3.8)	60 (3.4)	51 (1.4)	26 (1.8)	8 (1.6)	3 (1.4)
OR (95% CI)	1.00 (reference)	0.89 (0.76, 1.03)	1.00 (reference)	0.87 (0.73, 1.04)	1.00 (reference)	0.87 (0.62, 1.22)	1.00 (reference)	1.23 (0.72, 2.09)	1.00 (reference)	0.33 (0.04, 3.05)

(Continued)

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TABLE 4 Continued

	All		Normal weight		Underweight		Overweight		Obesity	
TG										
LGA										
n (%)	6693 (15.5)	2981 (20.8)	5204 (15.8)	2112 (19.7)	571 (8.5)	141 (10.9)	793 (25.6)	595 (29.0)	125 (30.7)	133 (42.8)
OR (95% CI)	1.00 (reference)	1.21 (1.15, 1.28)	1.00 (reference)	1.29 (1.22, 1.38)	1.00 (reference)	1.23 (0.98, 1.55)	1.00 (reference)	1.15 (1.00, 1.31)	1.00 (reference)	1.61 (1.15, 2.32)
Macrosomia										
n (%)	2267 (5.3)	991 (6.9)	1739 (5.3)	674 (6.3)	142 (2.1)	43 (3.3)	330 (10.7)	226 (11)	56 (13.8)	48 (15.4)
OR (95% CI)	1.00 (reference)	1.20 (1.10, 1.31)	1.00 (reference)	1.29 (1.16, 1.43)	1.00 (reference)	1.51 (0.98, 2.31)	1.00 (reference)	1.13 (0.93, 1.38)	1.00 (reference)	1.24 (0.79, 1.96)
LBW										
n (%)	1388 (3.2)	519 (3.6)	989 (3)	359 (3.4)	265 (3.9)	50 (3.9)	117 (3.8)	93 (4.5)	17 (4.2)	17 (5.5)
OR (95% CI)	1.00 (reference)	1.05 (0.99, 1.10)	1.00 (reference)	1.05 (0.99, 1.11)	1.00 (reference)	0.79 (0.49, 1.26)	1.00 (reference)	1.04 (0.91, 1.19)	1.00 (reference)	1.06 (0.73, 1.53)
SGA										
n (%)	1036 (2.4)	284 (2)	730 (2.2)	207 (1.9)	247 (3.7)	48 (3.7)	51 (1.6)	26 (1.3)	8 (2.0)	3 (1.0)
OR (95% CI)	1.00 (reference)	0.71 (0.61, 0.83)	1.00 (reference)	0.87 (0.73, 1.05)	1.00 (reference)	1.18 (0.83, 1.67)	1.00 (reference)	0.66 (0.38, 1.12)	1.00 (reference)	0.2 (0.02, 1.97)
LDL										
LGA										
n (%)	6988 (16.3)	2679 (18.4)	5332 (16.4)	1978 (17.9)	572 (8.7)	140 (9.5)	934 (27.9)	453 (25.1)	150 (35.9)	108 (36)
OR (95% CI)	1.00 (reference)	1.04 (0.99, 1.10)	1.00 (reference)	1.11 (1.05, 1.18)	1.00 (reference)	1.15 (0.94, 1.41)	1.00 (reference)	0.89 (0.71, 1.02)	1.00 (reference)	1.06 (0.76, 1.47)
Macrosomia										
n (%)	2329 (5.4)	926 (6.3)	1756 (5.4)	655 (5.9)	142 (2.2)	43 (2.9)	372 (11.1)	183 (10.1)	59 (14.1)	45 (15)
OR (95% CI)	1.00 (reference)	1.09 (1.00, 1.19)	1.00 (reference)	1.15 (1.04, 1.27)	1.00 (reference)	1.56 (1.08, 2.25)	1.00 (reference)	0.94 (0.77, 1.15)	1.00 (reference)	1.25 (0.80, 1.97)
LBW										
n (%)	1369 (3.2)	529 (3.6)	960 (2.9)	381 (3.5)	263 (4)	51 (3.5)	128 (3.8)	81 (4.5)	18 (4.3)	16 (5.3)
OR (95% CI)	1.00 (reference)	1.01 (0.97, 1.07)	1.00 (reference)	0.99 (0.82, 1.2)	1.00 (reference)	0.76 (0.5, 1.15)	1.00 (reference)	0.99 (0.6, 1.62)	1.00 (reference)	1.38 (0.37, 5.18)
SGA										
n (%)	986 (2.3)	328 (2.2)	698 (2.1)	234 (2.1)	241 (3.7)	53 (3.6)	41 (1.2)	36 (2)	6 (1.4)	5 (1.7)
OR (95% CI)	1.00 (reference)	0.86 (0.68, 1.1)	1.00 (reference)	1.00 (0.85, 1.18)	1.00 (reference)	0.85 (0.61, 1.18)	1.00 (reference)	1.59 (0.97, 2.6)	1.00 (reference)	1.71 (0.37, 7.88)
	≤ 25th	**> 25th**	**≤ 25th**	**> 25th**	**≤ 25th**	**> 25th**	**≤ 25th**	**> 25th**	**≤ 25th**	**> 25th**
HDL										
LGA										
n (%)	2268 (15.9)	7406 (17.1)	1686 (15.9)	5630 (17.1)	167 (8.3)	545 (9.1)	337 (24.2)	1051 (28)	78 (33.1)	180 (37.3)
OR (95% CI)	1.00 (0.94, 1.07)	1.00 (reference)	1.03 (0.96, 1.11)	1.00 (reference)	1.10 (0.88, 1.38)	1.00 (reference)	0.91 (0.78, 1.08)	1.00 (reference)	1.10 (0.75, 1.61)	1.00 (reference)
Macrosomia										
n (%)	839 (5.9)	2419 (5.6)	625 (5.9)	1788 (5.4)	52 (2.6)	133 (2.2)	137 (9.8)	419 (11.1)	25 (10.6)	79 (16.4)
OR (95% CI)	1.01 (0.91, 1.11)	1.00 (reference)	1.09 (0.97, 1.22)	1.00 (reference)	1.09 (0.71, 1.67)	1.00 (reference)	0.89 (0.70, 1.13)	1.00 (reference)	0.59 (0.34, 1.02)	1.00 (reference)
LBW										
n (%)	443 (3.1)	1464 (3.4)	310 (2.9)	1038 (3.1)	64 (3.2)	251 (4.2)	58 (4.2)	152 (4)	11 (4.7)	23 (4.8)
OR (95% CI)	0.90 (0.71, 1.13)	1.00 (reference)	1.05 (0.84, 1.31)	1.00 (reference)	0.94 (0.61, 1.46)	1.00 (reference)	0.65 (0.35, 1.21)	1.00 (reference)	0.24 (0.04, 1.54)	1.00 (reference)

(Continued)

TABLE 4 Continued

	All		Normal weight		Underweight		Overweight		Obesity	
SGA										
n (%)	378 (2.7)	942 (2.2)	268 (2.5)	669 (2)	88 (4.4)	207 (3.4)	18 (1.3)	59 (1.6)	4 (1.7)	7 (1.5)
OR (95% CI)	0.83 (0.68, 1.02)	1.00 (reference)	1.11 (0.93, 1.33)	1.00 (reference)	1.28 (0.92, 1.77)	1.00 (reference)	0.54 (0.29, 1)	1.00 (reference)	1.46 (0.27, 7.98)	1.00 (reference)

Data are shown as OR and 95% CI, adjusted for maternal age, mode of conception, parity, education attainment, consumption of cigarettes, infant sex, gestational diabetes, and gestational hypertension disorders. LGA, large for gestational age; LBW, low birth weight; SGA, small for gestational age; TC, total cholesterol; TG, triglyceride; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol.

increases to meet fetal growth and maternal circulating lipids directly impair placental vascular endothelium, leading to placental underperfusion and abnormal birth weight (53, 54). However, our study may indicate the important role of lipid levels in the first trimester. According to previous studies, elevated TG gives an increase in fatty acids, which may influence placental development and angiogenesis (42). In addition, fatty acids could act as growth factors and compete with hormones in binding to albumin, thus increase the free hormone levels such as sex hormones in circulation and subsequently impact intrauterine fetal growth (55). Moreover, maternal lipids in early pregnancy were found to be related to gestational complications such as gestational diabetes, which is a major contributor to LGA and macrosomia (56, 57).

The major strength of our study is the large study population from two centers and thorough and standardized medical records. In addition, the venous blood was drawn in a fasting state, which could reflect the lipid metabolic status better. Moreover, to our knowledge, this is one of the first studies to investigate the combined effects of pre-pregnancy BMI and maternal lipid profiles in early pregnancy on birth outcomes. However, the present study also has limitations,

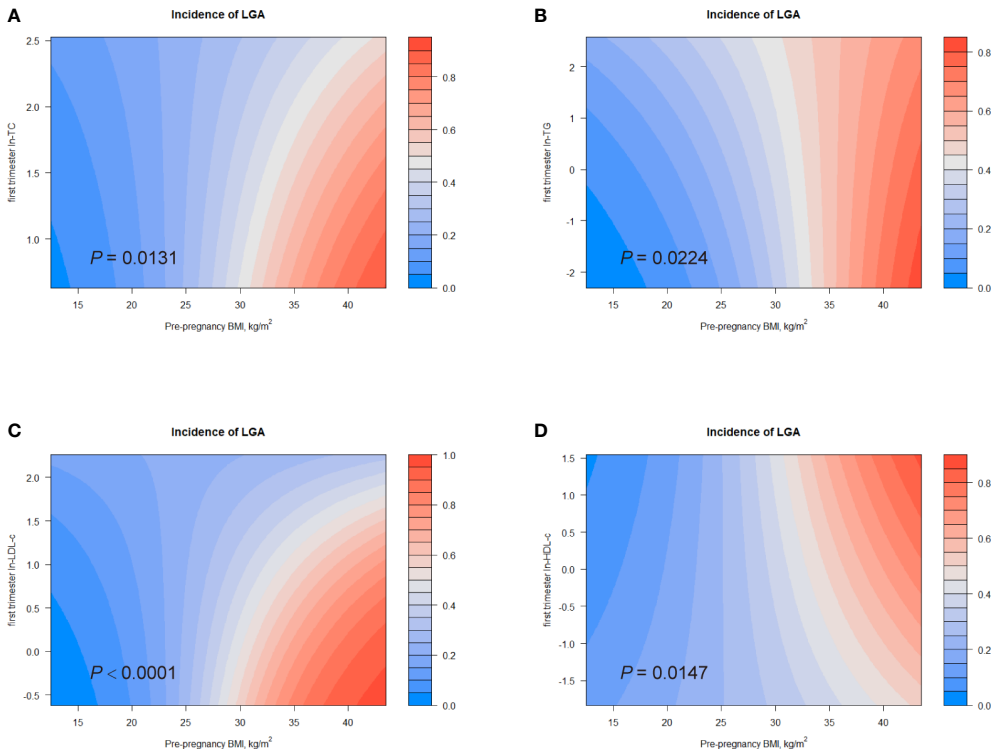

FIGURE 4
Combined effects of maternal pre-pregnancy BMI and lipid profiles in early pregnancy on incidence of LGA. Heat map for the correlation of incidence of LGA (red represents increased risks of LGA, blue represents decreased risks of LGA) according to the interaction of pre-pregnancy BMI and **(A)** ln-TC, **(B)** ln-TG, **(C)** ln-LDL-c, or **(D)** ln-HDL-c. Analyses were adjusted for maternal pre-pregnancy BMI, age, mode of conception, parity, education, consumption of cigarettes, infant sex, gestational diabetes and gestational hypertension disorders.

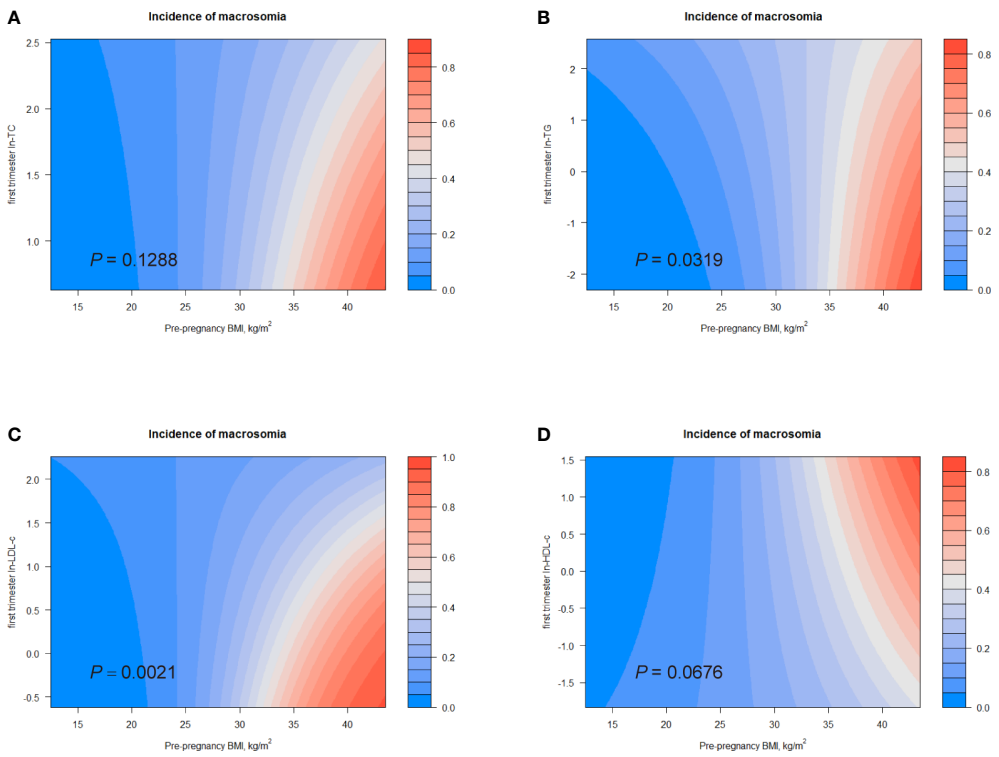

FIGURE 5
Combined effects of maternal pre-pregnancy BMI and lipid profiles in early pregnancy on incidence of macrosomia. Heat map for the correlation of incidence of macrosomia (red represents increased risks of LGA, blue represents decreased risks of LGA) according to the interaction of pre-pregnancy BMI and **(A)** ln-TC, **(B)** ln-TG, **(C)** ln-LDL-c, or **(D)** ln-HDL-c. Analyses were adjusted for maternal pre-pregnancy BMI, age, mode of conception, parity, education, consumption of cigarettes, infant sex, gestational diabetes, and gestational hypertension disorders.

such as some self-reported data (pre-pregnancy weight); a rather small ratio for pre-pregnancy overweight and obese women; lack of some important data, such as maternal blood pressure levels and assessment of placenta function; and not so strict exclusion criteria, as we did not exclude the pregnant women with gestational complications including gestational diabetes and hypertensive disorders. Therefore, we performed a sensitivity analysis to show the relationship between maternal lipid levels and birth weight in women without these conditions and found similar results (Table s4.). Also, the clinical significance of the reference values proposed in this study remains unclear, and we appeal more studies focusing on this issue. In addition, as a retrospective study, the unbalanced baseline data may lead to potential bias, although we have adjusted for confounders in statistical analysis.

In conclusion, maternal first trimester lipid profiles, especially TG, were associated with higher birth weight and increased risks of LGA and macrosomia in different pre-pregnancy BMI categories. Additionally, lipids screening during early pregnancy and pre-pregnancy weight status assessment should be essential to filter mothers who are prone to having infants of elevated birth weight. More studies focusing on the effect of gestational lipid profiles are necessary considering maternal and fetal health.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

Data interpretation, formal analysis, writing: S-MZ and H-QZ. Data curation: CL, CZ, and J-LY. Project administration,

funding acquisition, supervision and revision of the manuscript: Y-TW and H-FH. All authors have read the manuscript and agreed to the submitted version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.951871/full#supplementary-material

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Physiological and pathological roles of Ang II and Ang- (1-7) in the female reproductive system

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The local Renin-Angiotensin System (RAS) has been demonstrated to exist in a wide range of tissues and organs, In the female reproductive system, it is mainly found in the ovary, uterus and placenta. The RAS system is made up of a series of active substances and enzymes, in addition to the circulating endocrine renin-angiotensin system. The active peptides Angiotensin II (Ang II) and Angiotensin (1-7) (Ang-(1-7)), in particular, appear to have distinct activities in the local RAS system, which also controls blood pressure and electrolytes. Therefore, in addition to these features, angiotensin and its receptors in the reproductive system seemingly get involved in reproductive processes, such as follicle growth and development, as well as physiological functions of the placenta and uterus. In addition, changes in local RAS components may induce reproductive diseases as well as pathological states such as cancer. In most tissues, Ang II and Ang- (1-7) seem to maintain antagonistic effects, but this conclusion is not always true in the reproductive system, where they play similar functions in some physiological and pathological roles. This review investigated how Ang II, Ang- (1-7) and their receptors were expressed, localized, and active in the female reproductive system. This review also summarized their effects on follicle development, uterine and placental physiological functions. The changes of local RAS components in a series of reproductive system diseases including infertility related diseases and cancer and their influence on the occurrence and development of diseases were elucidated. This article reviews the physiological and pathological roles of Ang II and Ang- (1-7) in female reproductive system,a very intricate system of tissue factors that operate as agonists and antagonists was found. Besides, the development of novel therapeutic strategies targeting components of this system may be a research direction in future.

KEYWORDS

Angiotensin II, Angiotensin-(1-7), Renin-Angiotensin System, ovary, endometrial, placenta

Introduction

It is commonly known that the ovary, uterus, and placenta all contain local renin-angiotensin systems (1). However, its physiological and pathological significance in female reproduction is still unknown. Ang II and Ang- (1-7) are two of the RAS system's prominent active peptides. Ang II and Ang-(1-7) are bioactive substances involved in blood pressure regulation. These compounds are mostly linked to the incidence and progression of cardiovascular disorders (2). The biological effects of Ang-(1-7) are often in opposition to those of Ang II (3). With the depth of its research, recent studies have shown that it has a close bearing on physiological functions and pathological changes of the reproductive system (4–6).

The AT1 and AT2 receptors are the two main subtypes of Ang II in classical RAS, which is generated by Ang I in response to ACE (7). Ang II acts through AT1 using various signaling mechanisms, similar in some ways to those brought on by the EGF family and hormones mobilized by Ca 2+. On the contrary, phosphatases are primarily used to convey signals caused by activation of the AT 2 receptor. As a result, AT 2 receptor activation is thought to inhibit AT 1 receptor-mediated signaling (8).

Ang-(1-7) is generated through four metabolic pathways. The first is the hydrolysis of Ang I by enzymes such as enkephalin (NEP). Secondly, the angiotensin converting enzyme 2 (ACE2) cleaves Ang II to produce Ang-(1-7). Thirdly, ACE and NEP hydrolyze Ang-(1-9) to form Ang-(1-7). Additionally, Ang- (1-7) can be produced directly from Ang-(1-12) (9, 10). Among them, the cleavage of Ang II by ACE2 is the primary mechanism of production of Ang- (1-7) (11, 12). Although previous literature has demonstrated that Ang- (1-7) can act through AT1, AT2 and MAS receptors, it has radically differing affinities for AT1 and AT2 receptors (13, 14). MAS1 proto-oncogene G protein-coupled receptors are another route *via* which Ang- (1-7) can exert its effects (15), this is the main way that Ang- (1-7) plays its function. Ang II can contractile blood vessels, increase blood pressure, promote aldosterone secretion and promote cell proliferation through AT1 receptor. Ang II can inhibit cell proliferation, promote vascular dilatation, promote cell differentiation and induce cell apoptosis through AT2 receptor. Ang- (1-7) can diastolic blood vessels, reduce blood pressure, inhibit myocardial remodeling, improve endothelial function and inhibit cell proliferation through Mas receptor (Figure 1).

Changes in local Ang II and Ang- (1-7) activity can cause illnesses of the reproductive system, which in turn can lead to fertility issues. The content of this review is to describe the positive and negative effects of Ang II, Ang-(1-7) and their receptors on the biology of the ovary, the uterus, and the placenta. It provided an overview of Ang II and Ang- (1-7) expression, location, metabolism, and action in many reproductive organs, and elucidated their roles in physiological functions of the reproductive system. Finally, the variations in Ang II and Ang- (1-7) expression and activity in pertinent disorders were investigated. Additionally, their effects on the

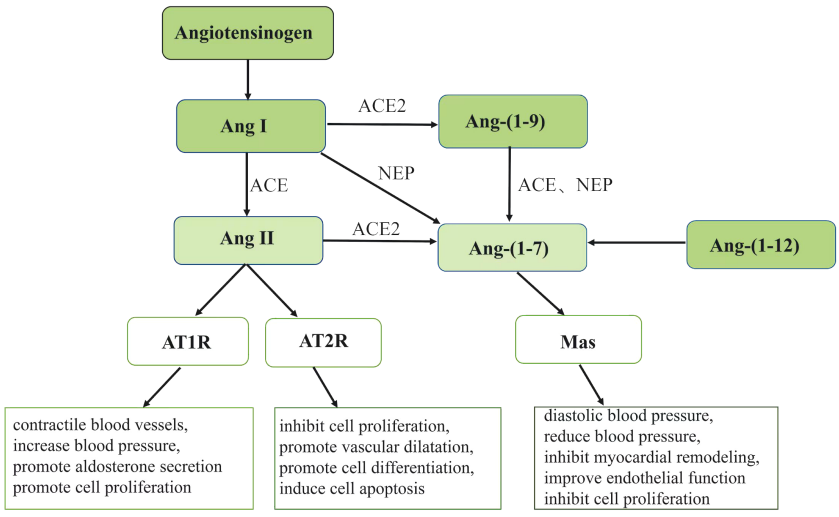

FIGURE 1
Ang, angiotensin; ACE, angiotensin converting enzyme; ACE2,angiotensin converting enzyme 2; AT1R, angiotensin receptor 1; AT2R, angiotensin receptor 2; NEP, enkephalin enzymes. The figure shows the pathway of Ang II and Ang- (1-7) generation. Angiotensinogen can generate Ang I and then hydrolyze Ang I to Ang II under the action of ACE. The generated Ang II can play different physiological roles through different receptors AT1R and AT2R.Ang- (1-7) can be generated through four pathways under the action of different enzymes and exert its physiological effects through Mas receptors.

onset and progression of illnesses as well as their potential for the therapy of connected disorders were explored.

Ang II, Ang- (1-7) and physiology of female reproductive system ovary

The primary human organ from which plasma reninogen is derived is the ovary (12).

Renin, which catalyzes the transformation of angiotensinogen into Ang I, is a precursor of prorenin. Ang I can either be immediately hydrolyzed to Ang- (1–7) or transformed into Ang II. Following that, Ang II was hydrolyzed by angiotensin converting enzyme 2 (ACE2) (16).

The follicular fluid contains Ang II and Ang- (1-7). While Ang- (1-7) is substantially less concentrated in the follicular fluid than it is in the plasma, Ang II is more abundant in the follicular fluid than it is in the plasma (2). This difference may be owing to the extra-ovarian origin of Ang II or the shorter half-life of Ang-(1-7) in follicular fluid. There was evidence that AT1R and AT2R were present in granulosa cells of primordial, primary, and secondary follicles (17). In the corpus luteum, microvascular endothelial cells express both AT1R and AT2R, although their expression varies during the course of the cycle: While AT1R expression does not change, AT2R expression decreases in the middle of the luteal phase and increases in the late luteal phase (14–16). All developmental stages of follicles are immune-responsive to Ang-(1-7) and Mas, except for the corpus luteum, ovarian stroma, and blood vessels (2, 18). The expression of Ang- (1-7) and Mas also varies in different developmental stages of follicles. In the granulosa layer of follicles, immunostaining can be observed in primary follicles, and strong immune response can be observed in secondary follicles. The Ang- (1-7) labeling is weak in preovulatory follicles, and Mas is moderately strong labeling. In secondary follicles, there was a significant immunoreactivity for Ang- (1-7) and Mas, and both were negative in preovulatory follicles. In the cytoplasm of large and small luteal cells, Ang- (1-7) labeling was weak, but Mas labeling was strong. Postmenopausal women's ovarian stromal cells stained moderately with Ang- (1-7) and Mas (2, 18).

Relevant studies have demonstrated that Ang II and its receptors have regulatory effects on oocyte maturation (19–22), *In-vitro* culture of bovine cumulus oocyte complex with Ang II can induce oocyte nucleus maturation (21, 23). Aside from that, Ang II also contributes to ovulation. Ang II can affect ovulation in cattle and rats through AT2R. Moreover, this effect can be prevented by inhibiting AT2R (24). Furthermore, the results showed that AT2R was also involved in follicular atresia through apoptosis (25, 26). Also, Ang- (1-7) participates in oocyte maturation and ovulation. Ang- (1-7) can promote the recovery of oocyte meiosis and induce ovulation in the absence of gonadotrophins, and an antagonist specific to Ang-(1-7), A779, inhibits these functions (18).

The plasma Ang-(1-7) levels in gonadotropin-stimulated women were shown to be elevated in a study of patients undergoing ovarian stimulation (COS) with human urine gonadotropin (HMG) and/or recombinant FSH (rFSH) (18) (2). Additionally, a linear relationship between Ang-(1-7) concentrations in human follicular fluid and the percentage of developed oocytes was discovered. However, in women with poor ovarian reserve function, AT1 receptor expression in granulosa cells was inversely linked with the amount of mature oocytes (27) (Table 1).

The expression and distribution of Ang II, Ang- (1-7) and their receptors are not the same among different species in ovary, which indicates that Ang II, Ang- (1-7) and their receptors may have different physiological effects. As the main bioactive substances in the renin angiotensin system, Ang II and Ang-(1-7) generally perform different functions and maintain a relative balance. Interestingly, in the ovary both seem to act in the same direction and play a crucial part in the physiological function of the ovary.

Endometrial

The endometrium undergoes periodic proliferation, differentiation and shedding during the menstrual cycle. Changes in the endometrium, driven by ovarian steroid hormones, play a crucial role in embryo implantation and pregnancy establishment (28). As reported by previous studies, Ang II and Ang- (1-7) and their related receptors can be detected in the endometrium throughout the menstrual cycle and change with the menstrual cycle (29). Ang II is most expressed in the proliferative phase, when it is predominantly located in glandular epithelium and stroma (30). Its expression is reduced during the secretory phase and is restricted to vascular stromal cells surrounding spiral arterioles in the endometrium (30, 31). The uterus has expression of both the Ang II receptor type 1 (AT1) and the Ang II receptor type 2 (AT2) proteins. The majority of AT1 is found in the endometrium, which has a high level of expression during the late proliferative phase but a lower level of expression during the secretory phase. AT2 was principally expressed in myometrium and uterine artery (32). Some studies have suggested that Ang II receptor activation can bring about apoptotic changes in the vascular wall (33). therefore, Ang II may be involved in endometrial vasoconstriction as well as vascular remodeling during menstruation (34). The expression of Ang- (1-7) was weak in endometrial glands at the early and middle stages of proliferation. Nonetheless, at the late stage of secretion, the expression of Ang- (1-7) increased and peaked. Ang- (1-7) was detected in the early stage of endometrial stromal hyperplasia, but not in the endothelium of endometrial vessels

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TABLE 1 Expression status of RAS components in different parts of ovary.

Ovarian structures	Ang II	Ang-(1-7)	AT1R	AT2R	Mas
Blood vessel	+	–	+	+	–
Primordial follicles		+		+	+
Oocytes	+		+		
Pregranulosa cells	+		–		
Primary/intermediate follicles		+		+	+
Oocytes	+		+		
Granulosa cells	+		+		
Secondary/Graafian follicles		+		+	+
Oocytes	+		+		
Granulosa cells	+		+		
Theca cells	+		+		
Ovarian cortex				n	
Epithelial cells	+	n	–		n
Stromal cells	+	+	+		+
Corpus luteum (early luteal phase)					
Large luteal cells	+	+	+	+	+
Small luteal cells	+	+	+	+	+
Corpus luteum (midluteal luteal phase)					
Large luteal cells	+	+	+	+	+
Small luteal cells	+	+	+	+	+
Corpus luteum (late luteal phase)					
Large luteal cells	+	+	+	+	+
Small luteal cells	+	+	+	+	+
Corpus luteum (in pregnancy)		n		n	n
Large luteal cells	+		+		
Small luteal cells	+		+		

+: express;-: negative; n: no data.

in the late secretory stage (30). The Ang- (1-7) receptor Mas, in contrast to Ang II receptor, only minimally enhanced in the glandular epithelium of the middle and late secretory stage, and did not change significantly with the fluctuation of ovarian steroid hormones. An experiment in which ovariectomized rats were given with estrogen or estrogen in combination with progesterone provided additional evidence that the expression of Mas receptors in the endometrium is not regulated by female sexual hormones (35, 36) (Figure 2).

However, there are still few studies bound up with the physiological function of local renin-angiotensin system in the uterus. Two studies on human endometrial cells cultured *in vitro* found that Ang II could induce cell proliferation and increase cell activity, but these effects could be offset by adding Ang- (1-7) (37, 38). Ang II can promote the transdifferentiation of endometrial epithelial cells into myofibroblasts. Except for this, Ang II has the ability to induce the activation of collagen type I as well as fibronectin. A recent study significantly prevented the biochemical and histopathologic changes resulted from endometrial hyperplasia by treating rats with angiotensin II receptor blockers. This means that the imbalance of local uterine RAS system and Ang II activation may trigger endometrial fibrosis, but this effect can also be inhibited by Ang- (1-7) (39, 40).

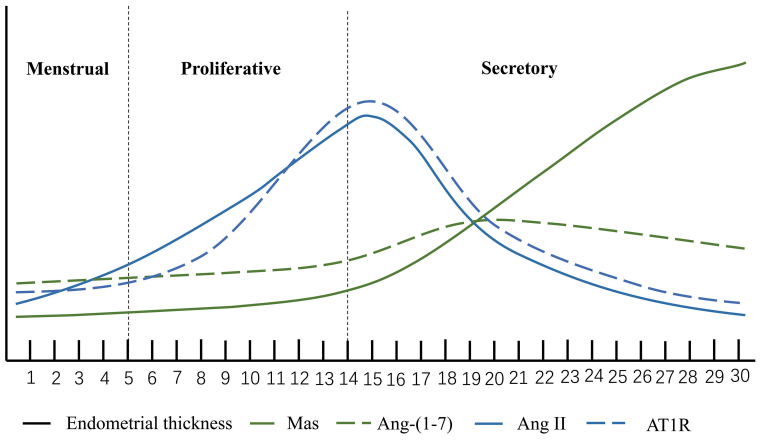

FIGURE 2
Ang II, Ang-(1-7), AT1R and Mas. The horizontal axis represents the menstrual cycle. The height of the vertical coordinate. The expression level of the molecule at each stage of the menstrual cycle.

Placenta

As a specialized connection for maternal exchange, the placenta supports hormone secretion, gas and nutrient exchange, as well as the establishment of an immune barrier (41). In the placental villi, high quantities of Ang II were found, and the expression of renin and ACE was also observed. AT1R and AT2R were detected in trophoblast cells (42). Different from the weak expression of AT2R, the expression of AT1R was quite high at the transcriptional level as well as the translational level throughout the whole pregnancy (42). All these circumstances suggest that placenta also has local RAS. As early as the second week of embryonic development, local RAS can promote blastocyst implantation, and Ang II can also heighten endometrial cell permeability by promoting decidual cell differentiation, thereby allowing trophoblast cell invasion into the maternal endometrium (43). Numerous studies have shown the pivotal function of uteroplacental RAS in spiral arterial remodeling and angiogenesis (44, 45). It has been shown that Ang II can influence the production of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in vascular endothelium and smooth muscle cells *via* activating AT1R in these cell types (46). In normal pregnancy, both VEGF and PlGF may be found in the trophoblast as well as the maternal decidua. In addition to this, VEGF and PlGF play a significant part in the development of the placental vasculature by controlling the growth of blood vessels in the chorionic villi (47). AT1R signaling in trophoblast cells also helps to enhance the synthesis of antiangiogenic molecules such as soluble Flt1 (sFlt1) and soluble endocrine hormone (sEng) (48), which helps to maintain a healthy equilibrium in placental angiogenesis. In addition to upregulation that occurs in trophoblast as a result of AT1R activation, sFlt1 expression also activates AT2R and Mas receptors *via* the binding of Ang II and Ang (1-7), respectively. This is not the case with sEng, indicating that the control of sEng is primarily dependent upon AT1R signaling and does not call for the involvement of AT2R or Mas receptors (49–51) (Figure 3).

It has been shown that angiotensin II is involved in the control of placental blood flow, hormone production, and immunological function. According to one research, Ang II may cause vasoconstriction in separate chorionic plate arteries (52). Pregnant mice lacking AT2R experience a gradual rise in blood pressure, indicating that AT2R signaling *in utero*placental arteries is crucial for maintaining a healthy placental perfusion balance (53, 54). Ang II may modify the impact of nutrition transport by affecting blood vessels. The enhanced secretion of hPL and SP1 and the synthesis of estrogen might both be boosted by Ang II, according to *in-vitro* tests on placental explants (48). The use of certain pharmacological blockers lends credence to the hypothesis that this function is mediated by AT1R and is associated with calcium signaling. In addition, it has been shown that Ang II, when co-cultured with trophoblast cells, affects monocyte adhesion *in vitro* and modulates immunity in the placenta (55–57).

There are few studies on the expression, localization and function of Ang- (1-7) in the placenta, and more evidence is needed to reveal its interaction with the placenta.

Ang II, Ang- (1-7) and the pathophysiology of female reproductive system ovarian hyperstimulation syndrome

The most dangerous side effect of ovulation induction is a condition called ovarian hyperstimulation syndrome (OHSS),

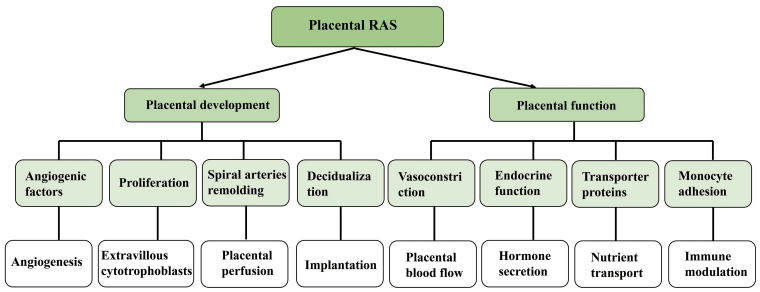

FIGURE 3
Effect and function of local renin angiotensin system on embryonic development.

which has a high fatality rate as well as a high morbidity rate. Ovarian enlargement, increased vascular permeability, extracellular fluid accumulation, and electrolyte imbalance are the hallmarks of severe OHSS (58).

High renin activity was found in both follicular fluid and plasma in patients with OHSS and was found to be directly associated with the severity of ovarian hyperstimulation syndrome (59–61). Several results showed that ascites from patients with severe OHSS had considerably greater amounts of reninogen and angiotensin II than pleural effusion and plasma did (62, 63). This result suggests that besides the peripheral circulation, the RAS system is also activated locally in the ovary. Activated ovarian RAS may cause ovarian enlargement and extracellular fluid accumulation in OHSS patients by inducing neovascularization and increasing capillary permeability.

Several researches have evaluated the effectiveness of medications that inhibit angiotensin converting enzyme and drugs that block angiotensin II receptors (64). Enalapril oral treatment was thought to have a 40% reduction in the occurrence of OHSS in a rabbit model, according to Morris et al. Nonetheless, Sahin et al. observed that neither hindering ACE nor blocking AngII receptor improved the ascites of OHSS in rabbits (65). Two clinical trials investigated the efficacy of dual renin-angiotensin blockade (a combination of angiotensin receptor blocker and angiotensin converting enzyme inhibitor) in preventing OHSS in IVF patients. It was found that this treatment may prevent some of the symptoms of OHSS and maintain a higher pregnancy rate after transfer of frozen-thawed embryos. However, OHSS cannot be completely eliminated by this treatment (66, 67).

Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is a complex illness that affects both the endocrine and metabolic systems. Its typical clinical phenotype includes Hyperandrogenism, insulin resistance, anovulation/anovulation and polycystic changes of the ovary are the most common causes of anovulation infertility (68).

There have been some studies on the expression changes of RAS components in patients with PCOS (69). According to Alphan et al., PCOS patients had greater blood total renin concentrations than non-PCOS patients (70). According to a survey, total renin concentrations were greater in obese PCOS women, and they were associated with fasting insulin and free testosterone levels. Although there was no discernible difference between PCOS women and controls in terms of plasma renin activity, ACE activity and Ang II levels were both markedly elevated in PCOS patients (71). Based on these findings, PCOS patients have an active renin-angiotensin system. A recent research, however, found that the ACE2-Ang- (1-7) -Mas axis in the ovary was hindered in polycystic ovarian rats. But in this study, a rat model characterized only by polycystic ovaries was used. Under the circumstances, RAS system expression levels are affected by multiple hormonal changes. As a consequence, the results of this experiment cannot preferably express the changes of RAS components in PCOS (72). In the endometrium of PCOS patients, a study of 15 endometrium of PCOS patients showed that the mRNA expression levels of ACE2, AT1, AT2 and Mas receptors in the endometrium of PCOS patients were higher than those in the control groups (73), These results make it clear that the elevated expression level of RAS components may affect the development of endometrium and play a role in its pathological process.

Insulin resistance and hyperandrogenemia are important clinical expressions of PCOS, and they are not independent. For example, insulin resistance can induce the increase of androgen level. As a multitude of studies have shown that Ang- (1-7) receptors can alleviate a myriad of metabolic diseases through Mas receptors and also counteract a range of adverse effects of Ang II, hypertensive drugs that block the action of Ang II can improve insulin resistance (74). Insulin resistance and hyperandrogenemia are the most common symptoms in PCOS patients. Studies have shown that Ang- (1-7) can not only improve the action of insulin, but also increase the delivery of

insulin to target organs through vasodilation (75). A range of evidence confirms that angiotensin receptor blockers and angiotensin converting enzyme inhibitors can improve insulin resistance (76). One study showed that lisinopril increased insulin sensitivity and reduced total and free testosterone in PCOS patients with hypertension (77). Angiotensin receptor antagonists and angiotensin converting enzyme inhibitors have positive effects on symptom improvement in PCOS patients. As mentioned above, both hyperandrogenism and insulin resistance are improved by hindering the RAS system, which may provide a new strategy for treating PCOS (77).

Additionally, some research have shown that the D allele of the ACE gene may be linked to acanthosis and a greater risk of insulin resistance in PCOS patients (78, 79). Other investigations have verified that, despite the ACE gene polymorphism not being linked to the pathophysiology of PCOS, it may be strongly linked to a number of metabolic diseases such insulin resistance, hyperlipidemia, and hyperandrogenemia (80, 81). And because it is bound up with steroid production in the ovary, it may also be connected with the exacerbation of clinical expressions of PCOS (82).

Ovarian cancer

Ovarian cancer (OC) is the most deadly kind of cancer that may develop in a woman's reproductive system. OC seriously affects life and health, but its pathogenesis remains unclear at present. The formation of tumors may be linked to the enhanced local expression of Ang II and its receptors in the ovary (83), Ang- (1-7), on the other hand, can reduce the growth, migration potential and invasiveness of tumors by several mechanisms against Ang II.

Takayasu et al. (84) Studies examining AT1R expression in human ovarian cancer and attempting to determine whether AT1R blockers could inhibit tumor progression showed AT1R expression in 85% invasive ovarian adenocarcinomas, 66% borderline malignancies, and 14% benign cystadenomas. In AT1R positive patients, there was a considerable increase in the amount of VEGF expression and tumor microvessel density. *In-vitro* studies proved that AT1R blockers could lessen the invasion potential and VEGF secretion of ovarian cancer cells. Previous research has shown that invasive and borderline diseased epithelial ovarian tumors had greater AT1R levels than healthy ovaries. Furthermore, earlier research has shown that individuals with ovarian cancer who have high AT1R levels had a poorer prognosis (83). Serum ACE levels are elevated in OC patients. As for epithelial OC patients, serum ACE levels do not vary according to patient stage and pathological subtype. This situation suggests that serum angiotensin I converting enzyme may be a marker of disseminated germinoma (85).

Ang II and ovarian cancer cells can hasten the metastasis of epithelial ovarian cancer (EOC) through interaction (86). Higher blood AT1-AA titers were shown to be related with the late advancement of EOC in patients, according to the findings of another investigation. What's more, higher serum AT1-AA titers may significantly contribute to the progression of EOC by facilitating cancer cell migration and angiogenesis (87). In ovarian cancer, high levels of AT1R expression are linked to increased rates of tumor angiogenesis and a poor prognosis, and AT1R blockers have been shown to inhibit peritoneal spread of ovarian cancer in a mouse model (88). *In vitro* and *in vivo* experiments on OC cell lines have shown that Candesartan, an AT1 receptor antagonist, can not only inhibit the invasion ability of tumor cells *in vitro*, but also inhibit the growth and progression of tumor and the formation of blood vessels in mice (84, 88). Therefore, a series of studies have confirmed that Ang II and Ang- (1-7) can participate in the pathological process of ovarian cancer by affecting the proliferation, migration and angiogenesis of tumor cells (6, 55, 89, 90).

Endometriosis

Endometriosis (Ems) is an estrogen-dependent condition marked by the development of adenocarcinoma and endometrial stromal cells outside of the uterine covered mucosa and myometrium. Although endometriosis is a benign disease, it can result in abdominal pain, menstrual abnormalities, and infertility, which disadvantageously affects about 10 percent of women in childbearing age (91).

The pathogenesis of endometriosis remains unclear. Worse still, the etiology and pathology of infertility are more complex. In the endometrium of people with endometriosis, there is enhanced expression of the Ang-1-7 receptor Mas, and the high expression of Mas may further the occurrence of endometriosis (92). Angiotensin type 1 receptor is involved in angiogenesis and affects endometrial growth, invasion and regression (93). In another trial using the AT1R antagonist telmisartan to treat mice endometriosis, it inhibited angiogenesis, immune cell content, and lesion growth in endometriosis (94). Tanshinone IIA not only significantly inhibits the growth of ectopic endometrium, but also alleviates EMs-induced pain syndrome by regulating RAS in neurons (95). However, on account of the physiological differences between human and mouse endometrium, a more complete theoretical and experimental basis is essential for applying this result to patients with endometriosis needs.

Studies on endometriosis and ACE gene polymorphism suggest that ACE gene polymorphism has some connection with the susceptibility and development of endometriosis. Furthermore, the ACE gene polymorphism can inhibit EMS-related pain by blocking the expression of Ang II and AT2R (95–98). On the other hand, some studies have borne out that ACE gene polymorphisms and haplotypes are not associated with endometriosis, and no association has been found between ACE

gene polymorphisms and the prevalence, progression, or number of lesions of endometriosis (99–101).

Endometrial cancer

Endometrial cancer (EC) is a primary epithelial malignant tumor of the endometrium, accounting for 20%-30% of female reproductive tract malignant tumors. It is generally believed that abnormal hormone secretion, genetic factors and environmental factors are connected to the onset and progression of the illness (102).

As we all know, the endometrium is responsible for the expression of all RAS components. The mRNA levels of AT1R, ACE1, and ACE2 were shown to be greater in tumor tissues of patients with endometrial cancer (103), compared to the levels found in neighboring non-cancerous tissues. The high expression of these factors supports the possibility that RAS contributes significantly to the emergence of endometrial cancer. The impact of Ang II on endometrial cancer cells was investigated in a study using endometrial cell lines. The results indicated that Ang II could augment the potential of cancer cell proliferation, metastasis and adhesion, and was associated with the highly differentiated state of cancer cells (104). However, to some degree, the capacity of tumor cells to migrate and invade may be impeded by silencing of the Ang II AT1R receptor, but this change is not absolute (105). Hence, Ang II may have a more complex network of effects in endometrial cancer, which needs further exploration. In endometrial cancer, Ang II increases the production of VEGF in a dose-dependent way and stimulate angiogenesis. Overexpression of Ang II-degrading enzymes and small interfering RNA were able to suppress both the immunoreactivity of VEGF as well as the number of blood vessels that were present in the tumor (106). One study observed that the AT1R blocker telmisartan significantly inhibited the growth of human endometrial tumors without toxic effects. This may provide a new strategy for future treatment of EC (105).

ACE gene polymorphism may be associated with the pathogenesis of endometrial cancer, and may be related to the development of the disease as well as the age of onset (31, 107, 108). Ang II may affect the progression of endometrial cancer by promoting the proliferation, differentiation and invasion of tumor cells. At present, few data can throw light upon the expression of Ang- (1-7) and its receptor in endometrial cancer and its effect on endometrial cancer, which may be the direction of future research, so as to further improve the research in endometrial cancer.

Preeclampsia

Preeclampsia (PE), which is a distinct pregnancy illness and a leading cause of maternal, fetal, and neonatal mortality with no proven cure other than delivery, is defined as the start of new-onset hypertension and proteinuria after 20 weeks of gestation (109). According to the performance of different pregnancy stages, PE is divided into early onset and late onset. Although the symptoms are similar, the causes of the two types are not the same (110). Early-onset PE is more commonly thought to be due to defects in placental development, so we are going to put our attention on early-onset PE.

Although the specific pathogenesis of PE is not adequately understood, numerous studies have shown that placental ischemia caused by reduced uteroplacental blood flow is the primary foundation of this pregnancy condition (111, 112). In PE pregnant women, the levels of Ang II and Ang- (1-7) were found to be much lower than those seen in normal pregnant women. Interestingly, the pressor response of Ang II and the amount of autoantibodies to the AT1 receptor are elevated (113). At1-aa can give rise to vasoconstriction through AT1R on vascular smooth muscle, and also promote the upregulation of various active substances (114–116), among which NADP upregulation enhances ROS formation, PAI-1(plasminogen activator inhibitor -1) reduces trophoblast invasion (117, 118). The increase of circulating sFlt1 level can result in the decrease of VEGF and PLGF bioavailability (50). Clinically, hypertensive individuals' risk of developing PE may be determined by assessing the ratio of circulating sFlt1/PIGF, which has the highest sensitivity and specificity in predicting and diagnosing PE. The decrease of free VEGF level can induce the expression of endothelin-1, touch off the decrease of renin secretion, further strengthen the negative regulation of blood volume, and thus cause the poor perfusion of placenta, which are associated with the development of the illness in its various stages (119). Aside from this, the increase of placental renin gene expression and protein level in transcription factor replacement and (Stox1) knockout mice gave rise to gestational hypertension through this change, which was alleviated by Ang II receptor blocker treatment, indicating that stox1 contributes to the development of PE through Ang II (120, 121).

There are different opinions on the expression of Ang- (1-7) in PE. One research revealed that early in pregnancy, PE patients' levels of Ang-(1-7) were greater than those of normal pregnant women, and this difference only existed in women with female fetuses (122). We believe that the pathogenesis of PE has a bearing on the imbalance of RAS. In future studies, we need to learn more about how Ang II and Ang- (1-7) are expressed and look into the reasons why PE patients have atypical Ang II and Ang- (1-7) expressions. Patients with PE have RAS disorder, especially the activation of AT1R can participate in the pathogenesis of PE by promoting the validation reaction-mediated oxidative stress and other pathways (123). Angiotensin antagonists and angiotensin-converting enzyme inhibitors can lower blood pressure, but cannot be recommended as oral drugs due to their teratogenic effects.

Coronavirus disease 2019

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It is associated with respiratory tract involvement and typical symptoms include dry cough, dyspnoea, fever, etc (124). In addition, the understanding of whether women's reproductive systems can be affected by the coronavirus continues to improve (125). ACE2 is a key enzyme in the RAS of this disease, not only regulating the expression of Ang II and Ang-(1-7), but also acting as a receptor to mediate infection of target cells by SARS-CoV-2 (126). ACE2 is present at all stages of follicular development. Ang II is mainly expressed in granulosa cells and Ang-(1-7) is expressed primarily in intermembrane cells. Both Ang II and Ang-(1-7) are involved in the development and maturation of oocytes (6). Two studies in women of childbearing age showed no significant effect of SARS-CoV-2 on ovarian reserve function (127, 128). Another analysis of 32 in vitro fertilization (IVF) patients revealed there were no obvious differences in follicular function, including but not limited to hormone synthesis, trigger response and embryo quality (129). However, it was also suggested that SARS-CoV-2 could cause a decrease in ACE2 expression, leading to an increase in Ang II and an imbalance in the expression of Ang II and Ang-(1-7) in the ovary. Consequently, it induced enhanced local inflammation, fibrosis, apoptosis and other pathological responses in the ovary, further affecting the reproductive function of the ovary. The most attention in research was paid to the effect of SARS-CoV-2 on menstruations. A series of studies indicated that SARS-CoV-2 has a significant impact on menstrual patterns, menstrual volume and dysmenorrhea (4, 130, 131). This might be because ACE2, Ang II and Ang-(1-7) can maintain cyclic changes in the endometrium by participating in vasoconstriction as well as cell renewal and proliferation. Since ACE2, which functioned as the SARS-CoV-2 receptor, was low in abundance in the endometrium, it did not show susceptibility to the coronavirus (132). Intriguingly, studies suggested that female age can be a risk factor for COVID-19. This was achieved because the expression of ACE2 in the endometrium increased with age (4). ACE2 was widely expressed in the placenta, including trophoblast cells, perivascular and vascular smooth muscle cells of decidual vessels as well as decidual stromal cells (133). This inferred that the placenta was more prone to the effects of SARS-CoV-2. Furthermore, the expression of ACE2 was not constant in the placenta. The level of ACE2 expression decreased with increasing gestational age, suggesting a greater likelihood of transplacental transmission of SARS-CoV-2 in early gestation (134). Although it was theoretically possible for SARS-CoV-2 to be transmitted to infants through the placenta. However, studies demonstrated that most newborns were not found to be infected after birth. It was hypothesized that this was possibly due to the barrier effect of the placenta, but the exact mechanisms of action remained unclear (135). Other than the possible effects mentioned above, SARS-CoV-2 could also affect endocrine organs such as the thyroid gland, thus impacting the endocrine system of patients (136, 137). This might further cause alterations in female fertility. Due to limited clinical data, the understanding of the impact of SARS-CoV-2 on the female reproductive system is not comprehensive and profound. Therefore, predicting the impacts of SARS-CoV-2 on the female reproductive system and the specific mechanisms of action will be an essential direction for future research.

Conclusion

In conclusion, the female reproductive system does in fact include a local renin-angiotensin system. Numerous studies have also demonstrated that angiotensin, including Ang II and Ang-(1-7), can be produced locally in the reproductive system. RAS affects oocyte maturation and excretion, endometrial periodic changes and hormone production. RAS is considered to be an important way to regulate physiological functions of the reproductive system. However, the functions that Ang II and Ang- (1-7) play in the physiology and pathology of the female reproductive system are still not fully understood. Furthermore, most of the previous studies remain at the level of nonfunctional description. The intricate dependency network in the peptide-hormone system in issue, together with species variations, are the main causes of the interpretation challenges. Undoubtedly, further study in this field is required to fully comprehend how RAS affects female fertility. Likewise, the intensive study in the future is indisputably essential to determine the contribution of RAS to a wide range of related diseases.

Author contributions

YL and ZZ contributed to conceived and designed the review. YL wrote the paper. RJ and HH did the document retrieval. LZ and MC polished the paper. ZZ and WP checked the paper. All authors listed have made a substantial and intellectual contribution to the review and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Characterization of placental endocrine function and fetal brain development in a mouse model of small for gestational age

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Conditions such as small for gestational age (SGA), which is defined as birthweight less than 10th percentile for gestational age can predispose to neurodevelopmental abnormalities compared to babies with normal birthweight. Fetal growth and birthweight depend on placental function, as this organ transports substrates to the developing fetus and it acts as a source of endocrine factors, including steroids and prolactins that are required for fetal development and pregnancy maintenance. To advance our knowledge on the aetiology of fetal growth disorders, the vast majority of the research has been focused on studying the transport function of the placenta, leaving practically unexplored the contribution of placental hormones in the regulation of fetal growth. Here, using mice and natural variability in fetal growth within the litter, we compared fetuses that fell on or below the 10th percentile (classified as SGA) with those that had adequate weight for their gestational age (AGA). In particular, we compared placental endocrine metabolism and hormone production, as well as fetal brain weight and expression of developmental, growth and metabolic genes between SGA and AGA fetuses. We found that compared to AGA fetuses, SGA fetuses had lower placental efficiency and reduced capacity for placental production of hormones (e.g. steroidogenic gene *Cyp17a1*, prolactin *Prl3a1*, and pregnancy-specific glycoproteins *Psg21*). Brain weight was reduced in SGA fetuses, although this was proportional to the reduction in overall fetal size. The expression of glucose transporter 3 (*Slc2a3*) was reduced despite the abundance of AKT, FOXO and ERK proteins were similar. Developmental (*Sv2b* and *Gabrg1*) and microglia genes (*Ier3*), as well as the pregnancy-specific glycoprotein receptor (*Cd9*) were lower in the brain of SGA versus AGA fetuses. In this mouse model of SGA, our results therefore demonstrate that placental endocrine dysfunction is associated with changes in fetal growth and fetal brain development.

KEYWORDS

placenta, mouse, pregnancy, prolactin, pregnancy specific glycoprotein, fetal growth, animal models, endocrinology

1 Introduction

Abnormal birthweight is one of the most common complications of pregnancy that has immediate and long-term consequences for offspring wellbeing (1). Compared to babies that have an appropriate weight for their gestational age (AGA), babies born small (<10th percentile) or large for gestational age (>90th percentile) (SGA or LGA, respectively) are at higher risk of obstetric and neonatal complications (2, 3). Moreover, studies have shown that SGA or LGA increases the risk for metabolic diseases and cognitive and neurodevelopmental abnormalities compared to AGA (4–8). The identification and subsequent classification of babies with abnormal weight is challenging, as it requires segregation of babies that are constitutively small or large due to parental genetics versus babies that grew abnormally due to intrauterine problems (9–11). Therefore, work is required to understand the causes and mechanisms by which altered fetal growth may arise.

Fetal growth is regulated, in part, by the placenta which is a fetal organ responsible for the transport of gases and nutrients between the mother and the fetus. Aberrant placental function related to failure of substrate transport or deficits in vascular growth have been linked to fetal growth abnormalities, including fetal growth restriction, as well as stillbirth (12–14). However, the placenta is also a powerful endocrine organ that secretes an abundance of hormones and growth factors into the maternal and fetal circulation that allow for fetal development and pregnancy maintenance (15–20). Indeed, there is now growing evidence suggesting that placental metabolism and function impact on fetal brain development (21–27). For example, in mice, there is genomic linkage between placental and the fetal hypothalamic development (28). In mice, placental metabolism of serotonin is critical for fetal forebrain development. Others have found that placental allopregnanolone deficiency in mice alters cerebellar white matter development and programmes postnatal autistic-like behaviour in the offspring (24). Therefore, these studies suggest a potential link between placental endocrine function, fetal growth and fetal neurodevelopment, which could have long last effects on neurocognitive health outcomes.

In this scenario, animal models in which fetal and postnatal outcomes can be monitored are essential. Much of the investigations focused on fetoplacental development have been conducted in rodents, rabbits, sheep and pigs (29–36). However, the use of mice is still preferred over the aforementioned animal species due to multiple reasons. For example, the short gestational period and the relatively easy, lower cost maintenance of mice compared to large animal species is great for investigating fetal growth control. In mice, the sequence of events for brain maturation are largely similar to in humans (37). Moreover, a similar to in human, the mouse placenta is haemochorial in structure. An additional key advantage of the mouse is that its placenta is structurally divided in two functionally specialised regions; transport is carried out in the labyrinth zone, while hormone production is principally performed by the junctional zone (Jz). This characteristic is very helpful when studying placental function, as both layers can be easy separated (38). Nonetheless, the vast majority of published studies assessing the importance of the placenta for fetal growth have focused on placental transport function [e.g (31, 39–43).], leaving the significance of placental endocrine function practically unexplored.

Numerous animal models of pregnancy complications have been created, including those that mimic environmental and maternal conditions (11, 29, 36). However, models based on constructing fetal growth curves and percentile cut-offs, as applied in human obstetrics, are not commonplace for experimental research on placental function and fetal physiology (44). Here, using the mouse as an experimental animal model, we took advantage of the normal fetal weight variation observed within the litter to compare placental endocrine function and fetal brain development for fetuses that were classified as AGA or SGA using percentile cut-offs. We therefore hypothesize that abnormal placental endocrine output is an additional factor contributing to the development of SGA. Moreover, using fetal growth curves and percentile cut-offs in a model in which maternal health is preserved provides us with a valuable opportunity to study endocrine interaction between the placenta and fetus in a controlled intrauterine environment. Indeed, our work identifies that the production of prolactins and pregnancy specific glycoproteins (PSGs) by the placenta is compromised in conjunction with reduced fetal growth and brain development of SGA fetuses. Our work reinforces the idea of a placental-fetal brain axis that is controlled, in part, by placental hormones (25).

2 Materials and methods

2.1 Animal work

All animal work was performed under the UK Animals (Scientific Procedures) Act 1986. Experiments were conducted with a total of 11 C57BL6/J wild-type female mice (4 months old) housed at the University of Cambridge Animal Facility under a 12/12 dark-light system and fed *ad libitum* with a standard chow diet (RM3; Special Dietary Services). Females were time-mated with C57BL6/J males and the day a copulatory plug was detected was designated as gestational day (GD) 1 (term occurs ~GD20). On GD16, pregnant females were killed by cervical dislocation, the uterus removed and fetuses cleaned from feto-placental membranes. Each fetus (and its corresponding placenta), was identified based on its position in the uterus, dried on tissue paper, weighed and immediately decapitated. Fetal brains were removed, weighed and immersed in liquid nitrogen for rapid freezing. The placental Jz was dissected from maternal decidua and placental labyrinth zone, and subsequently snap-frozen in liquid nitrogen. All frozen fetal and placental samples were stored at −80°C until tissues were powdered in individual pieces of foil using a hammer and dry ice for RNA/protein extraction (all samples were maintained in a frozen state, as far as possible). Only viable fetuses were used in the study and litter sizes ranged from 6 to 11 pups.

2.2 RNA extraction, cDNA reverse transcription and qPCR

Placental Jz and fetal brain RNA (8-9 samples per group) was extracted using the RNeasy Plus Mini Kit (Qiagen) and the quantity of RNA obtained was determined using a NanoDrop spectrophotometer (NanoDrop Technologies) as previously described (45). RNA was reverse transcribed using a high-capacity

cDNA reverse transcription kit (Applied Biosystems) according to manufacturer's instructions. Samples were analysed with a StepOne real-time PCR machine (Thermo Fisher Scientific) in duplicate using SYBR Green qPCR master mix (Applied Biosystems, Thermo Fisher Scientific) and primers described in Supplementary Table 1. Brain gene expression was normalized to the genomic mean of two housekeeping genes (*Actb* and *Gapdh*), while placental genes were normalized using *Actb* and *Ywhaz* as reference genes. These housekeeper genes remained stably expressed between the groups. Analysis was performed using the 2-ΔΔCt method (46).

2.3 Protein extraction and western blotting

Total protein was extracted from fetal brain and placental Jz (5 samples per group) using RIPA buffer (R0278-50M, Sigma Aldrich) supplemented with mini EDTA-free protease inhibitor cocktail mix (Roche), 1mM β-glycerophosphate (G-9891, Sigma Aldrich) and 1mM sodium orthovanadate (S65089891, Sigma Aldrich). Lysates were centrifuged at 2,500 rpm for 15 minutes at 4°C and protein concentration determined using the Pierce BCA protein assay kit (23225, Thermo Fisher Scientific). Samples were mixed with SDS gel loading buffer (L-4390, Sigma Aldrich) and protein denaturation was performed at 90°C for 5 minutes. After gel electrophoresis, proteins were transferred from the gel onto 0.45 µm nitrocellulose membranes (10600012, Amersham Protran). Membranes were then blocked either with 5% fetal bovine serum (A2153-100G, Sigma Aldrich) or semi-skimmed milk (Marvel) for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies described in Table 1. The day after, membranes were washed with TBS-T and incubated for 1 hour at room temperature with secondary antibodies (1:10,000 NA934 or NA931, Amersham). Membranes were exposed to ECL substrate (SuperSignal West Femto, Thermo Fisher Scientific) and images were taken with an Invitrogen iBright imaging system (Thermo Fisher Scientific). Pixel intensity of protein bands was analysed with ImageJ software and normalization performed against beta-actin levels. Phosphorylated proteins were normalized to its corresponding total protein abundance.

2.4 Sample size and statistical analysis

No sample size calculation was conducted prior to undertaking this study. For the purpose of this paper, the fetus, and not the mother, was designated as the experimental unit. GraphPad Prism software (version 9) was used to determine statistical differences between groups. The distribution of fetal weights (graphed as a histogram) was constructed with a non-linear regression (Gaussian distribution) and percentiles calculated with the descriptive statistical analysis tool of GraphPad Prism software. Then, data in Excel were organized according to fetal weight and separated in three experimental groups based on the fetal weight threshold (SGA fetuses: 10^{th} percentile ≤321 mg; AGA fetuses: 321-409 mg and LGA fetuses: 90^{th} percentile≥410 mg). ROUT test was used to identify outlier values, which were then excluded for statistical purposes. Normality and homogeneity of variance of variables for western blotting and qPCR data were performed using the Shapiro-Wilks test in GraphPad Prism. For comparisons between two groups, the Student's t-test or Mann-Whitney test were applied according to the normality of the variable. For data with more than two groups, one-way ANOVA coupled with Bonferroni *post hoc* test was employed. The relation between fetal intrauterine position as well as litter size with the presence of SGA, AGA and LGA was analysed by Chi square test using a contingency table in GraphPad Prism software. Pearson r correlations were performed with GraphPad Prism software and presented in a double gradient colour mapping graph that was subsequently modified with Adobe Illustrator to facilitate visualization. qPCR and western blotting data are expressed as individual data points and reported as mean ± SEM. P-values <0.05 were considered statistically significant.

TABLE 1 List of antibodies used for western blotting.

Protein	Catalogue number	Dilution
Insulin receptor	Santa Cruz, SC-711	1/200
AKT	Cell Signalling, 9272	1/1,000
Phospho-AKT (Ser473)	Cell Signalling, 9271	1/1,000
Phospho-AKT (Thr308)	Cell Signalling, 9275	1/1,000
mTOR GβL	Cell Signalling, 3274	1/1,000
FOXO1 (C29H4)	Cell Signalling, 2880	1/1,000
Phospho-FOXO1 (Ser256)	Cell Signalling, 9461	1/1,000
FOXO3a (D19A7)	Cell Signalling,12829	1/1,000
Phospho-FOXO3a (Ser318/321)	Cell Signalling, 9465	1/1,000
P44/42 MAPK (Erk1/2)	Cell Signalling, 4695	1/1,000
Phospo-MAPK-p44/42 (Erk1/2) (Thr202/Tyr204)	Cell Signalling, 4370	1/1,000
Beta-Actin	Cell Signalling, 58169	1/5,000

3 Results

3.1 Validation of the SGA model

The distribution of fetal weights across the 11 litters studied is shown in Figure 1A and litter sizes across the different litters used in this study can be found in Supplementary Table 2. A total of 9 fetuses were considered SGA (below the 10th percentile) and they were obtained from 5 different litters. On average, fetal weight was significantly lower by 19% in SGA fetuses when compared to AGA fetuses (Figures 1A, B). A total of 11 LGA fetuses (above the 90th percentile) were obtained from 3 different litters and these were on average, 12% heavier than the AGA group. All litters had at least 3 AGA fetuses. Analysis of placental weight did not show significant differences between the SGA, AGA and LGA fetuses (Figure 1C). However, the fetal weight to placental weight ratio, defined as grams of fetus produced by grams of placenta and also known as placental efficiency, was significantly reduced in the SGA fetuses compared to AGA and LGA fetuses. No differences in placental efficiency were observed between the AGA and LGA fetuses (Figure 1D).

Fetal and placental weights were not affected by the uterine position, as similar weights were found for fetuses positioned close to the ovary, cervix, and in between these two positions (Figures 1E, F). Nonetheless, placental efficiency was significantly lower by 10% for fetuses positioned close to the ovary compared to those in the middle of the uterus (Figure 1G). Individual group analysis demonstrated that there was not a clear pattern in the incidence of being SGA, AGA or LGA based on uterine position. Although a significant effect was observed between fetal position and being LGA or SGA (Figure 1H). Indeed, the number of SGA fetuses positioned close to the cervix was higher than for those proximal to the ovary (33.33% versus 22.22%; Figure 1H). This pattern was the opposite for LGA fetuses, with 22.22% located close to the cervix and 44.44% close to the ovary. AGA fetuses were primarily found in the middle part of the uterus (54.28%).

Finally, we divided our data according to the number of fetuses per litter to assess if litter size impacted the percentage of SGA, AGA and LGA (litters of 6 to 8 fetuses versus 9 to 11 fetuses; Figures 1I–L). This analysis revealed that there was no difference between fetal or placental weight, nor the proportion of fetuses classified as SGA, AGA or LGA between litters of 6 to 8 fetuses compared to those of 9 to 11 fetuses (Figure 1L). However, placental efficiency was significantly greater by 7.80% in litters with 9 to 11 fetuses compared to those with 6 to 8 (Figure 1K). Moreover, we then performed analysis of LGA and SGA fetuses by considering the litter size as a potential effect (comparison of LGA-SGA fetuses in litters of 6-8 fetuses versus LGA-SGA fetuses in litters of 9-11 fetuses). As displayed in Supplementary Table 3, we did not see differences in LGA or SGA fetuses in any of the aforementioned parameters.

3.2 Placental endocrine function in SGA fetuses

To understand if the reduced placental efficiency for SGA fetuses was related to alterations in the endocrine function, key growth and

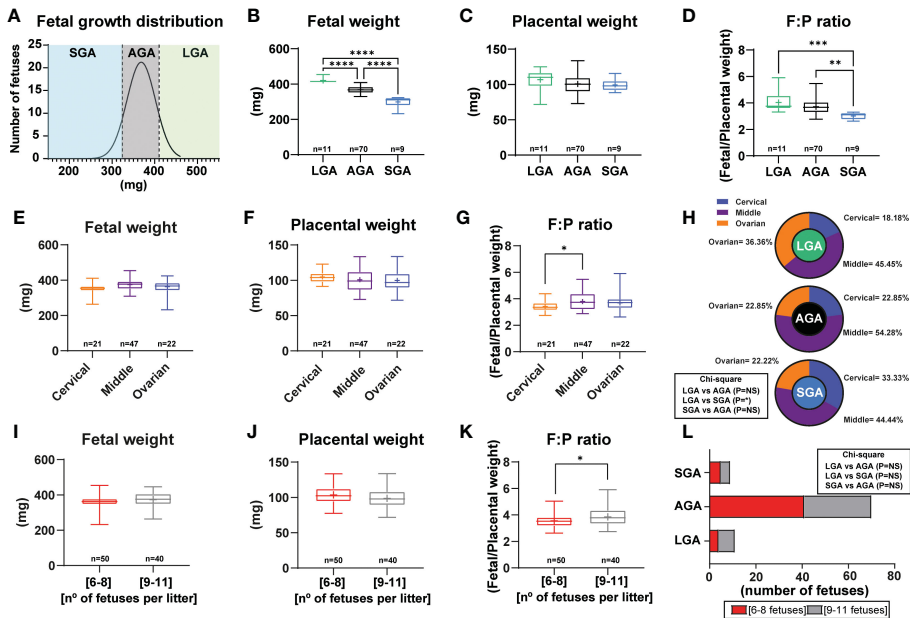

FIGURE 1
Fetal and placental weight in mice and establishment of a model to study small for gestational age. **(A)** Frequency distribution curve for fetal weights on GD16. Vertical line denotes the 10th (321mg) and 90th percentiles (410 mg). **(B)** Fetal weight for fetuses classified as SGA, AGA and LGA. **(C)** Placental weight classified as SGA, AGA and LGA. **(D)** Placental efficiency (defined as grams of fetus produced by grams of placenta) classified as SGA, AGA and LGA. **(E–G)** Feto-placental weights and placental efficiency based on their position in the uterus. **(H)** Distribution of LGA, AGA and SGA fetuses according to their position in the uterus. **(I–K)** Feto-placental weights and placental efficiency based on litter size. **(L)** Distribution of LGA, AGA and SGA fetuses according to litter size. Data are from 90 fetuses in total from 11 litters. Feto-placental weights are shown as box plots and whiskers. The rectangle shows the distribution, the line the median, the whiskers the maximum and minimum and the "+" is the mean of the group. Statistical analysis performed by one-way ANOVA, Student t-test, Mann-Whitney test and Chi-square test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. AGA (appropriate for gestational age), LGA (large for gestational age), SGA (small for gestational age), n° (number).

metabolism proteins were quantified by western blotting in the placental Jz of SGA compared to AGA fetuses (Figures 2A, B). This revealed that the abundance of FOXO1, which functions in cell cycle and differentiation (47), was significantly reduced by 27% in the SGA compared to AGA group without affecting phosphorylated site at serine 256 (ratio of phosphorylated to total FOXO1; Figure 2B). However, the abundance the insulin receptor β, AKT (total and phosphorylated at threonine 308 and serine 473 sites), FOXO3 (total and phosphorylated at serine 318/321 sites), ERK (total and phosphorylated at threonine 202 and tyrosine 204 site) and mTOR-GβL were unaltered between SGA and AGA fetuses (Figures 2A, B).

We next evaluated whether the expression of imprinted genes could be altered in placental Jz samples from SGA fetuses as previous studies have shown that, in mice, the imprinted loci *Igf2-H19, Ascl2* and *Peg3* are implicated in the control placental Jz formation and/or function (48–51). No changes were observed in any of the imprinted genes analysed in the Jz between SGA and AGA (Figure 2C). The Jz expression of *Tfap2c*, which has been shown to control the expression of certain imprinted genes such as *H19* and *Ascl2* (52), was also unaltered between the two experimental groups (Figure 2C). Moreover, mRNA levels of the IGF2 receptors, *Igf1r* and *Igf2r* remained similarly expressed in the Jz between SGA and AGA (Figure 2C).

We then assessed the placental capacity to produce hormones by measuring mRNA levels of genes involved in steroidogenesis (*Stard1*, *Cyp11a1*, *Cyp17a1* and *Hsd3b1*), pregnancy specific glycoprotein genes (PSGs; *Psg17*, *Psg18*, *Psg19* and *Psg21*) and prolactins (*Prl3a1*, *Prl3b1* and *Prl8a1*) in the Jz of SGA and AGA fetuses (Figures 2D–F). We observed that *Cyp17a1* expression was 28% lower in the placental Jz of the SGA group compared to AGA (Figure 2D). Moreover, *Psg21*

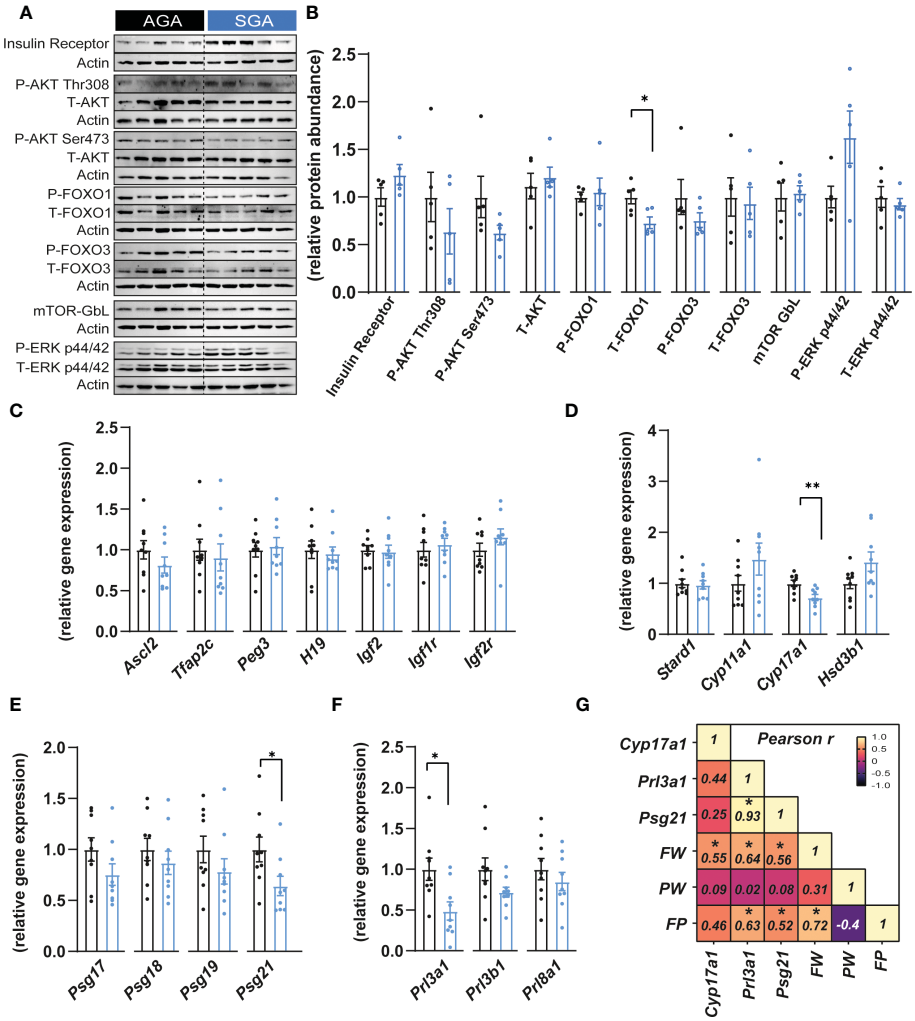

FIGURE 2
Small for gestational age is associated with altered expression of placental hormones. **(A, B)** Immunoblots and protein abundance of key growth and metabolic signalling proteins in dissected placental junctional zones (n=5 samples per group). **(C)** Expression of key genes involved in placental function and growth (n=9 samples per group). **(D–F)** Expression of steroidogenic pathway regulatory **(D)**, pregnancy specific glycoprotein **(E)** and prolactin **(F)** genes in dissected placental junctional zones (n=9 samples per group). **(G)** Heatmap of Pearson's correlations (number inside the box corresponds to the r value for the correlations that were significant) (n=18 samples). Data are shown as individual values and bars represent mean ± SEM. Statistical analysis performed by paired or unpaired t-test (Student t-test and Mann-Whitney test, respectively). *P<0.05, **P<0.01. AGA (appropriate for gestational age) and SGA (small for gestational age), FW (fetal weight), FP (feto-placental ratio, also known as placental efficiency), PW (placental weight), P (phosphorylated protein levels), T (total protein levels).

mRNA levels were reduced by 36% in the SGA versus AGA fetuses (Figure 2E). The expression of prolactin genes, *Prl3a1* was also downregulated by 51% in the SGA group versus the AGA (Figure 2F). However, the Jz expression of other hormone-related genes quantified, namely *Stard1*, *Cyp11a1*, *Hsd3b1, Psg17, Psg18, Psg19, Prl3a1, Prl3b1* and *Prl8a1* were not different between the two experimental groups. To understand the contribution of changes in placental Jz *Cyp17a1, Psg21* and *Prl3a1* expression for feto-placental size and placental efficiency, we performed Pearson r correlations (Figure 2G). This revealed that Jz expression of all three genes correlated positively with fetal weight. *Prl3a1* and *Psg21* correlated positively with placental efficiency. Both, *Prl3a1* and *Psg21* correlated positively with each other. Lastly, placental efficiency, but not placental weight correlated positively with fetal weight (Figure 2G).

3.3 Brain development in SGA fetuses

Short and long-term neurological impairments have been observed in offspring who were SGA (53). Therefore, we investigated if there could be any changes in fetal brain development in our mouse model of SGA. We found that SGA fetuses exhibited a 20% and 25% reduction in brain weight compared to the AGA and LGA groups, respectively (Figure 3A). In contrast, brain weight was similar between AGA and LGA fetuses (Figure 3A). Analysis of fetal brain weight relative to fetal body size revealed that the reduction in brain size was proportional to the reduced size of the SGA fetuses (brain to body weight ratio; not different between SGA, AGA and LGA fetuses; Figure 3B), which suggested that SGA fetuses were symmetrically smaller. We then determined the relationship between placental and fetal brain size by assessing the ratio of fetal brain weight to placental weight. This showed that fetal brain weight to placental weight ratio was significantly reduced by 18% in SGA compared to AGA fetuses (Figure 3C). However, this ratio was not different between LGA and AGA fetuses.

We also analysed the effect of uterine position and litter size on fetal brain development (Supplementary Figure 1). We did not see differences in the weight of the brain, although brain ratio was significantly increased in fetuses positioned closer to the ovaries compared to those in the middle position (Supplementary Figures 1A, B). No differences were found in the brain-placental ratio and litter size did not affect the weight of the brain, although brain ratio was significantly increased in litters of 6-8 fetuses

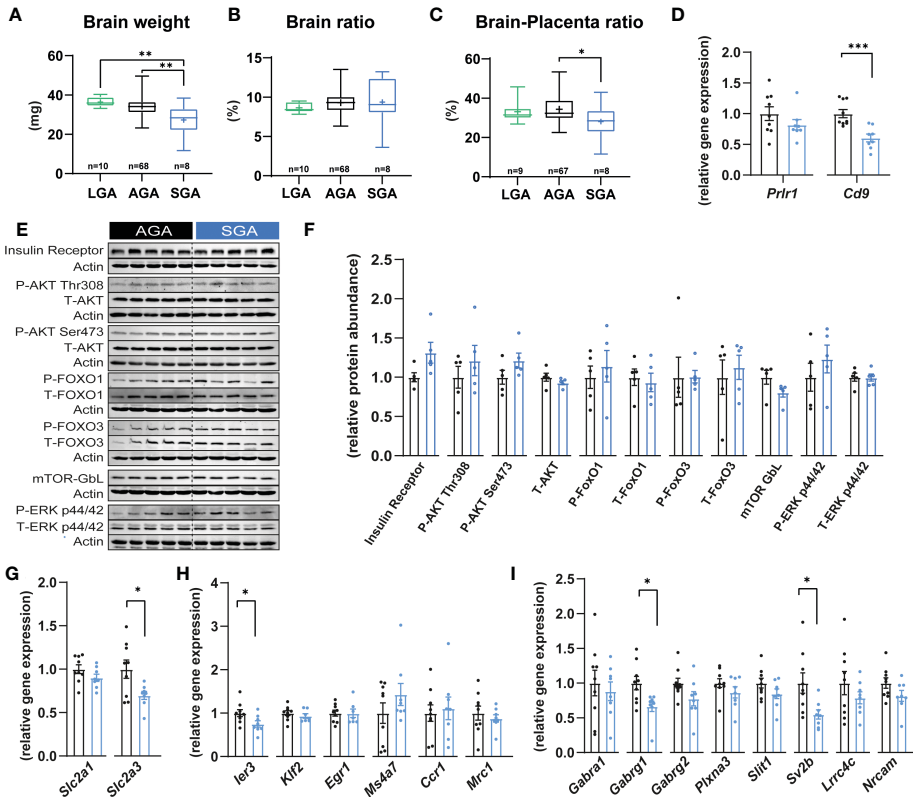

FIGURE 3
Small for gestational age and its impact on fetal brain. **(A)** Brain weight. **(B)** Brain weight expressed as a ratio of the brain weight divided by fetal weight. **(C)** Brain weight expressed as a ratio of brain weight divided by placental weight. **(D)** The expression of key prolactin and pregnancy-specific glycoprotein receptors in fetal brain (n=8-9 samples per group). **(E, F)** Immunoblots and protein abundance of key growth and metabolic signalling proteins in fetal brain (n=5 samples per group). **(G–I)** Expression levels of glucose transporters, microglia and axon developmental genes (n=8-9 samples per group). Data are from 90 fetuses in total from 11 litters (excluding outliers). Data are shown as individual values and bars represent mean ± SEM. Statistical analysis performed by one-way ANOVA followed by Bonferroni *post hoc* test (variables of three groups), paired or unpaired t-test (Student t-test and Mann-Whitney test, respectively). ROUT test was conducted to detect and eliminate outliers. *P<0.05, **P<0.01, ***P<0.001. AGA, (appropriate for gestational age); SGA, (small for gestational age); P, (phosphorylated protein levels); T, (total protein levels).

compared to those of 9 to 11 fetuses (Supplementary Figure 1C–F). Analysis of LGA and SGA fetuses by considering the litter size as a potential effect (comparison of LGA-SGA fetuses in litters of 6-8 fetuses versus LGA-SGA fetuses in litters of 9-11 fetuses) showed that LGA fetuses in litters of 6-8 fetuses had reduced brain ratio and brain-placental ratio compared to those LGA in larger litters (Supplementary Table 3). These effects were not found in the SGA fetuses (Supplementary Table 3).

To further investigate the relationship between placental function and fetal brain we quantified the mRNA levels of receptors that mediate the action of prolactins and PSGs, *Prlr1* and *Cd9*, respectively in SGA compared to AGA fetuses (54, 55). We found that the expression of *Cd9* was 39.5% lower in the brain of SGA versus AGA fetuses (Figure 3D). However, the mRNA expression levels of *Prlr1* in the brain were similar between SGA and AGA fetuses. To understand the potential molecular changes underlying brain weight differences in SGA versus AGA fetuses, we quantified the levels of key cellular signalling pathways involved in growth and metabolism and found that these remained similar in the brain of SGA and AGA fetuses (e.g. insulin receptor β, AKT, FOXO1-3, mTOR- GβL and ERK; Figures 3E, F). As the fetal brain is highly dependent on glucose metabolism for growth, we quantified the expression of genes encoding the main glucose transporters in the brain of SGA and AGA fetuses (56). Glucose transporter number 1 (*Slc2a1*) did not change between SGA and AGA fetuses; however, the mRNA levels *Slc2a3* were significantly reduced by 30% in SGA compared to AGA fetuses (Figure 3G).

We then explored whether our model of SGA and placental malfunction may be linked to changes in the differentiation and maturation of microglia. We analysed mRNA levels of 6 microglia markers (*Ier3*, *Klf2*, *Egr1*, *Ms4a7*, *Ccr1* and *Mrc1*) (57) and found that 5 out of 6 were stably expressed in the brain of SGA compared to AGA fetuses (Figure 3H). Interestingly, the expression of *Ier3* was significantly reduced by 25% in SGA compared to AGA fetuses. We then quantified the expression of 8 genes involved in axonogenesis (58) (*Plxna3*, *Slit1*, *Gabra1*, *Sv2b*, *Gabrg1*, *Gabrg2*, *Lrrc4c* and *Nrcam*). We found that *Sv2b* and *Gabrg1* were reduced by 45% and 12% in SGA compared to the AGA group, respectively (Figure 3I). The other axonogenesis genes analysed did not differ between SGA and AGA.

4 Discussion

In this study we have shown that the defects in placental endocrine function are associated with reduced fetal growth and brain development in a mouse model of SGA. In particular, we found that the placental capacity to respond metabolically (FOXO1) and produce sex steroids (*Cyp17a1*), prolactins (*Prl3a1*) and PSGs (*Psg21*) were compromised in fetuses classified as SGA. Moreover, SGA fetuses have a proportional reduction in brain weight, low expression of glucose transporter 3 (*Slc2a3*), axogenesis (*Sv2b* and *Gabrg1*) and microglia (*Ier3*) genes along with reduced expression of the PSG receptor (*Cd9*) in the fetal brain of SGA fetuses compared to AGA. Finally, we found a significant link between the placenta and fetal brain development, as shown by the reduced brain-placental weight ratio in SGA fetuses. Together, our data reveal that placental hormones could be additional regulators of fetal growth with implications for fetal brain development. Furthermore, miscommunication of placental hormones with the developing fetal brain may have immediate and long-lasting implications for neurocognitive outcomes.

In the mouse, we have recently shown that the placental secretome comprises more than 1,000 proteins, including prolactins (17). In rodents, the prolactin family consists of 23 closely related genes and their expression varies spatially and temporally in the placenta (59). Although the role of prolactins in modulating maternal physiology is well-defined, for instance in metabolic adaptations during pregnancy (15, 60, 61) and in promoting maternal nurturing behaviour and lactation (62), their role in embryo and fetal development is not fully understood. In humans, prolactin can be found in the fetal circulation from mid-gestation and its receptors are expressed in fetal tissues by 7.5 weeks of gestation (63). In mice, prolactin receptors are widely expressed by the fetus, including its brain, with *Prlr* mRNA levels detectable as early as on GD8 (64). Prior work has speculated that prolactin in the fetus may be important for the growth of the adrenal cortex, production of pulmonary surfactant and in the control of the immune system (65, 66). However, further research is required to understand how the prolactin hormone family drives fetal growth, especially the growth and development of the fetal brain, given that placental production of PRL was decreased in our SGA mouse fetuses. Consistent with our findings, in humans, there is a down-regulation of the growth hormone/chorionic somatomammotropin (*hGH/CSH*) cluster in the SGA placenta (67). Moreover, other work has shown that mothers carrying SGA male fetuses display lower concentrations of prolactin in their circulation when compared to women carrying AGA male fetuses (68).

In our mouse model, the expression of *Cyp17a1* was lower in the SGA *versus* AGA fetuses. Our findings are consistent with studies in humans that show polymorphisms in *Cyp17a1* can influence the risk of SGA (69). In humans, the expression of *Cyp17a1* is mainly localized in the syncytiotrophoblast, acting as a source of estrogen during pregnancy (70). In mice, placental *Cyp17a1* expression in the placenta is modulated by *Igf2* (48); however in the present study, *Igf2* and other imprinted genes that regulate placental endocrine capacity were not differentially expressed between SGA and AGA fetuses. Other work has indicated that sex steroids interact with FOXO1 signalling (71). In addition, FOXO1 is important for embryonic development and placental differentiation (72). Thus, understanding the link between reduced FOXO1 abundance and decreased placental endocrine capacity in our SGA mouse model would be valuable.

The expression of PSGs was also altered in our mouse model of SGA, with lower *Psg21* mRNA levels in the placental Jz and this was couple to decreased fetal brain expression of the PSG receptor, *Cd9*. In rodents, PSGs are synthesised by spongiotrophoblasts and giant cells, while in humans, PSGs are produced by the syncytiotrophoblast (73–75). PSGs belong to the carcinoembryonic antigen family (76) and although the functions of PSGs are not fully elucidated, they are proposed to modulate immune cells (77, 78) and angiogenesis (79). Moreover, low levels of PSGs are associated with the development of pregnancy complications, including early pregnancy loss, placental insufficiency, fetal growth restriction and fetal hypoxia (80–84). Reinforced by our findings that placental *Prl3a1* and *Psg21* expression are positively correlated with fetal weight, future work

should evaluate circulating levels of these hormones in fetal plasma. The placental transport labyrinth region in the mouse expresses receptors for such hormones (49, 85). Thus, future studies should evaluate whether the relationship between placental hormone production and fetal growth may also be mediated by potential local changes in the formation and function of the placental labyrinth as a result of altered paracrine signalling.

An additional objective of our work was to characterize the potential changes occurring in the fetal brain in our mouse SGA model. We did see a symmetrical reduction in brain weight and decreased mRNA levels of the glucose transporter 3 (*Slc2a3*) and impaired axogenesis markers (as inferred by the low expression levels of *Sv2b* and *Gabrg1*) in SGA fetuses. Of note, we did not find any changes in PI3K-AKT-mTOR signalling, which is critical for biological processes like nutrient uptake, cell growth and migration (86), in the brain of SGA compared to AGA fetuses. Changes in the PI3K-AKT-mTOR signalling cascade have been linked to microcephaly (87), due to reductions in neuronal cell division (88). We also did not identify any changes in FOXO1-3 and ERK signalling, and microglia markers (57) were similarly expressed between SGA and AGA fetuses, aside from the reduced mRNA levels of *Ier3*. Microglia are important specialised cell types in the nervous system that influence brain development, including neuronal proliferation or synaptic remodelling (89). SGA is a condition that can be linked to inflammation, as SGA newborns can exhibit elevated interleukin (IL) IL-1β, IL-8 and tumor necrosis factor in their circulation during the first postnatal weeks (90). The gene *Ier3* responds to changes in toll-like receptors (TLR3) (91) and its expression can be induced by different stimuli, including cytokines, infections and growth factors (92). In mice, low *Ier3* levels results in abnormal immune regulation and inflammation (92). Therefore, future work should explore the contribution of differences in *Ier3* in SGA fetus to the immune status, function and structural development of the developing brain. Regarding the changes in axogenesis markers, in the current study we employed whole brain lysates for our analyses and therefore cannot exclude the possibility of molecular changes in specific regions of the brain in the context of SGA. Nevertheless, in our SGA model, the expression of *Sv2b*, which encodes a synaptic vesicle protein and *Gabrg1*, which functions as a γ-Amino-butyric acid (GABA) receptor was reduced. Prior work has found that *Sv2b* is regulated by the maternal gut microbiota during mouse fetal brain development (58). This gene is broadly expressed in the central nervous system, with especially high expression in glutamatergic neurons (93, 94). Other investigations in mice, have also shown that intrauterine growth restriction is related to postnatal memory deficits that may be linked to abnormal proportions of maturing glutamatergic neurons at birth (95). Hence, study of the postnatal neurocognitive outcomes in our mouse model of SGA is warranted. In our study, the reduction in *Sv2b* and *Gabrg1* was in line with decreased expression of *Cd9* by the fetal brain in SGA fetuses, which may suggest a role for placental PSGs in shaping fetal axogenesis. Indeed, previous work in mice and humans has found CD9 is present in different cell types including astrocytes, sympathetic neurons and Schwann cells (96). Given that placental PSG production was also decreased in our SGA model, a greater understanding of how the placenta may influence fetal brain development through PSG signalling should be explored in future work.

A major limitation of the current study was that our study was not sufficiently powered to evaluate the influence of fetal sex on placental endocrine capacity and fetal brain development. This is important as previous work has demonstrated that fetal sex is an important determinant of placental endocrine capacity (48, 49). Furthermore, recent work in mice has revealed that placental steroid metabolism and metabolic signalling capacity differ depending on the size and the sex of the fetus (40). Moreover, the potential contribution of the labyrinth zone in the placental endocrine support of fetal development was not explored. This would be important in future work, as previous work has shown that the rodent labyrinth zone can assist in the production of steroids by the placenta (97). Moreover, steroids regulate glucose uptake through changes in *Slc2a1* and *Slc2a3* (98) and in our model we found reduced levels of *Slc2a3* in the SGA fetal brain. Moreover, the mouse is a litter-bearing specie and despite that our analysis of fetal intrauterine position and litter size composition show that there are no significant differences in fetal-placental weights (including fetal brain weight), we observed that placental efficiency is affected by both, the intrauterine fetal position and the number of fetuses per litter. Previous work performed in rats have identified greater blood flow at the cervical and ovarian ends compared to middle of the uterus, which may partly explain our findings (99). Work in multiple species has also found that placental efficiency is greater in monotocous species with multiple gestations and in polytocous species with larger litters, which is not surprising given the higher fetal demand on maternal resources (100, 101). Therefore, the analysis in combination of both, endocrine and vascular/transport function of the placenta, will provide a more accurate and detailed angle to understand the causes and short/long-term consequences of SGA.

In the current study, we intentionally studied unmanipulated, wildtype litters using fetal growth curves and percentile cut-offs to preserve maternal health and precisely study the role of placental endocrine interactions with the fetus in a controlled intrauterine environment. However, in doing so, we likely limited our ability to detect important pathways and proteins involved in placental endocrine function. Nonetheless, our work provides new evidence about the importance of placental hormones in the regulation of fetal growth and development. Moreover, the low levels of *Cd9* in the fetal brain suggest a direct link to changes in placental PSG production and sensitivity in the context of SGA. Further work is required to ascertain the specific contribution of the dysregulated placental hormones found in our SGA model (*Cyp17a1*, *Prl3a1*, *Prl3b1* and *Psg21*) and brain development. Moreover, animal studies deploying *in vivo* approaches to specifically delete genes in mouse placenta endocrine cells would be highly useful for decoding the communication between placental hormones and fetal organogenesis (48, 49, 102). This work could be complemented by experiments using fetal brain explants or cerebral organoids that are cultured with different combinations of placental hormones. To sum up, our results identify placental hormones as key regulators of fetal growth and may have relevance for understanding the aetiology of fetal growth disorders, and the mechanistic basis of associations between poor fetal growth and the subsequent increased risk of poor neurocognitive outcomes.

Data availability statement

The data presented in the study are deposited in the Gene Expression Omnibus repository, accession number GSE224682.

Ethics statement

The animal study was reviewed and approved by the United Kingdom Home Office under the Animals (Scientific Procedures) Act 1986 and underwent review by the University of Cambridge AnimalWelfare and Ethical Review Body.

Author contributions

JL-T performed research. JL-T and AS-P wrote the paper and designed the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1116770/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Brain phenotype based on the intrauterine position and litter size. Data are from 90 fetuses in total from 11 litters (excluding outliers). Data are shown as box plots and whiskers. The rectangle shows the distribution, the line the median, the whiskers the maximum and minimum and the "+" is the mean of the group. Statistical analysis performed by one-way ANOVA followed by Bonferroni *post hoc* test (variables of three groups), paired or unpaired t-test (Student t-test and Mann-Whitney test, respectively). *P<0.05.

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Expression of NFIL3 and CEBPA regulated by IFNT induced-PGE2 in bovine endometrial stromal cells during the pre-implantation period

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Prostaglandin E2 (PGE2) is considered as a luteoprotective factor, influencing the corpus luteum during the early pregnant period in the bovine species. Cyclic AMP (cAMP) is activated in response to PGE2 and plays a role in many physiological processes. The maternal recognition signal, interferon τ (IFNT), induces PGE2 secretion from the endometrial epithelial cells, the function of which in stroma cells has not been completely understood. In this study, PGE2 was found to activate cAMP in the bovine endometrial stromal cells (STRs). STRs were then treated with forskolin to activate the cAMP signaling, from which RNA extracted was subjected to global expression analysis. Transcripts related to transcription regulatory region nucleic acid binding of molecular function, nucleus of cellular component, and mitotic spindle organization of biological processes were up-regulated in cAMP-activated bovine STRs. An increase in the transcription factors, *NFIL3, CEBPA*, and *HIF1A via* the cAMP/PKA/CREB signaling pathway in the bovine STRs was also found by qPCR. Knockdown of *NFIL3*, *CEBPA*, or *HIF1A* blocked forskolin-induced *PTGS1/2* and *IGFBP1/3* expression. Moreover, NFIL3 and CEBPA were localized in endometrial stroma on pregnant day 17 (day 0 = estrous cycle), but not on cyclic day 17. These observations indicated that uterine PGE2 induced by conceptus IFNT is involved in the early pregnancy-related gene expression in endometrial stromal cells, which could facilitate pregnancy establishment in the bovine.

KEYWORDS

bovine, endometrium, stomal cell, prostaglandin E2, cyclic AMP

Introduction

Prostaglandins (PGs) are essential for reproductive processes such as ovulation, fertilization, and embryo implantation in autocrine, paracrine, or occasionally endocrine fashion (1, 2). The corpus luteum (CL) is formed after ovulation and secretes progesterone to maintain pregnancy. The luteolytic function of PGF2α, produced by the endometrium, has been verified in humans and other mammals, whereas endometrial PGE2 has a luteoprotective effect during early pregnancy. Luteoprotective factor PGE2 is produced by bovine endometrial epithelial and stromal cells (EECs, STRs) (3, 4). PGE2 directly acts on luteal cells through the luteinizing hormone receptor, which induces progesterone synthesis. Bovine embryonic interferon τ (IFNT; a pregnancy recognition cytokine) starts to increase on day 7 of pregnancy (day 0 = day of estrus) and peaks on days 19-20, just after the initiation of conceptus attachment to the uterine epithelium, followed by a rapid IFNT decrease (5–8). IFNT is secreted by mononuclear trophoblast cells and acts as the maternal recognition signal in the ruminants (9, 10). IFNT downregulates the expression of endometrial oxytocin receptors and then maintains the corpus luteum function *via* inhibition of the luteolytic pulse of endometrial PGF2α (11–15). Therefore, the increase in the ratio of PGE2/PGF2α synthesis in the endometrium induced by IFNT is essential for maintaining the CL function during the early stage of pregnancy in ruminants.

PGE2 binds to the type E prostanoid (EP) receptors containing four subtypes of G-protein-coupled receptors EP1, EP2, EP3, and EP4. PGE2 plays distinct functions in various physiological processes caused by the differences in cellular distribution and downstream signaling pathways of each EP receptor. The highly expressed EP1 in the uterus promotes cell proliferation by activating PLC/PKC signaling, and its expression decreases sharply in the luteal phase (16). EP1 can work with EP3 inhibitory G protein to inhibit the cAMP pathway (16). Although *EP1* or *EP3*-deficient mice do not show abnormal pregnancy, *EP2*-null mice exhibit anovulation and embryo implantation dysfunction (17). Activation of EP2 and EP4 stimulates the cAMP/protein kinase A (PKA) signaling pathway, cAMP-response element binding protein (CREB), and MAPK signaling, ERK1/2 extracellular signal-regulated kinase-1/2 (ERK1/2). Several studies have also shown that EP2 and EP4 can enhance PI3K/Akt signaling (18). PGE2 stimulates the synthesis and secretion of PGE2 to form a positive feedback loop in endometrium and increases VEGFA to induce endometrial angiogenesis (19). Furthermore, PGE2 induces the synthesis and secretion of estrogen, catenin, and intercellular adhesion factors in porcine conceptuses (20).

During the peri-implantation period in ruminants, the trophoblast, CL, and the endometrium synthesize and secrete PGE2 (15, 21, 22). Intrauterine injection of PG synthase (PTGS) inhibitors during early pregnancy could suppress the conceptus elongation in the ovine and reduce the pregnancy rate in the bovine (3). PGs derived from the uterus, rather than the embryo, play roles in embryo development and implantation (23). IFNT-induced PGE2 prevents luteolysis (15), and the PGE2/EP2 signaling pathway is essential for endometrial maturation for receptivity to the embryo (24). The transcripts and protein levels of EP2 are found in the endometrium during the estrous cycle and increase in the bovine endometrial stroma on day 18 of pregnancy (25). IFNT is observed to up-regulate PGE2 in cultured endometrial epithelial cells and stromal cells (26–30). Moreover, the signaling pathway downstream of EP2 in bovine endometrial stromal cells at the peri-implantation stage is not fully understood. However, the physiological significance of the high expression of EP2 in bovine endometrial stromal cells at the peri-implantation stage is not fully understood. We, therefore, hypothesized that IFNT-induced PGE2 in bovine endometrial stromal cells (STRs) may affect the receptivity of conceptus *via* EP2/cAMP signaling at the pre-implantation stage. To investigate the physiological role of PGE2/cAMP in STRs, we extracted RNA from STRs, which had been treated with an adenylate cyclase activator, were subjected to RNA-sequence analysis.

Materials and methods

Cell preparation and culture conditions

Isolation and culture of endometrial cells were carried out as previously described (31–35). In brief, the uteri of healthy Holstein cows were obtained from a local abattoir in accordance with protocols approved by the local Institutional Animal Care, Use and Ethics Committee at Okayama University, Okayama, Japan. Uteri of the early luteal phase (days 2-5) were excised and immediately transported to the laboratory. The uterine lumen was trypsinized (0.3% w/v), from which endometrial epithelial cells (EECs) were isolated. After collection of the epithelial cells, the uterine lumen was washed and cut transversely. Intercaruncular endometrial strips were dissected from the myometrial layer with a scalpel and washed once. The endometrial strips were minced into small pieces and then digested with 0.05% collagenase I. After stirring for 60 min, endometrial stromal cells (STRs) were dissociated, filtered, and washed. Endometrial cells were then cultured on collagen type IA-coated plates in Dulbecco modified Eagle medium/F12 (DMEM/F12) (1:1) medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% (v/v) newborn calf serum (Thermo Fisher Scientific), 2 mM glutamine (Thermo Fisher Scientific), and antibiotic/antimycotic solution (Thermo Fisher Scientific) at 37°C under 5% CO_2 in humidified air. EECs or STRs placed onto collagen type IA-coated 12-well plate were further incubated with or without IFNT (1 μg/ml), FSK (50 μM), PGE2 (30 μM), EPAC-selective agonist (8-[4-chlorophenyltio]-2'-O-methyl cAMP; 500 μM), or PKA-selective agonist (N^6-phenyl-cAMP; 500 μM) for 48 h.

RNA extraction and quantitative RT-PCR

Using the ISOGEN reagent (Nippon Gene, Tokyo, Japan), total RNAs were extracted from cultured EECs or STRs according to the manufacturer's protocols. The isolated RNA was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan), which was then subjected to qPCR amplification

using PowerUP SYBR Green Master Mix (Thermo Fisher Scientific). All primers are listed in Table 1. The qPCR amplification was carried out on an Applied Biosystems STEP One Plus real-time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification efficiencies of each target genes and the reference genes, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and actin beta (*ACTB*) were examined through their calibration curves and found to be comparable. Average threshold (Ct) values for each target were determined by Sequence Detection System software v2.3 (Applied Biosystems) (36).

PGE2 ELISA

Endometrial cells were treated with recombinant IFNT (1 μg/ml) for 24 h. The culture medium was centrifuged at 10,000 g for 10 min at 4°C, and the concentration of PGE2 in the supernatant was determined using a sandwich ELISA Kit (Prostaglandin E2 ELISA Kit, abcam, Tokyo, Japan) according to the manufacturer's instructions (37).

Cyclic AMP assay

Total cAMP levels in endometrial cells, treated with IFNT, forskolin or PGE2 for 48 h, were determined using a competitive EIA kit (Cyclic AMP EIA kit, Cayman Chemical Company) according to the manufacturer's recommendations. Briefly, cells were lysed for 10 min in 80 μl of 0.1 M HCl and were centrifuged at 1,000 x g at 4°C. The supernatant was used for the cAMP measurement (38).

TABLE 1 Primers for real-time qPCR analyses.

Name (Accession No.)	Sequence (5′—3′)	Product length (bp)
GAPDH NM_001034034	GCATCCCTGAGACAAGATGGTG	113
	CATTGATGGCAACGATGTCCAC	
PTGS1 NM_001105323.1	ATGAGCCGGCAGGGTATCT	115
	AGTAACAGCAGGGGTTCACTG	
PTGS2 NM_174445.2	CTCAGCGGTGCAGCAAATC	104
	CCTGTTCGGGTACAGTCACA	
IGFBP1 NM_174554.3	TGCTGGACAGATTAGCCAGG	112
	GACGTCTCACACTGTTTGCTG	
IGFBP3	GCTACAAGCGTTGTTGGACG	107
NM_174556.1	CCGACTCACTGCCATTTCCT	
HIF1A	TGCTCATCAGTTGCCACTCC	117
NM_174339.3	TCCAAATCACCAGCATCCAGA	
CEBPA	TCGACATCAGCGCCTACATC	134
NM_176784.2	GTAGTCAAAGTCGTTGCCGC	
NFIL3	GAGCGCCTTTGTGGATGAGC	134
NM_001075240.1	CAGGGCCCTCCTGTGAATGT	
VEGFA	CAAACCTCACCAAAGCCAGC	107
NM_174216.2	GCCCACAGGGATTTTCTTGC	

RNA sequencing and their gene ontology and pathway analyses

Total RNA for RNA-seq analysis was extracted from cultured EECs or STRs using Isogen (Nippon gene) according to the manufacturer's instructions. High-throughput sequencing libraries were prepared using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions, and the analysis was performed by Macrogen Japan (Kyoto, Japan). Primary sequencing data were deposited to the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (https://www.ddbj.nig.ac.jp/dra/index-e.html) (accession numbers DRR413427 to DRR413432). Data analysis was performed as described previously (39). Briefly, trimmed sequences were analyzed on the basis of the TopHat/Cufflinks pipeline based on the bovine genome (bosTau8) and reference annotations obtained from UCSC genome browser (https://genome.ucsc.edu). Differential and significant gene expression analysis was performed with the use of fragments per kilo-base of gene locus summarized mRNA per million reads (FPKM). Genes were selected with the criteria of an absolute expression level >1 FPKM. The gene ontology (GO) and enriched signaling pathway analyses were performed with the Enrichr tool (http://amp.pharm.mssm.edu/Enrichr/).

Western blot analysis

Cell lysates from the STR cultures were separated through SDS-PAGE and were then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After blocking with Block Ace reagent (DS Pharma Biomedical, Osaka, Japan), membranes were incubated with anti-CREB (1:2000, ab32515, abcam), phosphorylated (p-) CREB (1:2000, ab32096, abcam), p-ERK1/2 (1:2000, #4370, Cell signaling technology, Tokyo, Japan), p-p38MAPK (1:2000, #4511, Cell signaling technology), p-JNK (1:2000, #9251, Cell signaling technology), or ACTB (1:5000, ab1801, abcam) antibody. Immunoreactive bands were detected using enhanced chemiluminescence (EMD Millipore, Temecula, CA, USA) after incubation with horseradish peroxidase labeled anti-mouse, rabbit, or goat IgG (1:5000, Vector Laboratories, Burlingame, CA, USA). Signals were detected using C-DiGit Blot Scanner (LI-COR) and then band density was assessed with Image Studio DiGit software (version 5.2) (36).

Transfection of small interfering RNA

STR cells were transfected with either a non-targeting control, or with nuclear factor interleukin 3 (*NFIL3*) (Sigma-Aldrich),

CCAAT-enhancer-binding proteins alpha (*CEBPA*) (Sigma-Aldrich), or hypoxia-inducible factor (*HIF1A*) siRNA (Sigma-Aldrich) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) (40).

Immunohistochemistry

All animal procedures including uterine collections were performed in accordance with the guidelines of the Committee for Experimental Animals at Zennoh Embryo Transfer (ET) Center (Hokkaido, Japan) and the approval was also obtained from the Ethics Committee of the University of Tokyo (IRB number 7A-6-605). Paraffin sections of endometrial tissues were immunostained using antibodies targeting NFIL3, CEBPA and HIF1A, according to a previously described protocol (41). Briefly, the paraffin sections were rehydrated, boiled for 20 min in 10 mM citrate buffer (pH 6.0), and then incubated with an antibody against NFIL3 (1:100, Sigma-Aldrich, Saint Louis, MO, USA), CEBPA (1:100, ab15047, abcam), or HIF1A (1:100, ab463, abcam) overnight at 4°C. Subsequently, the paraffin sections were incubated with goat anti-rabbit IgG biotin conjugate (1:800 dilution, B8809, Sigma-Aldrich). The immunoreactivity was visualized by means of avidin-peroxidase (Sigma-Aldrich) and AEC substrate kit (Invitrogen) according to the manufacturer's instructions.

Statistical analysis

All experimental data from qPCR analyses represent the results obtained from three or more independent experiments each with triplicate assays. Data were expressed as the mean ± SEM. Significance was assessed using the Dunnett's test. A P-value < 0.05 was considered statistically significant. In RNA-seq analysis, a false discovery rate-adjusted *P*-value (*q*-value) <0.05 was considered to represent statistical significance.

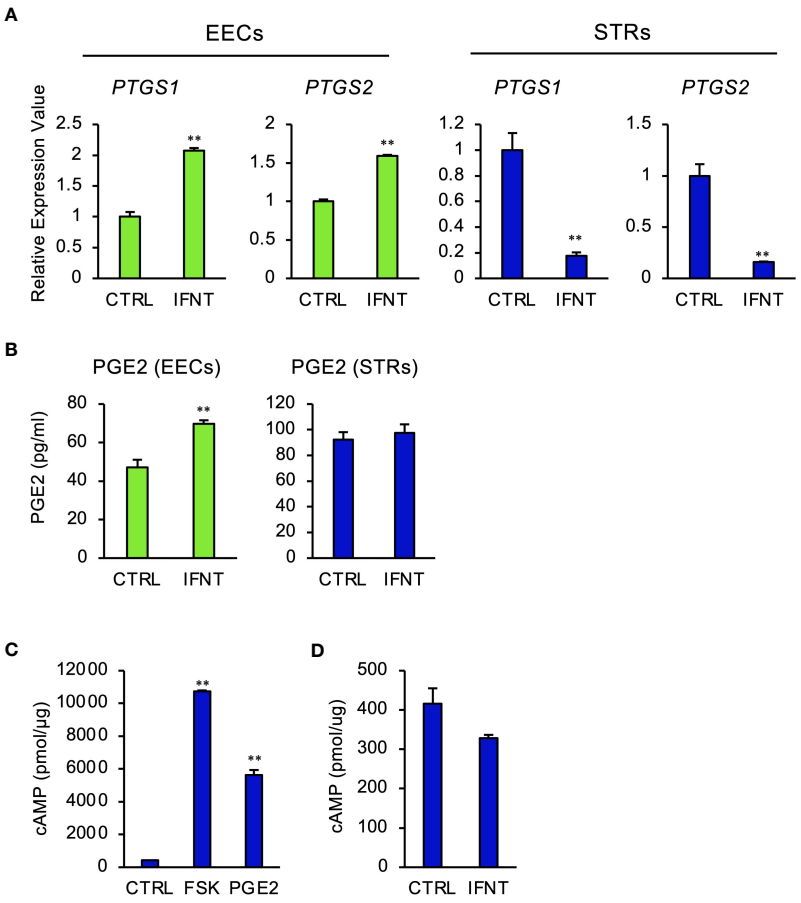

FIGURE 1
Regulation of PGE2 by IFNT in bovine endometrial cells. Bovine endometrial epithelial cells (EECs) or stromal cells (STRs) were treated with IFNT (1 μg/ml), FSK (50 μM), or PGE2 (30 μM) for 48 (h) **(A)** Transcript levels of prostaglandin synthase, PTGS1 and PTGS2, were measured by qPCR (n=3). *GAPDH* mRNA was used as the reference gene. Values represent the mean ±SEM of three independent experiments. ***P*<0.01. **(B)** PGE2 production in the culture media of the EECs or STRs was measured by the ELISA. Values represent the mean ±SEM of three independent experiments. ***P*<0.01. **(C, D)** The cAMP levels in the cell lysates were determined by EIA. The amount of cAMP was normalized to the amount of total cellular protein. The data from three independent experiments are presented. **p<0.01 vs. CTRL.

Results

2 IFNT induces PGEin bovine EECs

To investigate whether IFNT induced PGE2 in endometrial cells, EECs or STRs, were treated with IFNT. IFNT upregulated the expression of prostaglandin-endoperoxide synthase, PTGS1 and PTGS2 in EECs (Figure 1A), but those expressions were downregulated in STRs (Figure 1B). Secreted PGE2 in the culture media also increased when treated with IFNT in EECs, but not in STRs (Figure 1C). We next examined whether PGE2 stimulated STRs to generate intracellular cAMP. PGE2 increased intracellular cAMP level as well as FSK, an activator of adenylate cyclase (Figure 1D). However, IFNT did not stimulate the generation of cAMP in STRs (Figure 1E).

Cyclic AMP upregulates the expression of transcription factors and pregnancy-related genes in STRs

To explore the effect of PGE2 on gene expression through cAMP signaling pathway in STRs, we performed RNA-seq analysis. This identified 552 DEGs, of which 244 were downregulated and 308 were upregulated (Figure 2A). GO enrichment analyses of upregulated

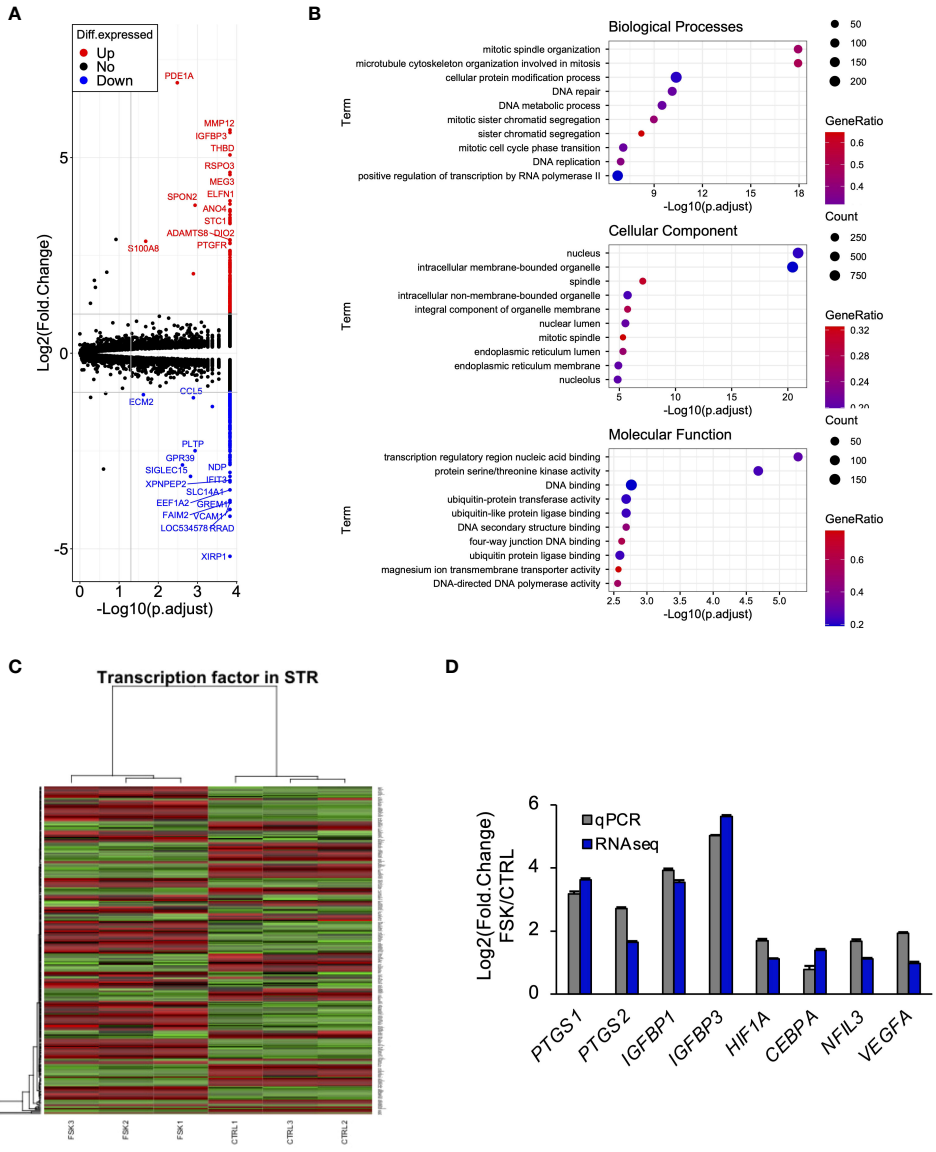

FIGURE 2
Cyclic AMP upregulates the expression of transcription factors and pregnancy-related genes in STRs. STRs were treated with FSK (50 μM) for 48 (h) Next, RNA was extracted and subjected to RNA sequencing (RNA-Seq; **(A–C)** or qPCR **(D)**. **(A)** Volcano plot showing expression of transcripts identified by RNA-seq. The transcripts highlighted in red or blue were differentially expressed by 2-fold ($P < 0.05$). **(B)** Differentially expressed genes were functionally classified using Gene Ontology analysis with the biological process, cellular component, or molecular function data sets. **(C)** Heat-map study of transcription factors in CTRL and FSK-treated STRs. Transcripts upregulated are shown in red, and those downregulated are shown in green. **(D)** Expression of *PTGS1/2*, *IGFBP1/3*, *NFIL3*, *CEBPA*, *HIF1A*, and *VEGF* genes in STRs was measured by qPCR (n=3). *GAPDH* mRNA was used as the reference gene. Values represent the mean ±SEM of three independent experiments.

genes identified those related to mitosis-, nucleus-, or transcriptional regulation-related terms (Figure 2B). GO analysis enabled us to suppose the involvement of transcription factors in STRs when stimulated with cAMP. The DEGs were further compared with the transcription factor database, from which a heat map was generated (Figure 2C). The results from the qPCR analysis also revealed an effect of an increase in cAMP on the expression of PGE2 synthase, pregnancy-related, and several transcription factors in STRs, which were similar to those found by RNA-seq analysis (Figure 2D).

PGE2 affects gene expression *via* cAMP/PKA/CREB signaling pathway in STRs

Intracellular cAMP activates two signaling pathways, PKA and exchange protein directly activated by cAMP (EPAC) pathways in STRs (42). To determine the effect of PGE2/cAMP on gene expression, STRs were treated with PKA- or EPAC-selective agonist. PKA-selective agonist upregulated the expression of *PTGS1/2*, IGF binding proteins (*IGFBP*) *1/3*, *NFIL3*, *CEBPA*, *HIF1A*, and vascular endothelial growth factor (*VEGF*) as well as FSK and PGE2 treatment, however, they were unaffected by the EPAC-selective agonist (Figure 3A). Further investigation of which intracellular signaling pathways were activated by PGE2/cAMP revealed that PGE2 increased phosphorylated CREB and decreased phosphorylated ERK1/2, but did not alter the activation of other p38MAPK and JNK in STRs (Figure 3B).

NFIL3, CEBPA, or HIF1A mediates cAMP signaling pathway in STRs

We next investigated whether NFIL3, CEBPA, or HIF1A significantly affected the expression of *PTGS1/2*, *IGFBP1/3*, and

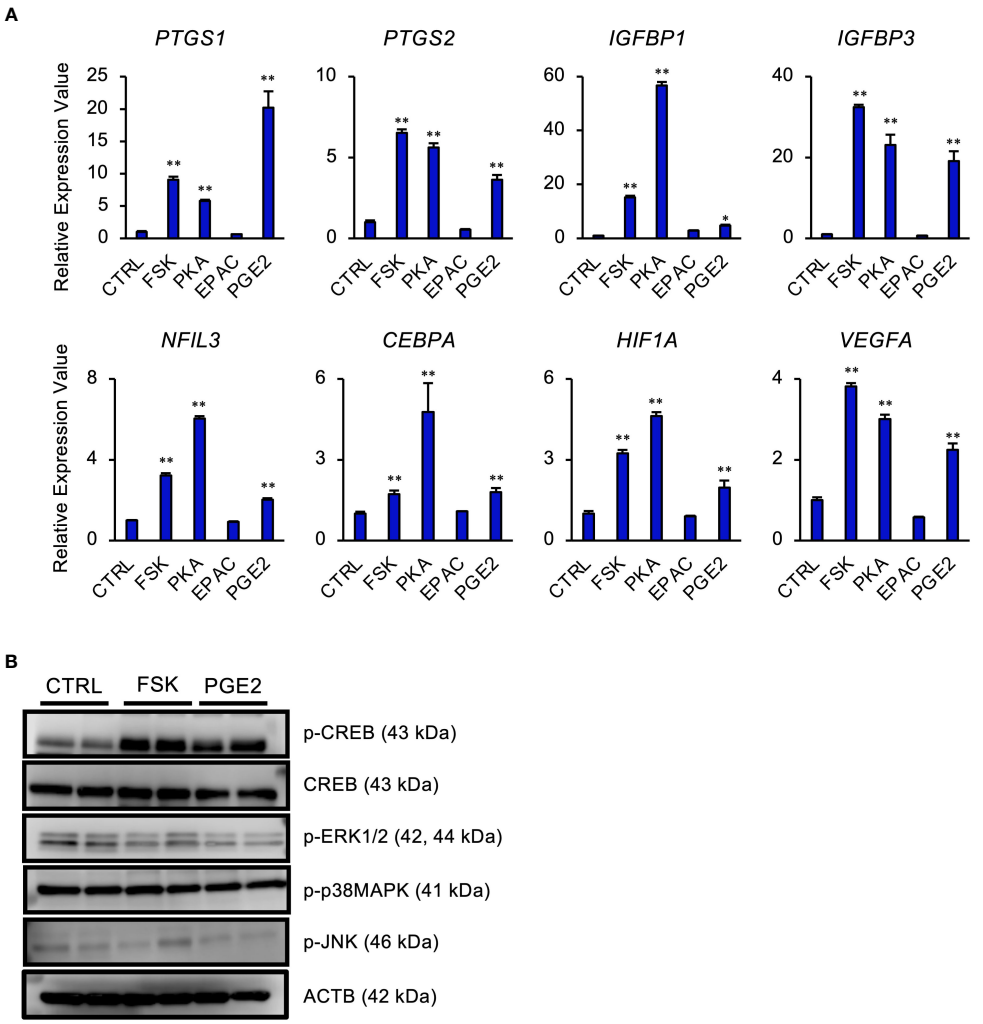

FIGURE 3
PGE2 affects gene expression *via* cAMP/PKA/CREB signaling pathway in STRs. STRs were treated with FSK (50 μM), PKA-selective agonist (500 μM), EPAC-selective agonist (500 μM), or PGE2 (30 μM) for 48 (h) **(A)** Expression of *PTGS1/2*, *IGFBP1/3*, *NFIL3*, *CEBPA*, *HIF1A*, and *VEGF* genes in STRs was measured by qPCR (n=3). *GAPDH* mRNA was used as the reference gene. Values represent the mean ±SEM of three independent experiments. **P*<0.05, ***P*<0.01 *vs*. CTRL. **(B)** Lysates prepared from STRs that had been treated with FSK (50 μM) or PGE2 (30 μM) for 24 h were subjected to immunoblotting to determine phosphorylated (p-) CREB, p-ERK1/2, p-p38MAPK, and p-JNK protein levels. ACTB served as a loading control.

VEGF in STRs (Figure 4). In the presence of FSK, *NFIL3* knockdown reduced the expression of *PTGS1/2* and *IGFBP1/3*. *CEBPA* knockdown also reduced the expression of *PTGS2* and *IGFBP3*. Furthermore, *HIF1A* knockdown reduced the expression of *VEGFA*, *PTGS2*, and *IGFBP1/3*.

NFIL3, CEBPA, and HIF1A are localized in endometrial stroma and epithelium during the pre-implantation period

Expression of NFIL3, CEBPA, and HIF1A in endometrial tissues on day 17, on which IFNT was maximally secreted from conceptuses, was examined by immunohistochemistry (Figures 5A–C). NFIL3, CEBPA, and HIF1A were expressed in stroma and luminal and glandular epithelium of pregnant animals, whereas NFIL3, CEBPA, and HIF1A were slightly expressed in only epithelium on cyclic day 17 in the absence of conceptus in the uterus.

Discussion

In this study, we demonstrated that PGE2 upregulated the pregnancy-associated transcription factors, NFIL3, CEBPA, and HIF1A, *via* the cAMP/PKA/CREB signaling pathway in bovine endometrial stroma (Figure 6). Upregulation of these transcription factors in stromal cells was further confirmed by the observations of their localization in the uterine tissue of day 17 pregnant cows as compared to those in day 17 cyclic cows. These results provided evidence that PGE2 regulated the expression of pregnancy-associated transcription factors in bovine endometrial stroma cells.

In the present study, IFNT increased the secretion of PGE2 and the expression of PGs synthase *PTGS1* and *PTGS2* (known as cyclooxygenase 1 and 2, respectively) in EECs, but not in STRs (Figure 1). It was found that the IFNT receptors, IFNAR1 and IFNAR2, shared by all of the type I IFNs, are expressed in the endometrial epithelium, and that these expressions are much higher than those in the stroma in ruminants. These results suggest that the epithelium may be more responsive to IFNT than the stroma (43–45). As PGE2 is generally known as an autocrine and paracrine factor (2) and has an ability to increase intercellular adhesion factors (20), these results indicated that PGE2 secretion from endometrial epithelial cells stimulated by IFNT might regulate the epithelial and stromal cells to facilitate conceptus adhesion to the endometrium at the early stage of pregnancy in the bovine.

The studies presented here also demonstrated that when the endometrial stroma cells were treated with PGE2, the production of cAMP increased over 10 times (Figure 1), which is consistent with the results of others (46), whereas cAMP level was not altered by the IFNT treatment. EP2 is one subtype of four PGE2 receptors and is the only one identified as critical for ovulation and embryo implantation in mice (47). The expression of EP2 in the bovine increases on day 18 of pregnancy primarily in the endometrial stroma, when the conceptus begins to attach to the uterus (10, 25). These results and ours suggest that the production of PGE2 from

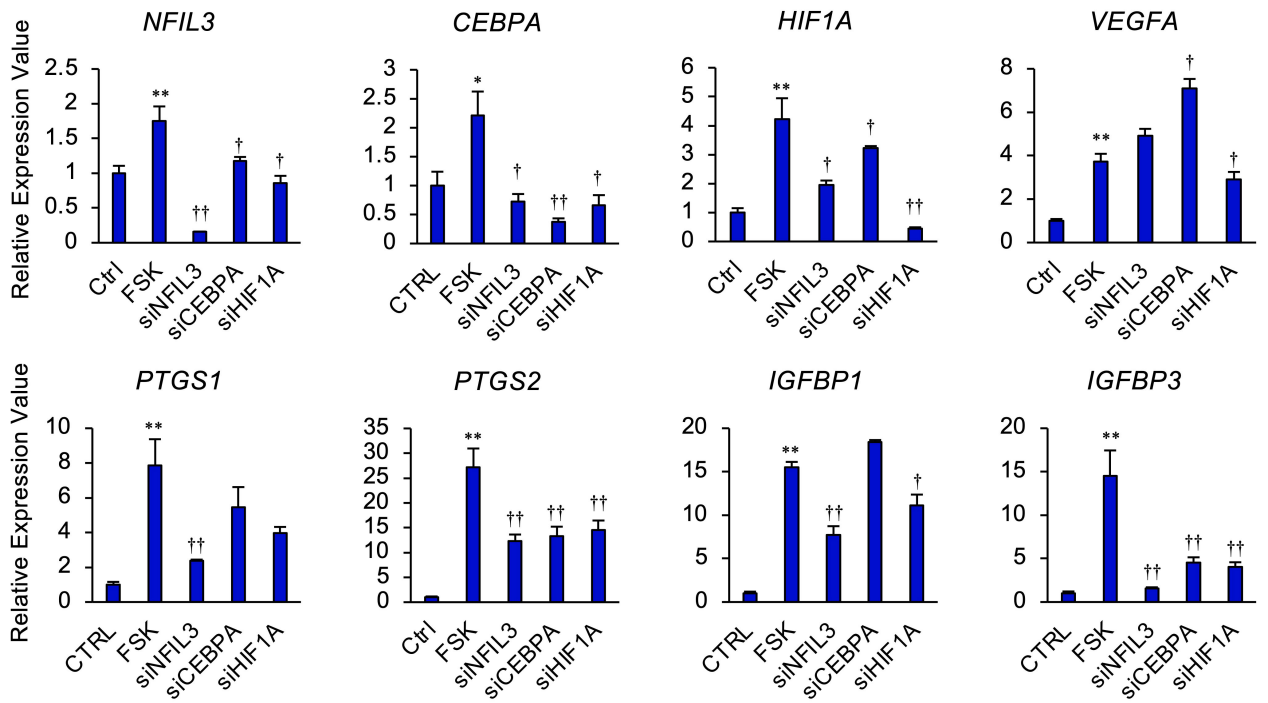

FIGURE 4
NFIL3, CEBPA, or HIF1A mediates cAMP signaling pathway in STRs. STRs transfected with *NFIL3*, *CEBPA*, or *HIF1A* siRNA were treated with FSK (50 μM) for 48 h. Expression of *PTGS1/2*, *IGFBP1/3*, *NFIL3*, *CEBPA*, *HIF1A*, and *VEGF* genes in STRs was measured by qPCR (n=3). *GAPDH* mRNA was used as the reference gene. Values represent the mean ±SEM of three independent experiments. **P*<0.05, ***P*<0.01 *vs.* CTRL, †*P*<0.05, ††*P*<0.01 *vs.* FSK.

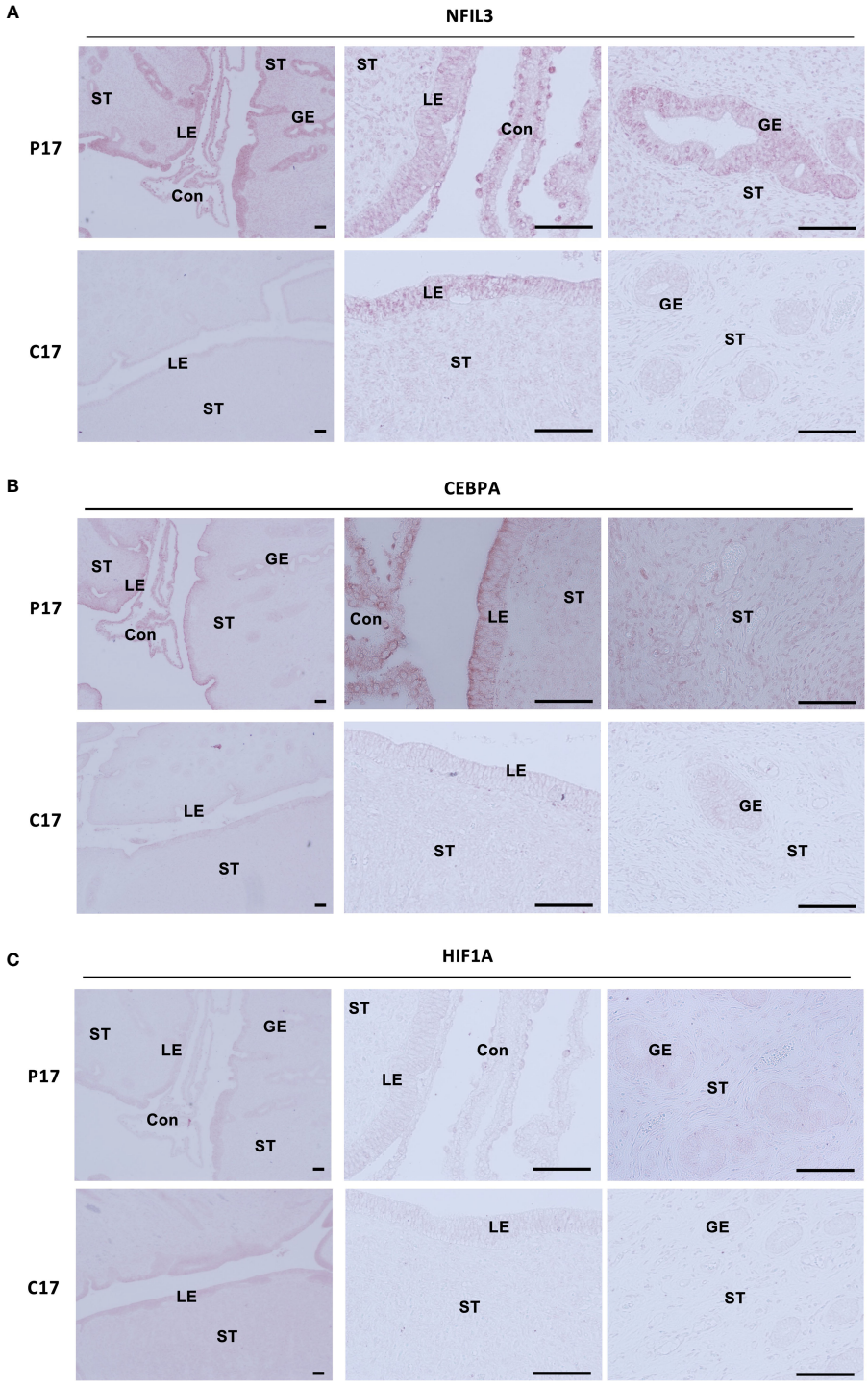

FIGURE 5
Localization of NFIL3, CEBPA, or HIF1A in the endometrial epithelium and stroma during pre-implantation periods. Sections of bovine endometrial tissues were immunostained for NFIL3 **(A)**, CEBPA **(B)**, or HIF1A **(C)**. P17, pregnant day 17; C17, cyclic day 17; ST, stroma; GE, glandular epithelium; LE, luminal epithelium; Con, conceptus. Scale bar = 100 μm.

endometrial epithelium might activate the cAMP signaling pathway through binding with the receptor EP2 in bovine stromal cells.

According to the functional GO analysis, the two most notable upregulation of biological processes in bovine endometrial stromal cells induced by cAMP were of the mitotic spindle organization and microtubule cytoskeleton organization involved in mitosis. Additionally, the nucleus and intracellular membrane-bounded organelle in the cellular component also increased significantly (Figure 2B). In ruminants, although decidualization in the endometrium does not occur, bovine endometrium is also remodeled in each estrous cycle, including cell proliferation in stroma cells (48). Our data indicated that the effect on the

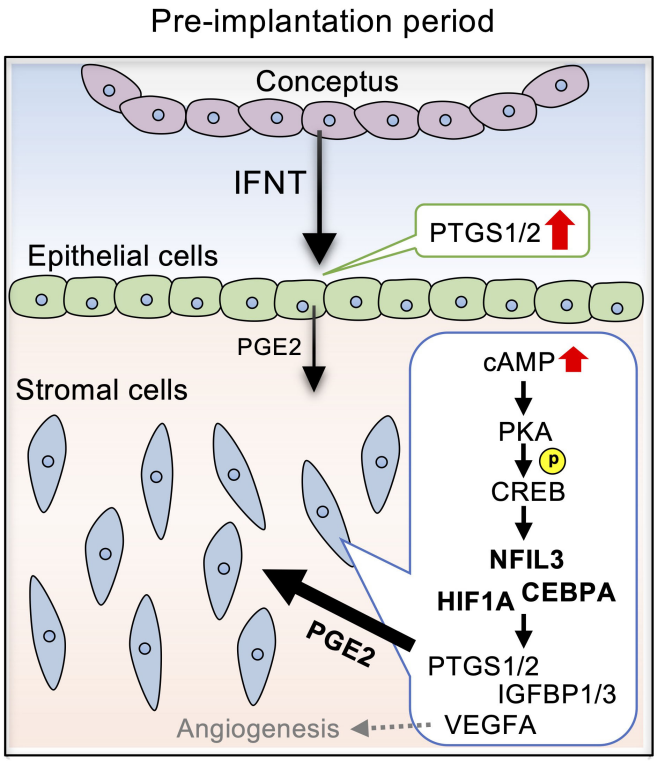

FIGURE 6
Diagram illustrating the effects of PGE2 on the expression of pregnancy-related factors *via* NFIL3, CEBPA, and HIF1A in bovine endometrial stromal cells. IFNT secreted from conceptuses acts on the endometrial epithelial cells, stimulating PGE2 production. PGE2 secreted from the epithelial cells activates cAMP signaling in the stromal cells, which is mediated by PKA/CREB. The cAMP signaling pathway upregulates the expression NFIL3, CEBPA, and HIF1A, which regulates PTGS1/2, IGFBP1/3, and VEGF.

proliferation within the endometrium may partly be regulated by PGE2/cAMP and steroid hormones. More importantly, the upregulated genes induced by cAMP were shown to be most associated with transcription regulatory region nucleic acid binding in molecular function. Previously, we found that an increase in intracellular cAMP is subsequently involved in the activation of PKA and EPAC in endometrial stromal cells (42). The cAMP has a different affinity for EPAC and PKA; furthermore, different signaling pathways conduct multiple beneficial and deleterious actions (49). In this study, the cAMP/PKA/CREB pathway played an important role in the regulation of endometrial stromal cell gene expression (Figure 3).

NFIL3, also known as adenovirus E4 promoter-binding protein 4 (E4BP4), is a ubiquitously expressed basic leucine zipper transcription factor and is reported as a master regulator of NK cell differentiation, ovulation, placentation, and embryonic development (50–52). Our histochemical results showed that NFIL3 was localized in the bovine trophoblast on day 17 (Figure 5A). It is consistent with the findings that *Nfil3* is expressed by early trophoblasts in mice and that deficiency of trophoblastic *Nfil3* causes abnormal implantation and placentation (52). The results presented in this study identified that endometrial NFIL3 was highly expressed in the endometrial epithelium, stroma, and glandular epithelium in day 17 pregnant cattle, but was not detectable in cyclic day 17 uterus except in the luminal epithelium (Figure 5). Moreover, the knockdown of *NFIL3* could significantly block FSK-induced *PTGS1/2* and *IGFBP1/3*. CEBPA exhibits a similar expression pattern as NFIL3 in this study. NFIL3 binds to CEBPs including CEBPA upon a C/EBP (CCAAT/Enhancer Binding Protein) DNA binding motif (53). CEBPA is an important transcription factor associated with hematopoietic differentiation (54) and upon interaction with CDK2 and CDK4, CEBPA inhibits these kinases and leads cultured cells to stop dividing or induce cell growth and proliferation (55, 56). CEBPA may play role in the regulation of *PTGS1/2* and *IGFBP1/3* in cooperation with NFIL3 in endometrial stromal cells. IGFBP1/3 are found as specifically expressed in the endometrium, and perform the functions of cell proliferation, migration, and attachment in the ruminants. IGFBP3 is expressed in the stroma of bovine endometria, and IGFBP1 functions as an adhesion molecule bridge of trophoblast and endometrium (57, 58). PTGS2 is induced by EP2 activation in bovine endometrial epithelium *via* PKA/ERK pathways (59). Thus, stromal NFIL3 and CEBPA might regulate the gene expression involved in conceptus elongation and the tight adhesion between conceptus and uterus at the early pregnancy stage in the bovine. Moreover, $Nfil3^{-/-}$ male$Nfil3^{-/-}$ female mating results in low fecundity, and $Nfil3^{-/-}$ females exhibit delayed uterine lumen closure and antimesometrial decidua differentiation in mice (52). Decidual NK cells have heterogeneity and are involved in neoangiogenesis

and immune modulation during the first trimester of pregnancy (60). Reduction in decidual NK cell numbers is detected in *Nfil3*$^{-/-}$ mouse. Considering that early pregnancy in the bovine is accompanied by a marked increase in the proportion of endometrial immune cells expressing markers for NK cells and cytotoxic T cells (61), stromal NFIL3 might play a role in maternal immune tolerance as well as endometrial cells proliferation. Interestingly, it is observed that the interface membrane separating maternal and fetal labyrinthine vessels is significantly thicker in *Nfil3*$^{-/-}$ mouse, potentially affecting the nutrient and waste exchanges in mice placentas (52). As a previous report has demonstrated, the inhibition of VEGF induces thickening of the endothelium with less fenestration in choriocapillaris (62). In the bovine endometrial stromal cells, suppression of NFIL3 could not block the induction of VEGFA stimulated by FSK. Not only NFIL3 but the other two transcription factors chosen in the present study also could not inhibit the increasing expression of stromal VEGFA stimulated by cAMP. In contrast to the haemochorial placenta of rodents, the synepitheliochorial placenta of bovine resembles a polarised uterine epithelial barrier facing the fetal chorionic (trophoblast) epithelium, including caruncular, the part fuses with embryo cotyledons to form a placenta during pregnancy, and intercaruncular. It is observed that significantly higher expression of VEGFA at the preimplantation stage in the caruncular endometrium area forms part of the embryo-maternal interface (63). Taken together, these results indicate NFIL3 might play a different role in bovine endometrium compared to that in mice, and the regulatory mechanism of VEGFA in the bovine stroma still needs to be elucidated.

In summary, we propose that PGE2 enhances NFIL3 and CEBPA expression in endometrial stromal cells *via* the cAMP/PKA/CREB pathway, which may facilitate conceptus development, cell proliferation, and immune tolerance, resulting in the establishment of pregnancy at the early stage in the bovine.

Data availability statement

The data presented in the study are deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive repository (https://www.ddbj.nig.ac.jp/dra/index-e.html), accession number DRR413427 to DRR413432.

Ethics statement

The animal study was reviewed and approved by Ethics Committee at Okayama University.

Author contributions

RB and KKu contributed to data acquisition. RB and KKu contributed to data analysis. YM, TS and HB prepared tissue sections. RB and KKi isolated primary endometrial stromal cells. RB, KKu and KI contributed to study conception, design, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1075030/full#supplementary-material

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The immune checkpoint molecule, VTCN1/B7-H4, guides differentiation and suppresses proinflammatory responses and MHC class I expression in an embryonic stem cell-derived model of human trophoblast

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The placenta acts as a protective barrier to pathogens and other harmful substances present in the maternal circulation throughout pregnancy. Disruption of placental development can lead to complications of pregnancy such as preeclampsia, intrauterine growth retardation and preterm birth. In previous work, we have shown that expression of the immune checkpoint regulator, B7-H4/VTCN1, is increased upon differentiation of human embryonic stem cells (hESC) to an *in vitro* model of primitive trophoblast (TB), that VTCN1/B7-H4 is expressed in first trimester but not term human placenta and that primitive trophoblast may be uniquely susceptible to certain pathogens. Here we report on the role of *VTCN1* in trophoblast lineage development and anti-viral responses and the effects of changes in these processes on major histocompatibility complex (MHC) class I expression and peripheral NK cell phenotypes.

KEYWORDS

embryonic stem cells, placental development, B7-H4/VTCN1, classical major histocompatibility complex class I molecules (MHC-I), natural killer cells, HLA-G, virus, anti-viral responses

Introduction

Study of the immunology of the human maternal-fetal interface has been hampered by several inherent characteristics of human placentation. First, the immunologic events at the maternal-fetal interface are dynamic, with features of pro-inflammatory responses at the initiation of pregnancy and near the time of delivery, and more pro-tolerogenic traits during much of the interval in-between (1). Immune characteristics also differ by location, as the placenta produces large amounts of immunomodulatory hormones and cytokines and presents high levels of allogeneic antigen load that decrease with distance from this source, i.e., antigenic load and immunomodulatory pregnancy hormone levels are generally lower in the maternal periphery than within the pregnant uterus (2–4). The human placenta is notably invasive, with fetal-derived, allogeneic placental trophoblast (TB) cells invading deeply into the maternal decidua and into the inner layers of the uterine myometrium. These invasive extravillous trophoblast cells (EVT) also remodel the decidual spiral arteries, where they are called endovascular trophoblast (endoTB) cells and are in direct contact with maternal peripheral blood, including its immune components. This level of invasion and subsequent interactions of 1) EVT with decidual immune cells, and 2) endoTB and the syncytiotrophoblast (STB) of the villous placenta with immune cells in the peripheral blood, is unusual among non-primate eutherian mammals, making small animal modeling of the human placenta problematic. Here, to address the immune events surrounding human implantation, we utilize two of a very limited number of *in vitro* models of preclinical human pregnancy: a human embryonic stem cell (hESC)-derived model of primitive TB (BAP cells) and, for validation, human blastocyst extended culture.

Two main models have been proposed to explain the immunology of human placentation (5–7). The first compared the implanting fetus to an allotransplant, since both represented an allogeneic mismatch between an immunologically competent host (mother/recipient) and foreign tissue (implanting fetus/transplanted organ) (7, 8). The second compared the implanting fetus to an invasive tumor (9), since both require robust cellular proliferation and "tumor" growth, rapid and extensive neovascularization, invasive capabilities, and local immunomodulation to avert rejection. One could even compare the long-term presence of fetal cells in the maternal periphery, a process called fetal-maternal microchimerism, to tumor metastasis. Although, for several reasons, we favor the tumor paradigm, neither fully reflects the nuanced immune changes that occur in the pregnant woman, particularly at the maternal-fetal interface. In short, the implanting fetus is neither an allogeneic transplant nor an invasive tumor, and its immunology is complex. For example, while a tumor can induce a local immunotolerant microenvironment, the immune cells at the site of the implanting fetus actively prepare the decidua for its implantation. Still, knowledge gained from transplant and cancer immunology can help to guide experimental direction and stimulate hypotheses during the study of pregnancy immunology. Accordingly, molecular regulators active in cancer and transplant immunology, may potentially play a role in immunomodulation at the maternal-fetal interface.

In RNA-seq analyses comparing BAP cells to term TB syncytialized in culture, we identified several genes that were differentially expressed at high levels in primitive TB (10) and verified that expression of the corresponding proteins was highest in early pregnancy but waned over gestation in primary human placental tissues (11). One of these genes was V-set domain-containing T cell activation inhibitor 1 (*VTCN1*), whose gene product is a cell surface-expressed protein belonging to the B7 costimulatory family, most often called B7-H4 in the immunology literature. *VTCN1* is typically expressed on the surface of antigen presenting cells, and although its precise receptor on the surface of T cells has not yet been determined, its role is likely as an immune checkpoint regulator. In most studies, the neo expression of *VTCN1*/B7-H4 on cancer cells is associated with cancer progression and poorer prognosis (12–16). Its presence in our model of peri-implantation human TB prompted us to hypothesize that it promotes maternal immune tolerance of trophoblast and/or trophoblast response to pathogens. Secondly, given the frequent comparisons of the implanting fetus to an invasive tumor (17, 18), we further hypothesized that *VTCN1*/B7-H4 could play a role in trophoblast invasion. Here, the BAP model of peri-implantation TB is used to test both hypotheses.

Materials and methods

Human ESC culture and differentiation

Human ESCs (H1, WA01) were cultured in Matrigel (BD Bioscience)-coated, six-well tissue culture plates (Thermo Scientific) under an atmosphere of 5% (vol/vol) CO2/air at 37°C in mTeSR1 medium (STEMCELL Technologies). The culture medium was changed daily and the cells were passaged every 5-6 days. The method for TB differentiation has been described previously (19). Briefly, on the day after passaging onto Matrigel-coated dishes at 1.2×10^4 cells/cm^2, the culture medium was changed to DMEM/F-12 medium (Thermo Scientific) with knock-out serum replacement (KOSR, Invitrogen) that had been conditioned by mouse embryonic fibroblasts (MEF) and supplemented with FGF2 (4 ng/mL). After 24 h, the conditioned medium was replaced with daily changes of nonconditioned DME/F12/KOSR medium lacking FGF2, but containing BMP4 (10 ng/mL), A83-01 (1 μM), and PD173074 (0.1 μM) (BAP treatment) for up to 8 days. Control cultures were maintained in conditioned medium containing 4 ng/mL FGF2.

PBMC isolation and coculture with TB cells

Human subjects were recruited for participation under an IRB approved protocol (University of Missouri, Columbia, Office of Human Research Protection Program, Medical IRB Committee-1 #2017804). Each subject provided written informed consent prior to enrollment. Peripheral blood was collected from 13 healthy women in the first trimester of pregnancy (6-12 wks estimated gestational age). The PBMC fraction was isolated from peripheral blood by using Ficoll-Paque Premium (GE Healthcare, Pittsburgh, PA) and centrifugation at 400×g for 30 min at 20°C; cells were cryopreserved until use.

PBMCs were thawed in pre-warmed (37°C) FBS. PBMC viability was over 80% as judged by Trypan blue (Sigma-Aldrich, St. Louis, MO) exclusion. After washing, PBMCs were resuspended at 5 X 10^5 cells/mL in nonconditioned DME/F12/KOSR medium containing IL-2 (100U/ml) and co-cultured with BAP treated TB cells for 72 h (BAP d5-d8) at 37°C.

Flow cytometry

BAP-primed hESC cells were dissociated with ACCUTASE™ (STEMCELL™ TECHNOLOGIES) at 37°C for 10 min. Cells were washed in culture medium and passed through a 40-μm cell strainer to remove the larger STB population (Fisher Scientific). Cells were placed in 3% (wt/vol) BSA in PBS for 30 min to minimize non-specific interactions with antibody and stained with APC-conjugated anti-human HLA-G monoclonal mouse antibody (BioLegend, 335910). After being washed twice with PBS, cells were fixed in 4% (vol/vol) paraformaldehyde (PFA) in PBS. APC-conjugated mouse IgG2a Isotype control (BioLegend, 40222) antibody was employed to account for nonspecific staining. Cells were analyzed in a BD Accuri™ flow cytometer (BD Biosciences, San Jose, CA) with subsequent FlowJo software (Tree Star, Ashland, OR). Suspended cells from the co-culture were harvested for flow cytometry 72 h after co-culture establishment. Cells were washed and stained with the following antibodies: PE/Dazzle™ 594 conjugated anti-CD3 Ab (BioLegend 300336), APC-conjugated anti-CD56 Ab (BioLegend 362504) and phycoerythrin (PE)-conjugated anti-CD16 Ab (BioLegend 302008) for 30 min at 4°C. After washing twice with PBS, cells were fixed in the *Foxp3*/transcription factor staining buffer (eBioscience) according to manufacturer's instructions. Matched fluorescence-labeled isotype antibodies were used as a negative control to account for nonspecific staining. Immunostained cells were analyzed by flow cytometry as described above.

RNA interference and crystal violet assay

For small interfering RNA (siRNA)-mediated knockdown of *VTCN1*, differentiated hESCs were transfected with either a *VTCN1* human siRNA oligo duplex (Origene, SR312516, 10nM) or a control, Trilencer-27 Universal scrambled negative control siRNA duplex (Origene, SR30004, 10 nM) on BAP treatment d 3 by using Lipofectamine 2000 (Invitrogen). After 6 h, the medium was replaced with BAP medium. Total RNA was collected 24 h, 48 h and 72 h after transfection. Protein was collected 72h after transfection. At BAP treatment d 6, i.e., 72 h after the BAP 3 days, cells colonies were stained with crystal violet solution [0.2% wt/vol crystal violet in an aqueous solution containing 20% (vol/vol) ethanol and 10% (vol/vol) formaldehyde] for 15 min. After washing with water, images of individual cell colonies were captured with a Leica M205 stereoscope.

VTCN1$^{-/-}$ hESC line

VTCN1 has five isoforms and our study showed CRISPR gRNA/Cas9 at a single site mutation was not sufficient to knockout the isoform products. To overcome it, two gRNAs were used to delete the whole coding sequence. The *VTCN1*$^{-/-}$ cell line was generated from the hESC by CRISPR nuclease-induced, targeted, double-strand breakage at the Genome Engineering and iPSC Center (GEiC), Washington University in St. Louis. Briefly, gRNAs were designed to target an exon common to all transcripts, where an indel can lead to nonsense mediated decay of the messenger RNAs. The parental H1 cells were nucleofected with the CRISPR gRNA/Cas9 ribonucleoprotein complex, and single cell H1 clones were screened for indels or biallelic deletions in the target gene (*VTCN1*). The whole coding sequence of *VTCN1* was targeted with two guide RNAs: sgRNA1: 5'-AGCCAGTACCCAGATACGCT-3' and sgRNA2: 5'-GAGATTAATCACAAATAGTG-3' (Figure 1). Genomic PCR determined biallelic deletion of *VTCN1* gene.

RNA extraction and quantitative real-time PCR

RNA was extracted in STAT60 containing 1-bromo-3-chloro-propane (Sigma-Aldrich), and DNA removed by using the TURBO-DNA-free kit (Ambion Inc.). A total of 650ng of each RNA sample was included for complementary DNA synthesis by using the iScript™ cDNA Synthesis kit (BioRad). Primers were designed based on published sources and synthesized by Integrated DNA Technologies; primer sequences are summarized in Table 1. Quantitative RT-PCR (qRT-PCR) was performed with a SYBR Green qPCR Master Mix (MedChemExpress) on the BioRad CFX Connect Real-Time PCR Detection System. Thermal cycling conditions were as follows: 95°C for 5 min, followed by 45 cycles of: denaturation at 95 °C for 10 sec, annealing at 56 °C for 10 sec and extension at 72 °C for 10 sec. PCR normalization of data to that of the endogenous control (GAPDH) and fold-change values calculated by the $2^{-\Delta\Delta CT}$ procedure have been described elsewhere (20). The final PCR results were expressed as relative expression compared to individual control sample in each assay. Data followed over time were subjected to two-way ANOVA, followed by the Bonferroni test for pairwise comparisons. Values of $p<0.05$ were considered to support the conclusion that differences were statistically significant.

RNA-seq analyses

RNA was obtained from *VTCN1* knock down or negative control siRNA transfected cells 24 h, 48 h and 72 h after transfection. The

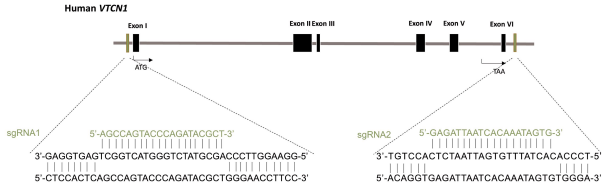

FIGURE 1
Generation of VTCN1-/- hESC using CRISPR/Cas9 genome editing. Schematic of the base pairing between guide RNAs (sgRNA1 for upstream and sgRNA2 for downstream) for VTCN1 and their targeting loci within VTCN1.

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TABLE 1 Primers used for Qpcr.

Gene	Primer sequence
VTCN1	5′GGG GAG GAT GGA ATC CTG AG
	5′CTC CGA CAG CTC ATC TTT GC
HLA-A	5′AAA AGG AGG GAG TTA CAC TCA GG
	5′GCT GTG AGG GAC ACA TCA GAG
HLA-B	5′GAC GGC AAG GAT TAC ATC GCC CTG AA
	5′CAC GGG CCG CCT CCC ACT
HLA-C	5′GGA GAC ACA GAA GTA CAA GCG
	5′CGT CGT AGG CGT ACT GGT CAT A
HLA-E	5′CCT ACG ACG GCA AGG A
	5′CCC TTC TCC AGG TAT TTG TG
HLA-G	5′CTC TCA GGC TGC AAT GTG AA
	5′CAT GAG GAA GAG GGT CAT GG
CGB	5′GTC AAC ACC ACCACC ATG TGT GC
	5′GGT AGT TGC ACA CCA CCT GA
ISG15	5′GAG AGG CAG CGA ACT CAT CT
	5′CTT CAG CTC TGA CAC CGA CA
OAS1	5′GCG CCC CAC CAA GCT CAA GA
	5′GCT CCC TCG CTC CCA AGC AT
MX1	5′TGG CAT AAC CAG AGT GGC TG
	5′CAC CAC CAG GCT GAT TGT CT
GAPDH	5′ CTG GGG CTG GCA TTG CCC TC
	5′ GGC AGG GAC TCC CCA GCA GT

experiment was performed three times to provide a total of 18 RNA samples. RNA quantitation and quality control was performed on a Fragment Analyzer (Advanced Analytical) and cDNA libraries were constructed by standard methods (Illumina TruSeq mRNA stranded kit) with index adapters (Illumina TruSeq indexes). DNA was then sequenced as single-end, 50 base-length reads on a NovaSeq6000 instrument (Illumina, Inc.) with an average read count of 24-34 million reads per sample. Initial base calling and quality filtering of the mRNA-seq reads generated with the Illumina analysis pipeline (fastQ format) were performed with the FastxToolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and adapters trimmed by using CutAdapt (https://cutadapt.readthedocs.org/en/stable/). Reads that mapped either to the human mitochondrial genome or to the internal Phix standard were removed through use of Bowtie software (http://bowtie-bio.sourceforge.net/index.shtml). Reads were mapped (TopHat v2.0.13; http://ccb.jhu.edu/software/tophat/index.shtml) to the reference genome (from Ensembl, v78; http://www.ensembl.org/index.html; Homo_sapiens.GRCh38.dna.primary assembly.fa with Homo_sapiens.GRCh38.78.gtf as annotation). Tests for differential expression were made by using cuffdiff v2.2.1. Uniquely mapped reads were counted with htseq-count v.011.1. Gene expression levels were calculated in FPKM (fragments per kilobase of exon per million mapped fragments), considering gene length as a sum of all exonic nonoverlapping sequences of all isoforms of a given gene. Regularized log transformed (rlog) counts output from DESeq2 to plot heatmaps and gene expression. Tests for differential expression (q-value < 0.01, Log2(foldchange) > 1.5; the Benjamini–Hochberg P value adjustment method was used with DESeq2 within Rstudio (RStudio, Inc.). To broadly analyze pathways affected by *VTCN1* suppression, regulated genes with identifiable alterations upon *VTCN1* knock-down were further analyzed by KEGG pathway enrichment with the web-based DAVID tool (Database for Annotation, Visualization, and Integrated Discovery v6.8). Further clustering and pathway analyses were performed by Ingenuity Pathway Analysis (Qiagen, Hilden, Germany).

Immunostaining

Cells were grown on coverslips coated with Matrigel and placed in six-well tissue culture plates as described previously (19). After fixing the cells in 4% paraformaldehyde (PFA) in PBS for 10 min and permeabilizing them in 1.0% Triton X-100/PBS for 30 min, coverslips were placed in 5% (vol/vol) goat serum/5% (wt/vol) BSA in PBS for 1 h. Cells were then incubated with appropriately diluted primary antibodies (Table 2) overnight at 4°C. Secondary antibody staining was performed with either Alexa Fluor 568-, 647-, or 488-labeled detection antibodies at a 1:300 dilution for 2 hours at room temperature (Table 2). Images were captured under a Zeiss Axiovert 200M with a Leica DFC290 color camera.

Western blotting

Proteins were extracted from cells in radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris·HCl (pH 7.2), 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and 100 mM NaCl], then fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane by means of a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% nonfat milk in Tris-buffered saline/Tween 20 (TBST; 10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membrane was washed once with TBST, and target proteins detected by incubating with the primary antibodies described in Table 3 at 4°C for 12 h. Membranes were washed three times for 10 min and incubated with a 1:3000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for 2 h. Blots were washed with TBST three times and developed with the UVP imaging system (AnalytikJena) according to the manufacturer's protocols. Pairwise comparisons were made with the Student's t test. Values of $p<0.05$ were considered to support the conclusion that differences were statistically significant.

Invasion assay

An aliquot of 5 x 10^4 H1 cells was transferred to invasion chamber wells as previously described (21). After 24 h from initial plating, the medium was changed (2 ml in each chamber) to BAP treatment for differentiation. The medium was then changed daily for 3 days. On the third day (4 days after initial plating), the cells were transfected with *VTCN1* knock-down or scrambled-control siRNA for 6 h. Then the medium was replaced with BAP treatment medium and the cells

TABLE 2 Primary and secondary antibodies used in immunostaining.

Antibody	Catalog no. (Source)	Dilution
B7H4	Ab209242(Abcam)	1:100
CGA	MAB4169(R&D)	1:100
CGB	Ab53087 (Abcam)	1:100
HLA-A	Ab52922(Abcam)	1:100
HLA-A	A2167 (ABclonal)	1:100
HLA-B (A, C)	Ab225636(Abcam)	1:100
HLA-B	A1285 (ABclonal)	1:100
HLA-C	Ab126722(Abcam)	1:100
HLA-G	sc-2179 (Santa Cruz Biotechnology)	1:50
Alexa Fluor 488 donkey anti-mouse IgG	A-21202 (Life Technologies)	1:300
Alexa Fluor 488 donkey anti-rabbit IgG	A-21208 (Life Technologies)	1:300
Alexa Fluor 555 donkey anti-mouse IgG	A-31570 (Life Technologies)	1:300

cultured for another 3 days. On the sixth day the cells on the lower surface of the membrane were fixed in 4% PFA and then stained with DAPI (diluted in PBS, 1:750). Each membrane was imaged by taking 12 images per membrane at 10 x magnification and the number of nuclei counted by means of Image J software. Pairwise comparisons were determined using the Student's t test, with a significance of $p<0.05$ indicated by asterisks.

Immunoassays

After 6 days of BAP treatment with siRNA transfection as above, spent culture medium was collected to measure hCG (human chorionic gonadotrophin) levels (Genway Biotech, LLC; GWB-BQK0F2). Separate but identically treated wells had spent culture medium collected on culture days 7 and 8. Total DNA was isolated (Promega, #A2360) from the respective cultures in order to normalize immunoassay results to cell density levels. ELISAs were performed by following the manufacturer-recommended protocols. Samples were collected in three independent experiments for each treatment. Data followed over time were subjected to two-way ANOVA, followed by the Bonferroni test for pairwise comparisons. Values of $p<0.05$ were considered to support the conclusion that differences were statistically significant.

Results

The inhibitory T cell co-receptor molecule, *VTCN1*, promotes syncytialization and inhibits invasion in BAP cells

H1 (WA01) ESCs were differentiated to TB with BMP4, A83-01, and PD173074 (BAP treatment) as previously described for up to 8

TABLE 3 Primary and secondary antibodies used in western blotting.

Antibody	Catalog no. (Source)	Dilution
B7-H4	14572(Cell Signaling Technology)	1:1000
HLA-A	Ab52922(Abcam)	1:1000
HLA-A	A2167 (ABclonal)	1:1000
HLA-B (A, C)	Ab225636(Abcam)	1:1000
HLA-B	A1285 (ABclonal)	1:1000
HLA-C	Ab126722(Abcam)	1:1000
HLA-G	sc-2179 (Santa Cruz Biotechnology)	1:500
MAPK	4695 (Cell Signaling Technology)	1:1000
pMAPK	4370 (Cell Signaling Technology)	1:1000
pSTAT1	9177 (Cell Signaling Technology)	1:1000
IFITM1	13126 (Cell Signaling Technology)	1:1000
GAPDH	5174 (Cell Signaling Technology)	1:2000

days (10 total days in culture) (19). Total protein was collected from BAP cells on treatment days 3 through 8 (Figure 2A). Western immunoblotting demonstrated marked increases in *VTCN1* in BAP treated cells beginning 4 days after exposure and continuing through d8 (10 days of culture) (Supplementary Figure 1A).

To determine possible roles for *VTCN1* in general TB development and function, we used siRNA to block *VTCN1* transcription (Figure 2A). *VTCN1* mRNA knock-down (KD) was confirmed by qPCR and decreases in protein expression demonstrated by western blotting and immunofluorescence histochemistry (IHC, Figure 2B, Supplementary Figures 1B-D). We began our analyses on the effects of *VTCN1* KD by assessing typical BAP-induced TB developmental milestones. To determine whether *VTCN1* regulated the syncytialization of TB cells, we transfected BAP cells with *VTCN1*-specific and scrambled control siRNA on treatment d3 (Figure 2A). Direct phase contrast imaging and crystal violet staining, which preferentially highlights syncytialized patches of cells within the colonies, demonstrated impairment of syncytialization in *VTCN1* siRNA- but not in scrambled control siRNA-exposed cells (Figure 2C). Fluorescence immunohistochemistry was used to assess the effects of *VTCN1* suppression on two protein markers of TB cell syncytialization, CGB and CGA (Supplementary Figure 1E, 2D, respectively). *VTCN1* KD did not affect CGB expression on BAP d6, which was expected, as CGB expression in BAP cells is typically first detected later in the differentiation progression (19). In contrast, CGA expression, which typically increases in the days prior to the initiation of syncytialization in BAP cultures, decreased upon *VTCN1* KD (Figure 2D), consistent with the visual reductions in STB areas within colonies discussed above (Figure 2C).

We next used time-course experiments beginning on d6 of BAP treatment (3 days after *VTCN1* KD) to document the effects of *VTCN1* KD on secretion of hCG, whose concentrations were normalized to DNA content of the respective cultures to adjust for possible treatment-related differences in cell growth and proliferation. Confirming the IHC results, BAP treated TB cells exposed to *VTCN1* siRNA produced similar amounts of hCG on d6 when compared to scrambled siRNA controls (Figure 2E).

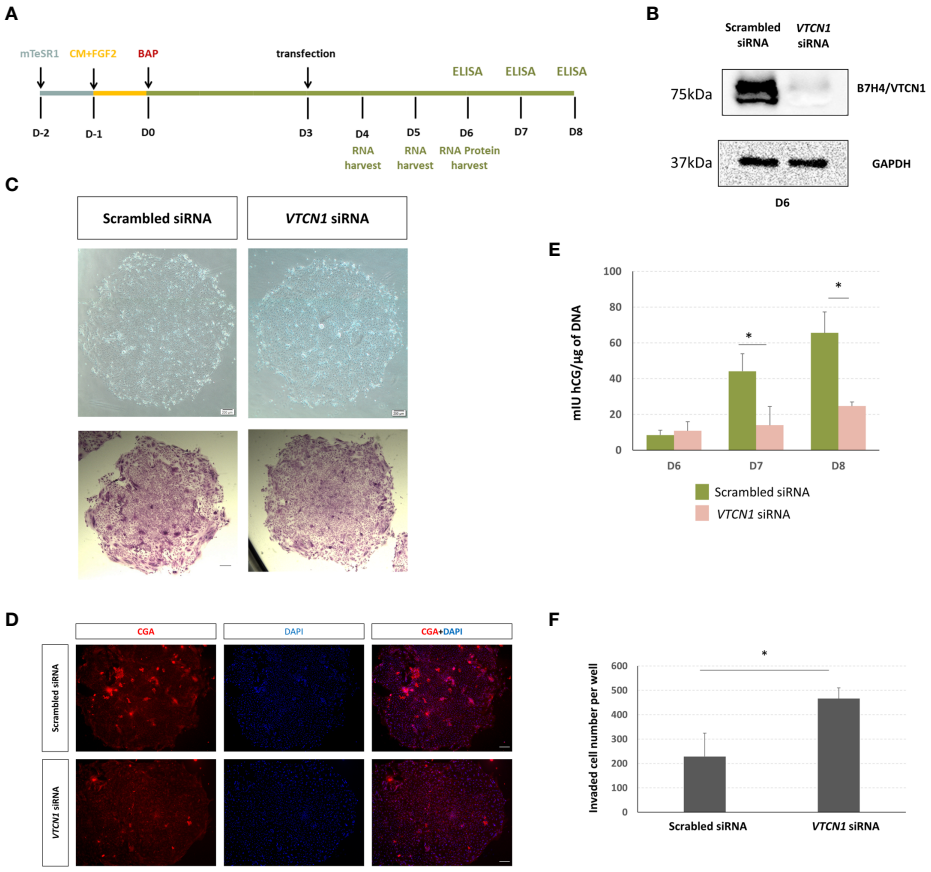

FIGURE 2
(A) Human ESCs (H1) were maintained on mTeSR1. Following the day of passaging, medium was changed to MEF-conditioned medium supplemented with FGF2 (4 ng/µL) (CM+FGF2). After an additional day, the medium was replaced with DME/F12 and 20% KOSR supplemented with BMP (10 ng/mL), A83-01 (1µM), and PD173074 (0.1 µM) (DME/F12/KOSR/KOSR+BMP4/A83/PD) for 8 d. BAP treated H1 ESC were transiently transfected with VTCN1 siRNA or scrambled siRNA (10nM) for 3 d beginning on d3 of BAP treatment. **(B)** VTCN/B7H4 protein expression was assessed by western blotting on BAP treatment d6 (3 days after transfection). **(C)** Phase contrast images of cell colonies on d6 (top). Cells were fixed and stained with crystal violet on D6 and images captured under bright field (bottom). **(D)** In separate experiments, VTCN1 KD and scrambled control cells were fixed on d6 of BAP treatment and stained for CGA (Scale bar, 100 µm). **(E)** Daily (24 h) hCG production was assessed by using ELISA on d6, d7 and d8 following initiation of BAP-induced differentiation and VTCN1 KD on d3 of differentiation. Protein concentrations by ELISA were normalized to the DNA content of each culture. Values are means ± SD for the individual experiments. The VTCN1 siRNA treated cultures secreted significantly lower levels of hCG by d7 and d8 when compared to controls (p=0.025 and p=0.04, respectively). **(F)** Comparison of TB invasion in BAP treated H1 ESC transfected with VTCN1 siRNA or scramble siRNA for 3 days. Values are the mean number of invaded cells ± SD in 12 randomly selected fields for three independent experiments, each in triplicate wells. Statistically significant comparisons to scrambled control siRNA are indicated by asterisks (p<0.05).

The noted decrease in syncytialization imparted by *VTCN1* KD led us to hypothesize that the BAP cells were differentiating through an alternative developmental pathway, most likely conversion into invasive EVT-like cells (22). In experiments replicating the BAP and transfection methods described above, BAP treated TB cells were exposed to *VTCN1* or scrambled control siRNA but were then cultured under conditions that allowed specific assessment of TB cell invasion (invasion assays). The invasive capacity of BAP cells increased after *VTCN1* KD when compared to scrambled siRNA-transfected cells (p=0.032) (Figure 2F).

The interferon response pathway is activated upon *VTCN1* repression in TB cells

We next performed RNA-seq on BAP cells at 24, 48 and 72 h after *VTCN1* KD to define pathways regulated by *VTCN1* better. A total of 61 genes were up-regulated and 155 were down-regulated 24 h after transfection. At this time point, 21 up-regulated pathways were enriched with a criterion of p<0.05 while no down-regulated pathways were enriched (Supplementary Figure 2A, Supplementary Table 1). Upregulated, differentially expressed genes at the 24 h time point were highly associated with pathways that included: herpes simplex infection, influenza A and cytokine receptor interaction. Of the 21, 20 were associated with immune responses. Analyses of the 48 h and 72 h post-transfection samples identified a further 12 and seven upregulated and enriched pathways, respectively (Supplementary Figure 2B, C, Supplementary Table 1), with upregulated differentially expressed genes highly associated with pathways that again included responses to virus.

To assess the effect of *VTCN1* KD at the single gene level in more detail, we generated a clustering heatmap of differentially expressed genes. The top 20 genes identified in this analysis are shown in the heatmap in Figure 3A, among which 16 genes are related to type I IFN responses, which are known to mediate innate immune responses to viruses. The genes most altered by *VTCN1* suppression when compared to scrambled siRNA exposed controls were next analyzed by using QIAGEN Ingenuity Pathway Analysis software (QIAGEN IPA). The affected functional categories with highest significance 24 h, 48 h and 72 h after transfection are presented in Figure 3A, B, Supplementary Figure 3A and Supplementary Figure 4A. Potential upstream regulators of the differentially expressed genes as predicted from the literature by IPA (Ingenuity Pathway Analysis) are shown in Figure 3C, Supplementary Figure 3B and Supplementary Figure 4B.

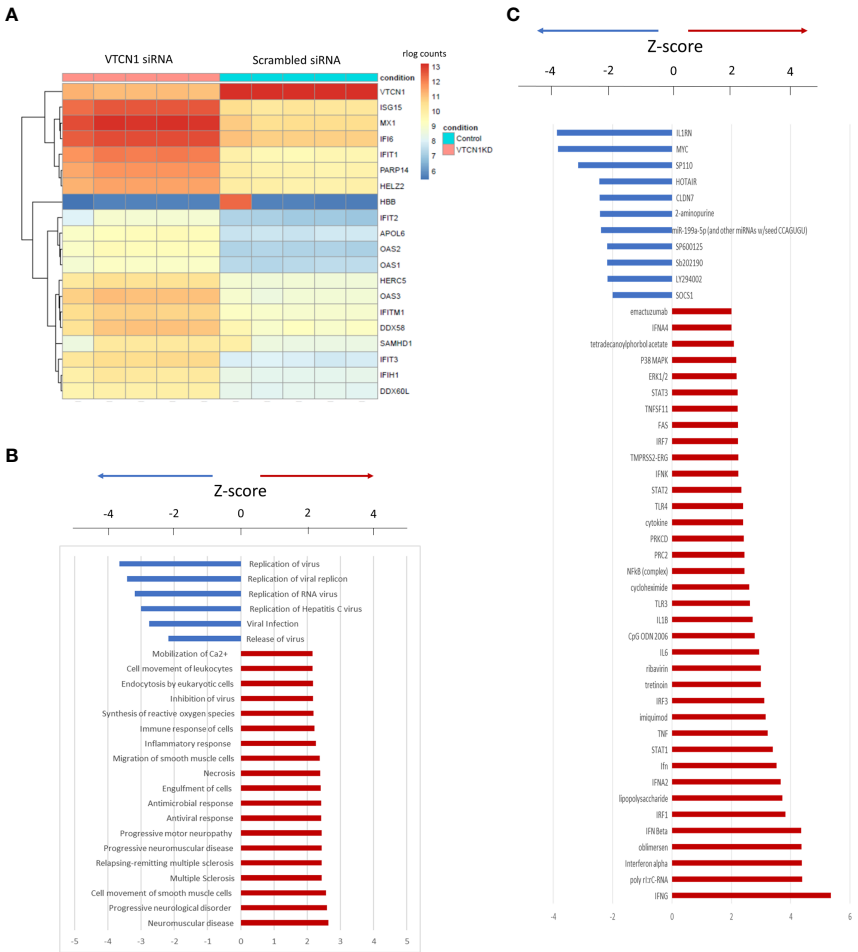

FIGURE 3
(A) Heatmap of the top 20 differentially expressed genes 24 h after VTCN1 KD on d3 of BAP treatment. rlog count (regularized logarithm transformed count) reflects gene expression normalized to the sequencing depth of each sample. **(B)** Affected functional categories 24 h after transfection. **(C)** Gene activation 24 h after transfection.

Signaling pathways involved during the promotion of invasion

RNA-seq data rlog counts normalized to scrambled siRNA controls demonstrated a decrease in *MAPK* and an increase in *STAT1* transcripts in response to *VTCN1* siRNA knock-down (Figures 4A, B). Additionally, the expression of EVT pro-invasion markers, Integrin Subunit Alpha 5 (ITGA5) and matrix metalloproteinases (MMP)-12, were significantly increased after *VTCN1* had been knocked down (Supplementary Table 2). To verify the documented transcriptional changes seen with bulk RNA-seq as well as activation of the MAPK/ERK1/2 and JAK/STAT signaling pathways upon *VTCN1* KD, protein levels of MAPK, phospho-MAPK (pMAPK) and phospho-Stat1 (pSTAT1) were examined by western blotting. As with the transcriptional changes in *STAT1*, pSTAT1 level increased in TB cells upon *VTCN1* KD (Figure 4D). While the direction of change in pMAPK protein expression upon VTCN1 KD followed that of the transcriptional changes, protein levels of pMAPK, using an antibody detecting p44/42 MAPK, were significantly elevated (Figure 4C).

IFITM1 overexpression inhibits TB cell fusion

RNAseq data from *VTCN1* KD cells also highlighted an increase in *IFITM1* transcripts when compared to scrambled control siRNA exposed cells. (Figure 4E). The IFN-induced transmembrane protein (IFITM) family includes members (IFITM1, -2, and -3) that protect multiple cell types from viral infections by preventing viral membrane fusion with cells and by inhibiting the syncytialization of infected cells (23–25). Since syncytialization is central to TB development, we compared IFITM1 protein dynamics by Western blotting to placental TB developmental changes in BAP primed TB with or without *VTCN1*. As expected, IFITM1 increased as syncytialization decreased when *VTCN1* was knocked down in BAP cells (Figure 4F).

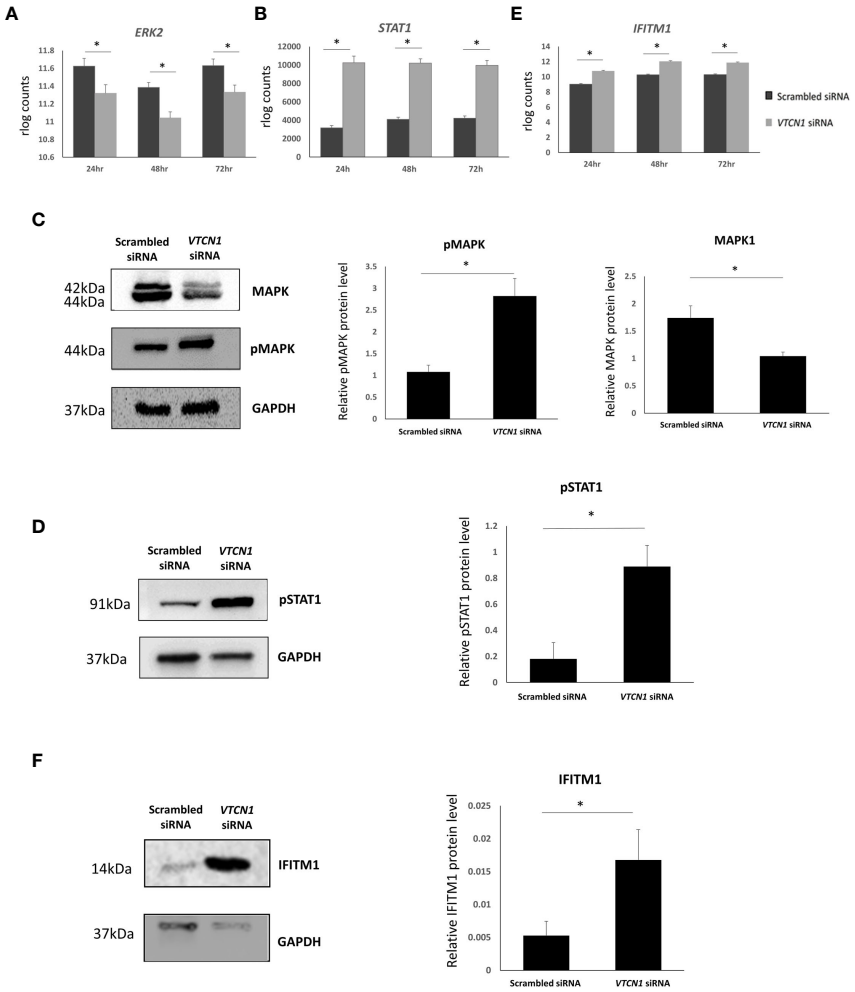

FIGURE 4
Transcript expression levels (in rlog counts) for ERK2 **(A)**, STAT1 **(B)**, and IFITM1 **(E)** in BAP differentiated TB cells exposed to scrambled siRNA or VTCN1 siRNA for 24h, 48h and 72h (BAP d4, d5 and d6). The hESCs were passaged and transfected as explained in the legend of Figure 1. Benjamini–Hochberg P value adjustment method were done with DESeq2. An adjusted p value less than 0.01 was considered to be statistically significant. Protein levels of MAPK and pMAPK **(C)**; pSTAT1 **(D)**; and IFITM **(F)** in BAP differentiated TB cells on d6 (3 days after transfection) were assessed by western blotting. Three independent western blots were quantified by densitometry using ImageJ Software. The protein expression of MAPK, pMAPK, pSTAT1 and IFITM1 was normalized to the corresponding GAPDH signals of appropriate samples. Pairwise comparisons were determined using the Student's t test, with a significance of $p < 0.05$ indicated by asterisks.

Transcription and translation of classical MHC class I molecules increase with *VTCN1* repression in BAP cells

RNA-seq analyses provided evidence that the mRNA levels of classical MHC class I molecules, *HLA-A*, *HLA-B* and *HLA-C* increase with *VTCN1* suppression, while *HLA-G* transcripts remain relatively unchanged. The changes in *HLA-A*, *-B*, *-C* and *-G* expression were validated by RT-PCR and correlated well with protein data acquired by western immunoblotting, fluorescence ICC and flow cytometry (Figures 5, 6). *HLA-A*, *HLA-B* and *HLA-C* transcripts increased on d5 and d6 of BAP treatment (d2 and d3 post *VTCN1* KD) (Figure 5A) and protein expression increased on BAP d6/*VTCN1* KD d3 (Figures 5B, C). The effects of *VTCN1* KD on the transcript and protein levels of *HLA-G*, a marker for EVT, were similarly analyzed and were not affected by treatment.

To identify possible off target gene silencing during siRNA treatment and/or the possibility of a purely interferon-stimulated increase in classical MHC class I expression secondary to a cellular response to the siRNA, we monitored HLA-A protein levels and IFN-stimulated genes expression in a *VTCN1* CRISPR-Cas9 knock-out (KO) hESC cell line after BAP treatment. HLA-A protein on d4, d6 and d8 of BAP treated *VTCN1* KO cells and IFN-stimulated genes including *ISG15*, *OAS1* and *MX1* mRNA levels on d6 of BAP treated *VTCN1* KO cells increased when compared to BAP-exposed wild type H1 cells (Figures 5D, E), replicating findings in our siRNA-induced transient knock-down models (Figure 3A, Figures 5A-C).

To further verify a relationship between *VTCN1* and *MHC class I* gene alterations during primitive TB differentiation, we used an alternative model and analyzed a publicly-available single cell RNA-seq database generated by West et al. for TB cells isolated from embryonic day (D) 8, D 10 and D 12 human embryos during blastocyst extended culture (26). Although the transcripts for *VTCN1*, *HLA-A* and *HLA-B* were all expressed at low levels in cytotrophoblast (CTB) at D 8, D 10 and D 12, the expression of *VTCN1* progressively decreased as the expression of *HLA-A* and *HLA-B* increased over time in culture, suggesting a possible association between B7-H4 and MHC class I during early placental development. In contrast, *HLA-C* and the non-classical MHC I molecules *HLA-E* and *HLA-G* genes were more robustly expressed throughout culture, and expression increased dramatically from D 10 to D 12 in culture (Supplementary Figure 5), likely a reflection of an increasing proportion of cells that are migratory (EVT-like) in the embryos.

VTCN1 in BAP cells shifts the phenotype of co-cultured peripheral NK cells towards that of decidual NK cells

We hypothesized that alterations in MHC cell surface expression in invasive primitive trophoblast of the implanting embryo might play a role in possible phenotype switching of peripheral maternal CD3-CD56^{dim}CD16^{+} NK cells toward the CD3^{-}CD56^{bright}CD16^{-} NK cells typical of the decidua (27). Since *VTCN1* KD increased the surface expression of classical MHC class I molecules, including HLA-C, on BAP primed TB cells, we examined the effects of such changes on peripheral immune cells from pregnant women in a co-culture system. Cryopreserved PBMCs isolated from peripheral blood of pregnant women (5-12 weeks gestation) were thawed and cocultured for 3 days (d5 – d8 of BAP differentiation/d3–d5 post *VTCN1* KD) with control and *VTCN1* KD BAP-treated human ES cells. Non-adherent cells in the co-culture, largely PBMCs, were then isolated and subjected to flow cytometry. Lymphocytes were identified by FSC/SSC light scatter. The gating strategy for CD56 and CD16 expression on all CD3-negative cells is presented in Figure 7A. The percentage of CD3-CD56^{dim}CD16^{+} peripheral-type NK cells among CD3 negative lymphocytes increased (p=0.043, Figure 7B) and the percentage of CD3^{-}CD56^{bright}CD16^{-} decidual NK-like cells decreased (p = 0.019, Figure 7C) in PBMCs cocultured with *VTCN1* KD TBs when compared to those cocultured with scrambled control siRNA-exposed TB.

Discussion

We previously demonstrated that *in vivo* protein expression of the B7 family checkpoint inhibitor, *VTCN1* (B7-H4), is at its highest in villous trophoblast during the first few weeks of pregnancy (11). It is also expressed in trophoblast differentiated from pluripotent stem cells that we believe may correspond to primitive trophoblast associated with the implanting conceptus (10, 11). *VTCN1* is known to mediate immune homeostasis at several sites of inflammation, including those in the tumor microenvironment (28), and we hypothesized that it might also play an analogous role in maternal-fetal immune regulation. Here, we've studied the role of *VTCN1* and its translation product in early human placental development and in immunologic interactions at the human maternal-fetal interface.

Downregulation of *VTCN1* expression in our *in vitro* model of peri-implantation human TB development shifted cells away from syncytialization (Figures 2B, E) and toward invasion (), consistent with findings in some cancers. The cancer literature contains inconsistent findings on the associations between *VTCN1* (12, 13) and metastatic behaviors. High levels of *VTCN1* have been observed in invasive breast cancer (13), and high tumor cell expression has been linked to disease progression and poor prognosis in renal cell cancer (12), suggesting that *VTCN1* promotes tumor invasion. On the other hand, high levels have been positively correlated with improved survival in breast cancer patients and smaller tumors in a B7-H4 $^{-/-}$ murine model of breast cancer (29), seemingly supporting a converse relationship with invasion. Our study supports the former role in the human placenta. We suggest that *VTCN1* may limit invasion and promote syncytialization at the earliest stages of gestation but permit a shift toward invasion as pregnancy progresses and *VTCN1* expression decreases. This early bias toward STB lineage development is consistent with the essential role of hCG, a primary secretory product of STB, in the support of early pregnancy when the conceptus must signal the mother to avoid a return to ovarian cyclicity. It is also consistent with the observation that peak levels of hCG occur during the first trimester and subsequently decline (30, 31). The shift away from syncytialization and toward invasion upon *VTCN1* suppression in our model system was not associated with a simultaneous change in HLA-G expression, a marker of the invasive

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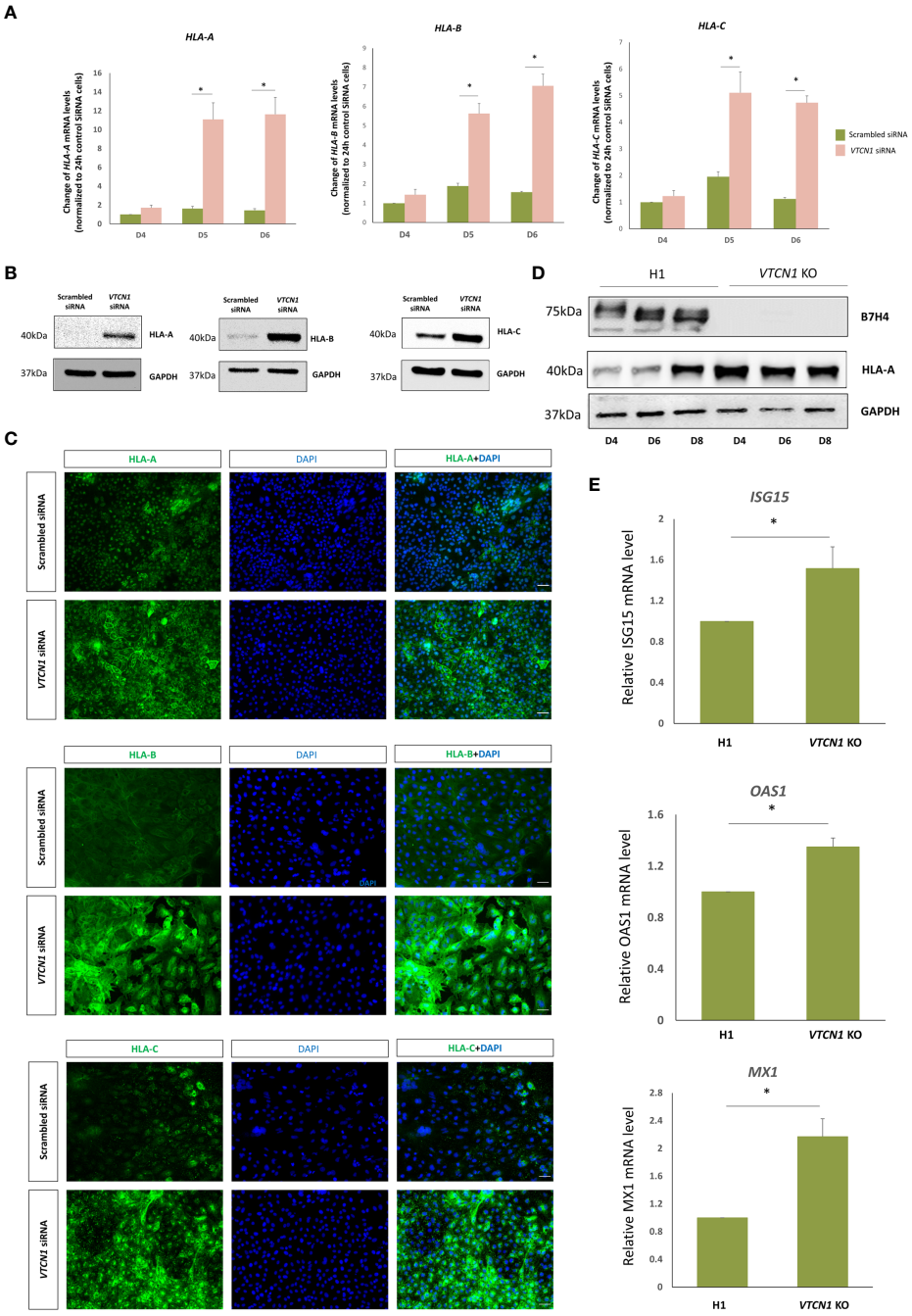

FIGURE 5
BAP treated H1 ESC were transiently transfected with VTCN1 siRNA or a scrambled siRNA control for 3d. **(A)** The effects of VTCN1 KD on HLA-A, HLA-B and HLA-C transcription were assessed by RT-PCR. Data represents means ± SD of three independent experiments; statistically significant comparisons to scrambled control siRNA are indicated by asterisks ($p<0.05$). **(B)** Protein was collected on BAP d6 (transfection d3) and the expression of HLA-A, HLA-B and HLA-C were assessed by western blotting **(C)** Immunofluorescence microscopy was performed to assess the expression of HLA-A, HLA-B and HLA-C on BAP d6 (transfection d3) (Scale bar, 100 µm). **(D)** Western blotting analysis of HLA-A protein expression in BAP treated H1 cells or VTCN1 KO cells on BAP d4, d6 and d8. **(E)** ISG15, OAS1 and MX1 mRNA levels for BAP treated H1 cells (WT) and VTCN1 KO cells were assessed by qPCR on BAP d6.

trophoblast subtypes. It is possible that changes in *VTCN1* expression uncouple invasion/migration and HLA-G expression.

To investigate *VTCN1*-mediated inhibition of invasion in the human placenta, we assessed the effect of *VTCN1* knock-down on signaling pathways known to be central to the maintenance and/or differentiation of human TB subtypes (32). Activation of the MAPK/ERK1/2 pathway promotes invasion in the transformed human cell line HTR-8/SVneo, which models the EVT lineage (33), while inhibition of STAT1 limits invasion in HTR-8/SVneo and JEG-3 cells (34, 35). The latter are commonly used to model CTB proliferation and invasion (36). We demonstrate that *VTCN1* knock-down activates the MAPK/ERK1/2 and JAK/STAT pathways and increases BAP-primed TB invasion (Figure 2F, Figures 4C, D). Although these same signaling pathways are also reported to regulate TB cell fusion in primary term TB cell cultures and during forskolin-mediated syncytialization in BeWo cells (37, 38), upregulation of

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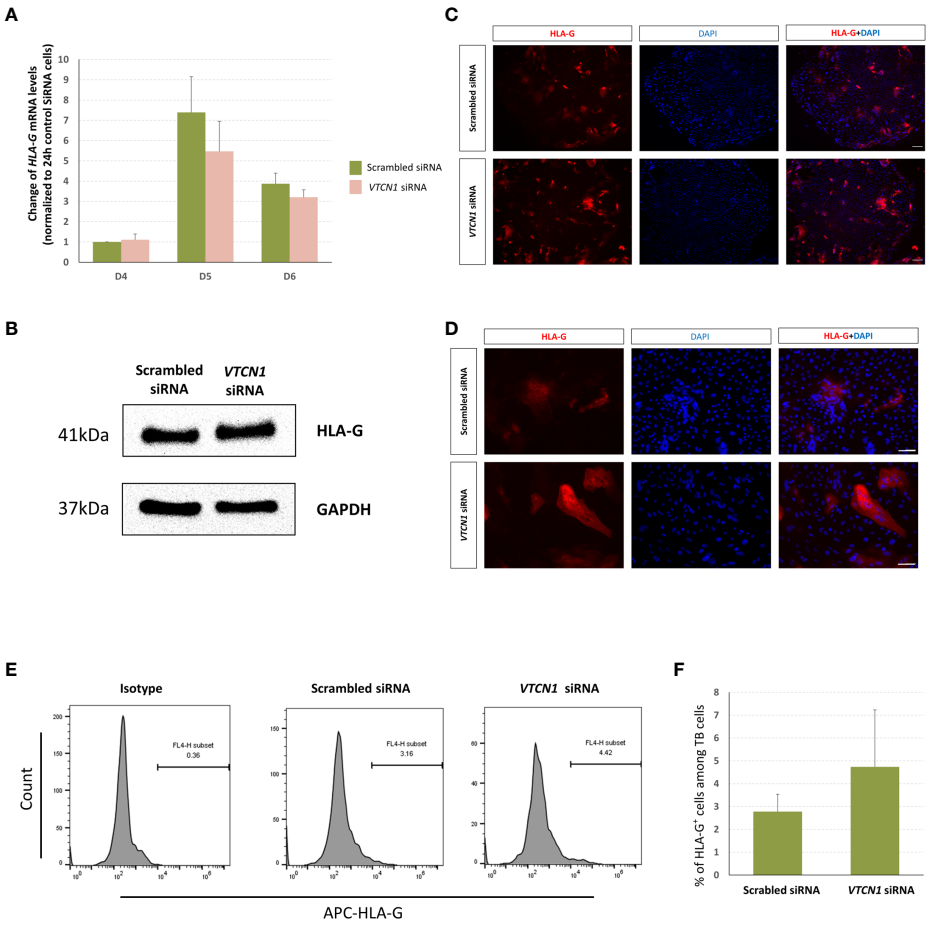

FIGURE 6
H1 ESC on D3 of BAP differentiation were transiently transfected with VTCN1 siRNA or a scrambled siRNA control for 3 d, **(A)** HLA-G mRNA was assessed by RT-PCR. Data represents means ± SD of three independent experiments; pairwise comparisons were made using Bonferroni methods. No statistically significant differences were detected in HLA-G transcripts in VTCN1 KD cells when compared to scramble siRNA transfected cells. **(B)** Protein was collected on BAP d6 (KD d3) and the expression of HLA-G was assessed by western blotting. **(C, D)** Immunofluorescence microscopy was performed to assess the expression of HLA-G on BAP treatment d6 (KD d3) (Scale bar, 100 μm). **(E, F)** Cells were collected on BAP treatment d8 (KD d5) and flow cytometry was used to track the cell surface expression of HLA-G.

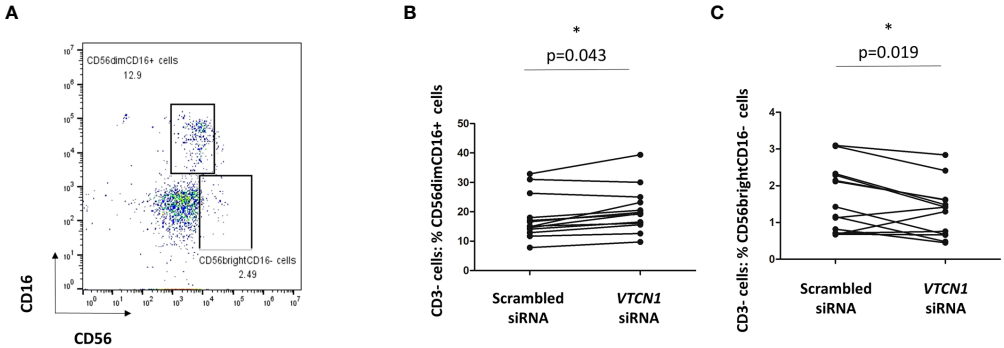

FIGURE 7
(A) PBMCs isolated from pregnant volunteers were co-cultured with TB cells with or without VTCN1 KD from BAP d5 to d8 (KD d2-d5). Flow cytometry was used to analyze the CD56 and CD16 expression on CD3- lymphocytes in co-culture. **(B, C)** Flow cytometry was used to analyze the effects of VTCN1 KD in TB cells on human CD3- CD16+ CD56dim cells **(B)** and CD3- CD16- CD56bright **(C)** peripheral blood cells after coculture with TB cells. Results are expressed as percentages. p-values were calculated using the paired t-test (n=13); significant differences are indicated by asterisks.

pMAPK and pSTAT1 by *VTCN1* knockdown was not associated with spontaneous cell fusion in our study.

IFN-inducible transmembrane 1 (IFITM1) expression was significantly increased upon *VTCN1* KD in our model of primitive TB development ((Figure 3A, Figures 4E, F; also see below). Although IFITM1 is known to protect uninfected cells from viral infection *via* blocking virus-cell fusion and opposing entry by many enveloped viruses (24, 25, 39), IFITM1 also impairs syncytin-mediated fusion (23, 40). We have shown previously that IFN responses are blunted in primitive TB when compared to term TB counterparts (41), so the increase in IFITM1 upon knockdown of *VTCN1* in our model is consistent with this. We hypothesize that the increase in IFITM1 upon *VTCN1* knockdown in primitive TB may account, at least in part, for the reduction in STB formation.

During pregnancy, there is a shift from the marked inflammatory attachment reaction at the initiation of pregnancy (implantation) to a more pro-tolerogenic, non-inflammatory state for the majority of the remainder of pregnancy (1, 42, 43). Unlike TB derived from term placenta, BAP-generated primitive TB of the early first trimester has diminished levels of 1) interferon-stimulated genes (ISG), such as *ISG15*, *MX1*, *DDX58* (*RIG*-1), 2) interferon induced proteins with tetratricopeptide repeats (*IFIT*) family members, 3) and oligoadenylate synthase-like (*OAS*) family members, all of which augment the innate immune response to viral infection (44, 45). Such diminished responses have been linked to a greater risk of vertical transmission of viruses in early gestation including transmission of CMV, HSV, Zika virus and SARS-CoV-2 (46–50). Our RNA-seq analysis revealed that expression of several ISGs were upregulated when *VTCN1* expression was repressed (Figure 3A). Pathway enrichment and clustering analyses indicated that *VTCN1* expression negatively regulates innate immune response and inflammation in hESC primed TB. These activities uphold its known role as an immune checkpoint regulator but might also confer on early pregnancy an increased susceptibility to virus infection (Figure 3A, Supplementary Figure 2-4), which is consistent with our prior findings (41, 44).

We also demonstrated that transcription and translation of classical major histocompatibility complex (MHC) class I molecules are increased in TB when *VTCN1* expression is knocked down (Figure 5). MHC expression patterns in the human placenta are unique, and the control of MHC class I expression is poorly understood (51–54). Villous CTB and STB do not express the classical MHC class I molecules, HLA-A, -B and -C. EVT also lack HLA-A and -B, but express HLA-C and several non-classical MHC class I molecules, including HLA-G (48, 53–55). We have shown that *VTCN1* knock-down upregulates mRNA and protein levels of HLA-A, -B and -C, but has little effect on HLA-G expression. These findings are supported by TB single-cell RNA-seq data derived from human blastocysts grown *in vitro* for up to 12 days post-fertilization (extended blastocyst culture) (26). These embryos allow the first 5 days of placental growth to occur in the absence of maternal signals. In such an extended human blastocyst culture model, low *VTCN1* expression correlated with an elevation in classical MHC-I expression in early TB cells (Supplementary Figure 5). Together, these data are consistent with an extensive literature demonstrating that *VTCN1* is expressed in a large variety of malignancies (12–14, 55) and that MHC class I products are simultaneously often poorly expressed in many (estimates range from 40-90%) of such tumors (56–58), thereby enhancing evasion of immune responses by the host.

Since HLA-A, -B and -C (but not HLA-G) expression is responsive to IFNA and -G exposure (59, 60), and siRNAs are notorious for causing off-target antiviral IFN responses, we determined whether similar changes were observed when CRISPR-Cas9 was used to achieve a biallelic knockout of all variants of *VTCN1* in undifferentiated H1 cells. Again, increases in MHC class I expression and modest but significant upregulation of certain interferon-inducible genes were noted during BAP-induced differentiation of the mutant H1 cells, suggesting that the changes observed after siRNA treatment were not artifactual.

While it is clear that human decidual NK cells have marked phenotypic and functional differences from their peripheral counterparts (61–72), what remains less clear is their origin, with some scholars suggesting they derive from expansion of an *in situ* endometrial population (61, 62, 73, 74) and others suggesting many are called to the site of implantation from a pNK cell population and either expanded or differentiated in the local microenvironment (63–67). It is likely that both are involved. In this study, we have reported that the presence and absence of TB-expressed *VTCN1* can shift the phenotype of peripheral NK cells isolated from pregnant women from the $CD56^{dim}CD16^{+}$, classically cytotoxic subtype (68, 69) to the $CD56^{bright}CD16^{dim}$ subtype more typical of the decidua (63–68). This finding suggests that the expression of the immune checkpoint regulator, B7-H4, on the surface of TB could be involved in local differentiation of pNK cells, possibly those called into the decidua in response to the hormonal changes of ovulation and corpus luteum formation, toward a decidual phenotype. While changes in surface MHC expression may be involved,. parthenogenesis embryonic stem cells and animal experiment will be conducted to dissect the machanisms underlying this phenotypic shift and its role in the nuanced innate and adaptive immune interaction at the maternal-fetal interface of early human pregnancy ((70, 71)).

The BAP-derived TB and extended human blastocyst culture systems have recognized limitations. Still, as two of a very limited number of approaches to the study of the earliest stages of human pregnancy, their concordant results around *VTCN1* are reassuring. Recently, the derivation, culture and differentiation conditions for human trophoblast stem cells (hTSCs) were reported (72) and hTSC were subsequently generated from pluripotent stem cells including hESC and iPSC (75–82). Future experiments will utilize hTSC-based models to differentiate among trophoblast sublineages, co-culture systems that allow study of TB interactions with maternal decidual and vascular cells and expanded study of the *VTCN1* CRISPR-Cas9 knock-out (KO) hESC cell line. Future investigation on *VTCN1*-related changes in NK cells will also be expanded to analyze NK cell function in addition to the phenotypic changes reported her.

In summary, we suggest that: 1) VTCN1 is a critical regulator of TB syncytialization, TB invasiveness and possibly other aspects of differentiation in the early human placenta; 2) *VTCN1* limits upregulation of classical MHC Class I genes and an array of proteins involved in interferon responses in TB, including that of IFITM1, a protein whose expressions counteracts syncytialization; and 3) *VTCN1* presentation by TB cells can induce phenotypic changes in peripheral natural killer cells that resemble those characteristics of the maternal-fetal interface. In sum, the immune checkpoint regulator, *VTCN1*, likely plays an important role in early placental development and therefore diseases of abnormal placentation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by University of Missouri, Columbia, Office of Human Research Protection Program, Medical IRB Committee-1 #2017804. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JZ and DS designed research. JZ, YT and MW performed research. YQ conducted the bioinformatics analyses. JZ performed statistical analyses. JZ and DS analyzed data. DS recruited patients. JZ and DS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1069395/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) Time course for protein expression of B7H4 in BAP treated H1 hESCs from BAP d3 to d8. **(B)** BAP treated H1 hESCs were transiently transfected with scramble siRNA (control) or VTCN1 siRNA (VTCN1 KD) and cultured for up to 72 h. Assessments were made 24 h, 48 h and 72 h after transfection. Relative concentrations of VTCN1 transcripts were assessed by real-time PCR (n=3; i.e., three RNA preparations from three independent experiments). Data represents means ± SD, statistically significant values compared to scrambled control siRNA-exposed cells are indicated by asterisks ($p<0.05$). **(C–E)** BAP treated H1 hESCs were transiently transfected with scramble siRNA (control) or VTCN1 siRNA (VTCN1 KD) and kept in culture for 3 d after transfection. B7H4 **(C, D)** and CGB **(E)** protein expression was assessed by immunofluorescence microscopy (Scale bar, 100 μm).

SUPPLEMENTARY FIGURE 2
KEGG pathway analysis of differentially expressed genes after exposure to VTCN1 siRNA and a matched scrambled siRNA control. **(A)** Genes upregulated 24 h after transfection, **(B)** genes upregulated 48 h after transfection, **(C)** genes upregulated 72 h after transfection.

SUPPLEMENTARY FIGURE 3
Assessment of gene activation patterns for differentially expressed genes after exposure to VTCN1 siRNA and a matched scrambled siRNA control. **(A)** Affected functional categories 48 h after transfection. **(B)** Activated genes 48 h after transfection.

SUPPLEMENTARY FIGURE 4
Assessment of gene activation patterns for differentially expressed genes after exposure to VTCN1 siRNA and a matched scrambled siRNA control. **(A)** Affected functional categories 72 h after transfection. **(B)** Activated genes 72 h after transfection.

SUPPLEMENTARY FIGURE 5
Data were generated in a model of human extended blastocyst culture (26, 83) and were analyzed by using the publicly-available databases generated from that work. **(A-F)** MHC-I expression in early pregnancy. FPKM values of the MHC-I genes: HLA-A **(A)**, HLA-B **(B)**, HLA-C **(C)**, HLA-E **(D)**, HLA-G **(E)** and of VTCN1 **(F)** in small cells of human embryos on d8, d10 and d12. The authors suggest these small cells represent mononucleated CTB.

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Modulation of fetoplacental growth, development and reproductive function by endocrine disrupters

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Maternal endocrine homeostasis is vital to a successful pregnancy, regulated by several hormones such as human chorionic gonadotropin, estrogen, leptin, glucocorticoid, insulin, prostaglandin, and others. Endocrine stress during pregnancy can modulate nutrient availability from mother to fetus, alter fetoplacental growth and reproductive functions. Endocrine disrupters such as bisphenols (BPs) and phthalates are exposed in our daily life's highest volume. Therefore, they are extensively scrutinized for their effects on metabolism, steroidogenesis, insulin signaling, and inflammation involving obesity, diabetes, and the reproductive system. BPs have their structural similarity to 17-β estradiol and their ability to bind as an agonist or antagonist to estrogen receptors to elicit an adverse response to the function of the endocrine and reproductive system. While adults can negate the adverse effects of these endocrine-disrupting chemicals (EDCs), fetuses do not equip themselves with enzymatic machinery to catabolize their conjugates. Therefore, EDC exposure makes the fetoplacental developmental window vulnerable to programming *in utero*. On the one hand prenatal BPs and phthalates exposure can impair the structure and function of the ovary and uterus, resulting in placental vascular defects, inappropriate placental expression of angiogenic growth factors due to altered hypothalamic response, expression of nutrient transporters, and epigenetic changes associated with maternal endocrine stress. On the other, their exposure during pregnancy can affect the offspring's metabolic, endocrine and reproductive functions by altering fetoplacental programming. This review highlights the latest development in maternal metabolic and endocrine modulations from exposure to estrogenic mimic chemicals on subcellular and transgenerational changes in placental development and its effects on fetal growth, size, and metabolic & reproductive functions.

KEYWORDS

in utero exposure, endocrine-disrupting chemicals, fetoplacental growth, bisphenol, glucocorticoids, leptin, bps

1 Introduction

Pregnancy hormones such as estrogen, human chorionic gonadotropin (hCG), glucocorticoid, leptin, insulin, prostaglandin, and others are vital to a successful pregnancy. There are several risks associated with pregnancy-related complications, but one among them is the exposure of the mother and fetus to the modulators of pregnancy hormones by endocrine disruptors (1). Endocrine-disrupting chemicals (EDCs) can mimic the body's hormonal response over life-long exposure. Among these thousand chemicals, endocrine disruptors such as bisphenols (BPs) and phthalates are abundant and present in substances contaminated daily, including food surfaces, cosmetics, and many more. The mechanisms underlying the endocrine stressors during critical development phases on predisposing adult disease risk are evolving (2). Epigenetic modification ensures developmental plasticity that allows adaptive regulation of the developing fetus at the expense of altered placental functions in response to EDC exposure. Normal placentation is the key to a healthy fetus. At the same time, exposure to EDCs can modulate placental functions by altering placental invasion and angiogenesis required for successful placentation (3). Placenta is the active barrier between the mother and fetus and involves waste disposal, gaseous exchange, immunogenic barrier, and hormone production. EDCs can affect placental barrier functions, structure, and stability of gene expression due to maternal exposure during pregnancy that modulate maternal endocrine balance, implantation processes, and fetus organ development (4).

BPA is an estrogenic chemical that disrupts the development programming of reproductive function through changes in gonadal hormones (5). Prenatal phthalate exposure disrupts the hormonal balance in pregnant women and transmits its risks to their offspring (6). In addition, exposure to EDCs predisposes the risk of preterm birth and altered intrauterine growth, gestational length, birth weight, ponderal index, femur length, abdominal circumference, head circumference, and anogenital index in newborns. Scientific investigations have consistently shown the interplay between epigenetics, lifestyle exposure, and the gestational environment. The epigenetic changes due to exposure to EDCs during critical windows of gestation and its effects on gastrulation, fetal organogenesis, and overall growth and development are emerging. The critical window of EDC exposure during pregnancy can disrupt the programming of organ development that could have long-lasting effects. Fetal exposure to these EDCs, such as BPs (bisphenol A, bisphenol S), Phthalates (diethylhexyl phthalate, DEHP and their metabolites) are suspected of altering the programming of normal biological functions, including endocrine imbalance and fertility. This review consolidates pregnancy-linked hormonal balance in response to EDC exposure, the endocrine effects on placentation, epigenetic changes, and maternal endocrine regulation on fetal growth and reproductive development.

2 Exposure to endocrine disruptors affects maternal endocrine balance and fetal growth, development, and pregnancy outcomes

Pregnancy is a complex physiological process regulated by various hormones and signalling molecules within the endocrine system. The maternal endocrine system changes during pregnancy to support fetal growth and development that involves changes in several axes, including hypothalamic-pituitary-adrenal (HPA), thyroid, and gonads. The HPA axis is crucial in regulating the maternal endocrine system during pregnancy. The hypothalamus secretes the corticotrophin-releasing hormone, which stimulates the pituitary to release adrenocorticotropic hormone (ACTH). ACTH, in turn, stimulates the adrenal glands to produce cortisol, an essential hormone for fetal development. However, excessive cortisol production can lead to adverse pregnancy outcomes, such as premature birth and low birth weight (7). The thyroid regulates metabolism, growth, and development during pregnancy. The thyroid gland produces two hormones, triiodothyronine (T3) and thyroxine (T4), essential for fetal brain development. The demand for thyroid hormones increases during pregnancy, and the maternal thyroid gland enlarges to meet this demand. In addition, the placenta produces hCG, which stimulates the maternal thyroid gland to produce more thyroid hormones. However, excessive thyroid production can lead to hyperthyroidism, negatively affecting the mother and fetus. Therefore, tight regulation of the thyroid axis is essential in protecting the mother during pregnancy (8).

The reproductive organs undergo significant changes during pregnancy to support fetal growth. The ovaries stop producing eggs, and the placenta takes over the production of estrogen and progesterone, essential for maintaining pregnancy. Placenta does not have all the necessary enzymes to convert cholesterol to estrogen or make progesterone (9). Estrogen and progesterone promote the growth of the uterus and help maintain the pregnancy by relaxing the uterus and preventing contractions. In addition, estrogen promotes the development of the fetal lungs, liver, and immune system. However, excessive estrogen production can lead to adverse outcomes, such as preeclampsia and gestational diabetes. Therefore, tight estrogen regulation is essential for optimum pregnancy outcomes (10).

Placenta acts as an endocrine organ during gestation, secretes several hormones essential for adapting maternal physiology, and transfers nutrients and gases to the growing fetus, thereby supporting fetal development (11). The trophoblast of the developing placenta produces hormones to ensure that the endometrium's uterus lining can receive them for embryo implantation. As the gestation progress, the placental hormones control maternal physiology to supply adequate oxygen and

nutrients to support fetus growth and development. Syncytiotrophoblasts are the cells that produce placental hormones in a tightly regulated manner. Several hormones regulate fetoplacental growth, including glucocorticoid, leptin, gonadotrophin, insulin, insulin growth factor, prostaglandin, and others.

Bisphenols can cross the placenta from maternal blood into the fetal compartment and are detectable in the amniotic fluid and cord blood, indicating exposure to developing fetuses (12). BPA exposure during a critical developmental window can affect trophoblast proliferation, migration, invasion, fusion, apoptosis, and placental morphology. *In-vitro* studies have shown that placental JEG-3 cells treated with low doses of BPA altered steroidogenic genes CYP11A1 and CYP19 (13). In addition, trophoblast cells (BeWo) increased β-hCG secretion and trophoblast cell fusion upon treatment with BPA (14). The exposure to low doses of BPA in pregnant mice from day 1-7 caused degenerative changes in the placenta's trophoblastic giant cells and spongiotrophoblast layers.

Moreover, the placenta's intervillous spaces were narrower in BPA-exposed mice (15). The pregnant CD-1 mice administered with BPA from gD 1 to 11 affected placental angiogenesis and caused necrosis and degeneration of giant cells in early pregnancy (16). In mice, exposure to BPA during preimplantation affects nutrient transport in embryo, preimplantation embryo development, and uterine receptivity (17).

Additionally, the progeny of mothers exposed to BPA was growth-restricted compared to the controls (18). Female rats orally administered with BPA showed reduced lumen diameter, acetylcholine-relaxation, and expressions of endothelial nitric oxide synthase 3 (NOS3), estrogen receptor α (ERα), and peroxisome proliferator-activated receptor ɣ (PPARɣ) in the uterine arteries (19). The low levels of BPA exposure have decreased the trophoblast invasion by altering the balance between the MMPs/TIMP in BeWo cells (20). Similar findings were reported in the *in-vivo* study conducted on pregnant mice exposed to BPA, which exhibited pre-eclampsia-like features like decreased trophoblast cell invasion, increased retention of smooth muscle cells, and a decreased vessel area at the junctional zone of the placenta (21). BPA exposure to the placenta is also reported to alter its metabolic state by altering the levels of GLUT1 expression in the villous explants (22) and placental HTR8/SVneo cells (23).

Increased expression of glucose transporters in the placenta promoted excess glucose transverse to the fetus, induce fetal hyperglycemia, and cause associated developmental defects. Pre- and post-conceptional exposure to bisphenols decreased the area occupied by a spongiotrophoblast relative to trophoblast giant cells within the junctional zone and markedly reduced placental serotonin (5-HT) concentrations. In addition, they lowered the 5-HT GC immunoreactivity (24). BPS-exposed placentas had lower E-cadherin expression, fewer binucleate cells, and higher glial cell missing-1 protein expression. Interestingly, BPA exposed placentas were unaffected, highlighting the intrinsic differences among bisphenol chemicals (25). This evidence suggests that exposure to BPA during pregnancy may adversely affect placental function and fetal development. Thus, due to EDC exposure, fetal development could be impaired due to altered placental response involving its growth, hormonal balance, gene expression, epigenetic modification, and angiogenic and invasive activities.

Placenta is the critical endocrine organ whose formation and development dictate the fate of a successful pregnancy. Placenta plays a vital role during gestation, including implantation of the fetus to the uterine wall, placentation, hormone secretion, uterine artery remodelling, gaseous exchange, transport of nutrients, removal of waste materials, and parturition. Any disruption or imbalances in hormonal production can adversely affect fetoplacental development. Data show that exposure to endocrine-disrupting chemicals like bisphenols A and their substitutions (BPS, BPF, TBBPA, BADGE) affect early development stages. Early developmental involves implantation, trophoblast invasion, and remodelling are modulated by BPA (16, 17, 26–32), BPS (33, 34), BPF (32, 34), and TBBPA (35) exposure. The trophoblast Syncytialization and nutrient exchange across the placenta are affected by BPA (22, 23), and BPS (36) exposure. Feto-placental growth outcomes such as intrauterine and fetal growth, & weight, ponderal index, femur length, abdominal circumference, head circumference, and ponderal index are altered by several EDC exposure, including BPA (23, 24, 37–41), BPS (24, 25, 39, 42), BPF (39), TBBPA (40, 43, 44), and BADGE (45).

2.1 Effects of maternal glucocorticoids on the fetoplacental growth and development

Endocrine stress elicits metabolic responses through the centrally coordinating neuroendocrine by activating the HPA-axis through hormonal signalling, leading to the systemic release of glucocorticoid (GC) from the adrenal (kidney) in humans (46). GCs act as maturation signal to sense EDC exposure and regulates intrauterine programming of adult physiological phenotype. Excess exposure to endogenous GCs activates maternal HPA by augmenting nutritional and stress response during pregnancy and increases fetal GC concentration. Fetal GC can be elevated independently of the mother to compensate reduced supply of nutrients and oxygen or by the changes in the placental activity of the 11HSD enzymes. The placenta produces an enzyme called 11-beta hydroxysteroid dehydrogenase type 2 (11β-HSD2), which inactivates cortisol in the placenta and prevents it from reaching the fetus.

Maternal GC affects the growth and development of the placenta by modulating surface areas for nutrient exchange, formation of fetal blood vessels, and expression of nutrient transporters across the placenta. Glucocorticoid receptor (GR) is ubiquitous in the cellular system and highly expressed in the placenta. Maternal GC has multiple ways of controlling fetoplacental growth, including its effects on the placental vascularization (47), expression of nutrient transporters in the placenta (48), cardio-metabolic maturation of the newborn heart (49), birth weight of the offspring (50), change in body fat deposition and body composition of the primate offspring (51), and others. Excess GC exposure during early fetal life in humans results in glucose intolerance later in life due to the reduced ability to secrete insulin at adult age (52). Placental 11β-HSD2 is a key GC

metabolizing enzyme that controls fetal GC levels and mineralocorticoid receptors specificity in the kidney. In the kidney, the enzyme acts as a receptor-modulator of metabolically active GCs, such as cortisol (human) and cortisone (animal), to convert to their derivatives. In the physiological state, maternal GC levels are relatively higher than fetus but protected from excess GC exposure by the placental 11β-HSD2 enzymes. Inhibition or mutation of 11β-HSD2 is reported to lead to low birth weight (LBW) (53, 54). During human pregnancy, cortisol levels are elevated in the maternal circulation (55). However, the placental HSD11β protects the fetus from exposure to high levels of maternal GCs (56). The reduced placental activity and expression of $HSD11\beta_2$ correlates with lower birth weight (57, 58), and dysregulated placental $HSD11\beta_2$ expression is reported in intrauterine growth restriction (57, 59). Therefore, optimal placental HSD11β balance is critical for the organ growth of the fetus and its maturation.

2.2 Placental leptin and its roles in fetal growth

Leptin is a fat cell-produced hormone that regulates satiety, signalling, and energy balance (60). Leptin (16KDa) is the novel placenta-derived hormone in humans (61); it plays an endocrine role in controlling maternal satiety, energy metabolism, and fat metabolism and supports trophoblast proliferation and survival (62). Leptin produced by the placenta and adipose tissue has a similar size, charge, and reactivity, but their upstream regulations differ. The human placenta expressed a comparatively higher leptin mRNA and protein than rodents (63). Both placental and maternal circulatory leptin increases with gestation, indicating leptin's role in maintaining pregnancy.

Expression of the leptin gene in placental vascular endothelial cells in contact with fetal blood first highlighted its potential role in placental angiogenesis. Leptin is co-localized with syncytiotrophoblasts on the maternal side in humans. In contrast, it is co-localized at both the maternal side and labyrinth of the cytotrophoblast of the developing fetus in rodents. The leptin receptor gene (OB-R) in mouse placenta has different variants, including signalling receptor (OB-Rb), transporter receptor (OB-Ra), and soluble receptor (OB-Re). The placental leptin content is regulated by changes in maternal hormones like progesterone and cortisol, which can affect leptin production in maternal adipose tissue in humans (64). A positive correlation exists among circulatory leptin, hCG, and estradiol concentration in pregnant women (65). Leptin and hCG act as a potential regulators of angiogenesis by controlling VEGF secretion at the maternal-fetal interface (66). The leptin receptor was upregulated by hypoxia in placental cells (67). Placental leptin is a biomarker of placental hypoxia in severe preeclampsia (68). Maternal fat deposition increases during pregnancy, but the mother's BMI does not correlate with the gestational increase in circulatory leptin, indicating the contribution of leptin from the placenta, not from maternal adipose tissue (69). The leptin binding to soluble leptin receptors secreted by the placenta increased circulatory leptin and prevented leptin from binding to signalling receptors, leading to leptin resistance (70). The soluble receptor of leptin concentration was decreased rapidly in the later gestation, around 20-30 weeks (71), suggesting leptin's role in the catabolic phase of pregnancy in regulating maternal appetite and fat metabolism. It was proposed that leptin could promote placental development by stimulating angiogenesis (72). Although leptin's role in placental angiogenesis is unclear, *in vitro*, data showed that leptin stimulated tube formation independent of VEGF and upregulated expression of genes associated with angiogenesis in the placental cells (73). In addition to its angiogenic effects, placental leptin showed anti-inflammatory roles by producing excess in GDM and PE to counter the effects of proinflammatory cytokines (74). Leptin is essential in fetoplacental growth and development as cord blood leptin positively correlates with birth weight (75). Maternal hyperglycemia in insulin-dependent diabetes during pregnancy enforces the fetal pancreas to release insulin that increases fetal and placental leptin concentration in paracrine and induces fetal overgrowth and macrosomia (76). Maternal hyperglycaemia was associated with DNA methylation of the leptin gene, which could risk childhood obesity (77). Placental leptin gene expression was reduced due to BPA exposure (38). Leptin level was increased in BPA-exposed overweight mice in the non-fasting state, indicating the involvement of leptin resistance in these animals (78). BPS exposure potentiated the obesity induced by a high-fat diet and was linked with a higher fat mass, food intake, and hyperleptinemia (79).

Overall, the placenta regulates its growth by its leptin in an autocrine manner since cord blood leptin concentration correlates with placental size. Again, placental leptin executes paracrine regulation of maternal energy balance by increasing its concentration later in the pregnancy, thus sustaining fetal growth and development.

2.3 Maternal chorionic gonadotrophin and placental trophoblast functions

Chorionic gonadotrophin (hCG) is produced by the placenta. During early pregnancy, trophoblasts secrete hCG, as the first placental hormone (80). hCG promotes the production of ovarian progesterone and estrogen from the corpus luteum. Later, the placenta takes over the progesterone production as steroid biosynthesis occurs throughout the gestation (81). hCG is a glycoprotein with α and β subunits linked by a non-covalent bond. The α-subunit is encoded in chromosome 6 and is similar to other glycoproteins such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyroid-stimulating hormone (TSH). The β-subunit is encoded by chromosome 19 and is different for each hormone. hCG exists in different isoforms, such as classical hCG, hyperglycosylated hCG, and the free β unit of the hyperglycosylated hCG (82). The hCG levels peak during the first trimester, later decrease, and remain at basal levels throughout pregnancy (83). The biological functions of hCG include angiogenesis, vasculogenesis (84), and differentiation of cytotrophoblasts into syncytiotrophoblasts (85, 86). A

retrospective cohort found that in the first trimester, low free β-hCG levels significantly increased the risk for preterm birth, LBW, intrauterine growth restriction (IUGR), and low APGAR score (87). Immunostaining of placental tissues reveals α-hCG staining rates decreased significantly in IUGR cases over the control (88).

In contrast, the high free β-hCG group had a significantly decreased risk of GDM and preterm birth. In the second trimester, low and high β-hCG had substantially higher risks of the most common adverse outcomes, i.e., spontaneous abortion, IUGR, and preterm birth (87). Moreover, free β-hCG was associated with an increased risk of spontaneous fetal loss (89). Serum β-hCG levels of the missed abortion were lower than those of the control group. Decreased hCG production in early pregnancy down-regulates the expression of the VEGF-MEK/ERK signal pathway, ultimately reducing the angiogenesis (90).

Moreover, studies also show a relationship between gestational trophoblastic disease and hCG. Thus, maternal hCGs involved in several gestational events during pregnancy while their abnormal levels are associated with adverse pregnancy outcomes. Higher exposure to BPA and phthalates was associated with a lower hCG in pregnancy, suggesting altered production or secretion of hCG by the placenta (91).

2.4 Insulin, placentation, and nutrient transport

The peptide hormones such as insulin, IGF1, and IGF2 perform mitogenic and metabolic effects by specifically binding to their surface receptors in target tissues. In normal physiology, insulin and IGFs bind to their receptor, such as IR or IGF1R, in a rapidly growing embryonic tissue (92), thereby regulating placental growth and transport, trophoblast invasion, and placental angiogenesis. Insulin (1ng/ml) stimulated angiogenesis by increased KDR expression in the extravillous HTR8/SVneo cells (93), which is required for vessel formation to promote angiogenesis in the placenta. Maternal diabetes could result in hypervascularization and excess angiogenesis in the placenta due to dysregulated insulin, growth factors, and excess pro-angiogenic effects of insulin in the endothelial cells (94). Placental IGF1 and IGF2 expression are regulated by compartment and gestational stages. For example, IGF2 was not detected at term but was highly expressed in the first trimester villous and extravillous cytotrophoblasts, indicating its significant roles in the early embryonic development involving the placentation (95–97). During the early trimester, these IGFs regulate the uterine-trophoblast invasion (98), migration (99), and placental LPL activity (100). Moreover, IGFs regulates FABP4 expression (101), transplacental glucose and amino acid transporters (102) via activation of mammalian target of rapamycin (mTOR) signalling (103), and increased basal membrane GLUT1 content and trans epithelial glucose transport (104). Several factors, including mTOR signalling, regulate the nutrient transport mechanism. In trophoblast cells, mTOR senses the nutrient signal for maternal supply and fetal demand. mTOR senses various maternal endocrine signals (such as insulin, leptin, and others) to regulate fetal growth by activating gene transcription and protein expression, resulting in modulated expression of nutrient transporters. The maternal hormones such as insulin, leptin, and IGF1 stimulate, while hypoxia and adiponectin downregulate the mTOR signalling (105). Protein restriction during pregnancy affects insulin signalling and glucose metabolism in the muscle tissue of the offspring by involving mTOR (106). Pregnancy complications associated with obesity and endocrine stress due to EDC exposure can affect the mTOR pathway, leading to altered placental nutrient transporter efficiencies and influencing the fetus and development.

2.5 Prostaglandin and placental trophoblast development

Prostaglandins (PGs) are lipid-derived hormone-like molecules that modulate several fetoplacental growth and development functions. Unlike hormones produced by endocrine glands released into the bloodstream, prostaglandins are produced by specific tissues at the site of action. Prostaglandins are produced from arachidonic acid, 20:4n-6 (ARA), by phospholipase A2, followed by cyclooxygenase (COX). A higher ARA level in the early placenta supports prostaglandin's roles in early development, i.e., decidualization, vascularization, angiogenesis, organogenesis, and others (107–110). Moreover, ARA does not accumulate in the placenta near term since its derivative can trigger parturition. In contrast, the precursor of the vasorelaxant and anticoagulant PGE1 levels are increased at period, facilitating blood flow to support fetal growth spurt. Prostaglandin E2 (PGE2) is abundantly produced by the decidua that helps the migration of first-trimester extravillous trophoblast by increasing the intracellular calcium concentration (111). Upregulating PGE2 production and PGE2 receptor expression associated with increased levels of leukemia inhibitory factor favors EVT invasion during the placentation (112). Early stages of spiral artery remodeling mimic angiogenesis, are facilitated by several cytokine-producing cell types and therefore remain critical for early placentation that involves EVT (113, 114). PGE2 is involved in angiogenesis (115) by acting on various cell types that produce proangiogenic factors, such as VEGF, CXCL1, and bFGF, which act on target endothelial cells to promote the angiogenesis (116). Multiple signaling mediators by the various PGE2 receptors facilitate the PGE2-induced angiogenic response. PGE2 stimulated *in vitro* angiogenesis comparable with VEGF in trophoblast cells. However, PGE2-induced angiogenesis was inhibited by COX2 inhibitors. In contrast, VEGF-induced tube formation was the least affected (110), indicating that PGE2 may have an independent effect on angiogenesis in the placenta. Another PG, like PGF2α levels, increases during the window of implantation in the uterine lumen of humans and other mammals, suggesting its role in embryo implantation. PGF2α reported stimulating invasion and migration of the human trophoblast HTR8/SVneo cells (117). PGF2α increased decidual gelatinolytic activity in fetal membranes since its inhibition helps arrest the labor (118). Prostaglandins and their derivatives perform multiple roles associated with fetoplacental growth and development, including embryonic implantation, vascularization, angiogenesis, trophoblast invasion, migration, and labor.

3 Roles of placental steroid hormones in pregnancy

Placenta acts as the primary organ for steroid hormone synthesis during gestation. Placenta contains all the necessary enzymes for steroid biosynthesis. The cytochromes P450s (CYPs) and the hydroxysteroid dehydrogenases (HSD) are the enzymes in the placenta where hormonal biosynthesis takes place from the maternal cholesterol (119). The placenta converts cholesterol into pregnanolone (PREG) and finally to progesterone by a series of enzymatic reactions. In addition to the placenta, fetal tissues can convert PREG to dehydroepiandrosterone sulfate (DHEAS) and finally to 16α-OH-DHEAS. Hydroxylated DHEAS and DHEAS again enter the placenta and eventually transform into androstenedione and testosterone. In the placenta, the aromatase enzyme (CYP19A1) converts androstenedione and testosterone into estrogen [estrone (E1), estradiol (E2), or estriol (E3) (119). Progesterone predominantly mediates its actions by binding to the nuclear receptor (PR-A and PR-B) and membrane-bound receptor causing non-genomic actions.

High progesterone levels are correlated with the development of glucose abnormalities in pregnancy. Recent studies have shown that progesterone receptors knocked out in female mice (PR-/-) but not male had lower fasting glycemia than PR+/+ mice and showed higher insulin levels on glucose injection. Moreover, pancreatic islet cells were larger and secreted more insulin due to increased β-cell proliferation (120). Placental estriol, estradiol, and progesterone levels were lower in preeclampsia's placental tissues than in healthy pregnant women (121). In preeclampsia, a severe hypertensive disorder of pregnancy, the sirtuin1 expression was lower in serum and placenta. Progesterone alleviates preeclampsia-like symptoms mediated by SIRT1 deficiency (122). Progesterone prepares the uterus for pregnancy and prevents preterm births by inhibiting the contraction of uterine muscles. Vaginal progesterone was associated with a significant reduction in the risk of preterm birth (<33 weeks of gestation), spontaneous preterm birth, and admission to the neonatal intensive care unit in pregnancies with short cervix (123). Vaginal progesterone administration significantly decreased the risk of preterm birth, neonatal death, composite neonatal morbidity, and mortality in women with twin gestation and short cervix (124).

As mentioned, the enzyme aromatase converts androgen precursors from fetal and maternal adrenal glands into estrogen in the placenta. The classical estrogen receptors (ER-α & β) and the plasma-bound G protein-coupled ER (GPR30/GPER) are expressed in the uterine artery endothelial cells (UAECs) and smooth muscle of the uterus, mediating their actions through genomic or non-genomic pathways. Activation of ERs by estrogen enhances UAEC's nitric oxide production, thereby causing uterine vasodilation during pregnancy (125). Knockout of ER-α in the female mice decreased litter size and maternal nurturing behavior (126). Estrogen receptor-β is involved in implantation and parturition in the mature uterus (127). Insulin resistance associated with hyperestrogenemia occurs in GDM and PCOS. Estradiol can bind to insulin and may interfere with binding to its receptor, causing insulin resistance (128). Recent studies have shown a possible link between estradiol and glucose homeostasis. Cord blood estradiol in the GDM was significantly lower than in the control group.

Moreover, the cord blood estradiol concentrations were negatively correlated with birth weight (129). Estrogens are well known to modulate angiogenesis in both physiological and pathological conditions. Exogenous estrogen stimulates cell proliferation and angiogenesis in the pregnant ewe UAECs (130). Female ovariectomized ER knockout mice showed impaired angiogenesis, suggesting the role of ER in the angiogenesis (131). As estrogens promote angiogenesis and vasodilation, preeclampsia may be associated. Plasma 17β-estradiol levels were significantly reduced in severe PE subjects (132). Similar outcomes were found in patients with mild to severe PE (133). Placental tissue levels of estriol and estradiol were significantly lower in PE compared to healthy pregnant women (121). Overall data indicate the role of estrogen in the pathophysiology of preeclampsia, which could be caused due to aromatase deficiency in the placenta.

4 Endocrine modulating factors during pregnancy and gestational disease risks

Several studies mentioned in this section have reported a relationship between placental hormones and the development of IUGR. Low hCG concentrations during the late first trimester were associated with decreased fetal growth, birth weight, and small for gestational age (SGA) (134). A similar study was conducted on 9450 singleton pregnant women. SGA was associated with low maternal serum levels of pregnancy-associated plasma protein-A (PAPP-A), free β-hCG, and slow early fetal growth (135). β-hCG levels were significantly decreased in SGA cases compared to the control (136). Sex hormones also regulate IUGR. 17-β Estradiol (E2) is critical to a physiological pregnancy and synthesized by the conversion of androgens by the CYP19A1 gene. Maternal E2 level in plasma was significantly decreased in the IUGR. Although the placental expression of CYP19A1 was considerably higher than in control (137), serum levels of estriol (E3) were significantly lower in the IUGR (138). Moreover, the estrogen-related receptor gamma expression was reduced in the FGR placenta compared to the control (139).

Gestational diabetes mellitus is characterized by spontaneous hyperglycemia during pregnancy. The Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study found that maternal hyperglycemia independently increased the risk of preterm delivery, cesarean delivery, infants born large for gestational age, and neonatal hypoglycemia (140). Although GDM is a pregnancy disorder, it resolves following delivery but can have long-term health consequences on maternal and fetal life. Risk factors include obesity, diabetes, a sedentary lifestyle, genetics, and advanced maternal age. The primary pathophysiological condition in GDM is β-cell dysfunction. It is caused by an inability of β-cells to sense blood glucose concentration and release insulin adequately. Due to this condition, high blood glucose prevails, leading to hyperglycemia. Glucose is the primary energy source for both

placenta and fetus development. As glucose is always required, the placenta expresses insulin-independent glucose transporter GLUT1. GLUT1 plays a role in implantation and angiogenesis. The expression of GLUT1 on the basal plasma membrane of the placenta is positively correlated with birth weight (141). However, the expression and function of GLUT1 are down-regulated in pre-eclampsia and IUGR (142).

Moreover, insulin-stimulated glucose uptake of the first-trimester trophoblast cells, HTR8/SVneo, is partially mediated via GLUT1 (93). Trophoblasts and endothelial cells of the placenta also express insulin receptors, which can be activated by insulin and alter the placental metabolism of nutrients (143). Higher hCG levels in early pregnancy are associated with a lower risk of GDM (144). In contrast, elevated hCG levels were especially predominant among women who developed GDM (145). The relationship between hCG and GDM was inconsistent and varied with the duration of pregnancy. Estradiol is an essential mediator of glucose homeostasis. *In-vivo* studies have shown that aromatase knockout mice (ArKO-/-) develop glucose intolerance and insulin resistance. Treatment of ArKO males with 17β-estradiol improved the glucose response (146). Cord blood estradiol was significantly lower in GDM than in control. Moreover, cord blood estradiol was negatively correlated with birth weight. Estrogen protects pancreatic β-cells from apoptosis and prevents insulin deficiency (147). A recent study has also shown a relationship between progesterone and GDM. Compared to controls, GDMs have significantly lower progesterone at weeks 10-14 (148).

Hyperglycemia in mothers can lead to macrosomia due to the excess glucose available to the fetus via the placental glucose transporter GLUT1. In addition to β-cell dysfunction, insulin resistance and several maternal hormones produced by adipose, liver, and placenta can develop GDM. Gestational hCG levels are associated with predictive risk with the development of GDM but inconsistently.

5 Endocrine-disrupting chemicals induce endocrine dysfunction and fetal programming of development and reproductive disease

Endocrine-disrupting chemicals interfere with the typical endocrine system and produce adverse effects. EDC was thought to exert its action primarily through nuclear hormone receptors such as ER, androgen receptors (AR), thyroid receptors (TR), and retinoid receptors. However, recent data suggest that EDC can also act on non-nuclear and orphan receptors. EDC can be synthetic or natural chemical compounds. Synthetic chemicals are used as plasticizers [bisphenols, phthalates], pharmaceutical agents [diethylstilbestrol (DES)], industrial solvents [polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins], agrochemicals [methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)], fungicides (vinclozolin)]. Natural chemicals include phytoestrogens such as genistein and coumesterol.

Humans are exposed to EDC by various routes such as ingestion, inhalation, and transdermal. EDCs have been detected in maternal blood, amniotic fluid, and cord blood (149). The EDC's influence on maternal response may program several adult diseases, particularly during gestation. Figure 1 describes the life course exposure risk of EDCs on the diverse functional effects in modulating fetoplacental development that are mediated by maternal effects spanning from hormonal imbalance, epigenetic modification, dysregulated metabolic responses and risks of gonadal and reproductive functional deformities in the offspring.

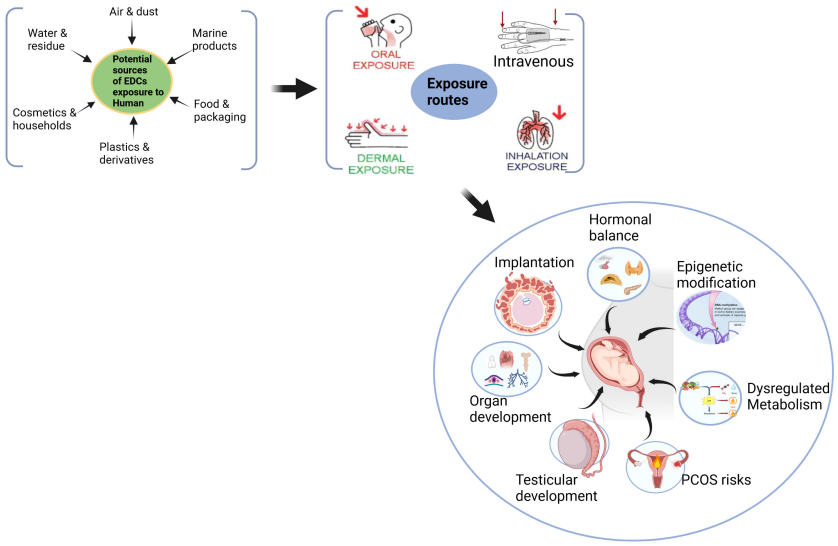

FIGURE 1
Maternal exposure risk of EDCs in modulating fetoplacental development that they might risk programming adult metabolic and reproductive diseases.

Adults can perform rapid first-pass metabolism to convert these into biologically inactive metabolites and rapidly clear them in the urine. Still, the unborn developing fetus and placenta do not have the enzymatic machinery to catabolize these compounds. As these chemicals cross the placenta, EDC threatens the unborn fetus. Placental trophoblast invasion, proliferation, and its several functions are affected due to exposure to bisphenol A (BPA) and their substitution chemicals (Figure 2). Research has shown that EDC exposure during the critical developmental window can have long-term health impacts on the fetus and its later adult life (2). Moreover, successful pregnancy depends on the physiological secretion of several hormones. Any changes in the hormonal milieu during development can cause pregnancy-related complications and diseases.

Humans are constantly exposed to synthetic EDCs such as bisphenols and phthalates. The plastic-derived chemicals such as BPA and its substitute bisphenol S (BPS) are the major endocrine disruptors exposed ubiquitously since it mimics hormones and modulates the endocrine system, affecting the metabolic outcome. The food contaminated with packaging and chemical agents mixed with bisphenols is continuously exposed in micron levels through leaching, especially plastic-derived residual contaminants (153). Semisynthetic and plasticizer-laden food surfaces (154) are exposed to heat and change in pH, resulting in chemical hydrolysis of polyester bonds, releasing chemicals directly exposed to living systems (155). BPs are absorbed through the skin, and first-pass metabolized to their conjugates, resulting in a greater endocrine risk to human disease burden (156).

BPA is the most abundant in consumer products, including food and beverage containers, medical devices, toys, and thermal receipts. BPs are detected in human blood irrespective of gender. However, the findings on gender-specific exposure to BPA are inconsistent, as studies have shown that BPA concentrations were higher in men than women (157, 158). At the same time, others found equal concentrations of BPA in both males and females. The discrepancies in the data could be multifactorial, including but not limited to age, sample size, geographical location, and technique. The concentrations of BPA detected in the cord blood are closely related to levels in maternal blood, indicating that BPA readily crosses the placenta. Epidemiology and clinical evidence of EDCs exposure suggest a profound association between the exposure of BPA and its analogs during pregnancy and altered fetal and neonatal growth outcomes, including intrauterine growth, gestational length, birth weight, ponderal index, femur length, abdominal circumference, head circumference and others (Table 1).

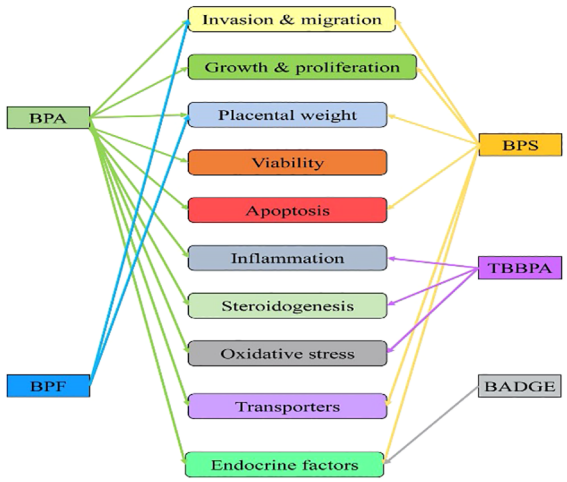

FIGURE 2
Influence of endocrine-disrupting chemicals (bisphenols) exposure on trophoblast invasion, development, and placental functions. Single-headed arrow indicates an independent study reported so far available from the PubMed database. BPA, Bisphenol A (23, 24, 26–28, 38); BPS, Bisphenol S (25, 33, 36, 42, 150, 151); BPF, Bisphenol F (34, 39); TBBPA, Tetrabromo Bisphenol A (35, 40, 43, 152, (44); BADGE, Bisphenol A diglycidyl ether (45).

Trans maternal exposure to BPA in non-obese diabetic mice increased the severity of insulitis and the incidence of diabetes in female offspring (172). Perinatal exposure to BPA alters the expression of genes involved in β-cell growth regulation, incrementing β-cell mass/area and β-cell proliferation during early life. Excess insulin production in early life may impair glucose metabolism in later life (173). Additionally, the metabolic changes caused by *in-utero* BPA exposure were found to be inheritable. i.e., maternal exposure to a low dose of BPA impaired insulin secretion induced islet inflammation and increased β-cell death in the offspring. Surprisingly, subsequent generations (174) and third-generation (175) inherited these changes, indicating that BPA-induced epigenetic modifications were inherited.

Although excess GC exposure can modulate fetus growth and development, little is known about how the GC system is vulnerable to BPs exposure, particularly during pregnancy. Data suggest that BPs significantly inhibited HSD11β2 activity in humans and rats, possibly by steroid binding site, as evidenced by docking analysis (176). It is well established that BPA exposure modulates cellular function by their estrogen agonist or androgen antagonist function. Still, their preference for binding GR or ER in the placenta is unknown. BPA may mediate its effects via a non-classical ER pathway without GC, where ER antagonists inhibit BPA-induced adipogenesis but not GR antagonists (177). BPA at lower concentrations increased the expression of both protein and mRNA of HSD11β_2 in placental trophoblast cells (38). Our recent data showed that prenatal BPs exposure lowered HSD11β2 expression in the rat testis (178), indicating reduced protection against cortisol-mediated stress induced by bisphenols in these offspring.

BPA possesses pseudo-estrogenic and anti-androgenic effects that might interfere with the developmental programming of the male reproductive system in the offspring. Prenatal exposure to BPA and BPS at much lower concentrations than TDI, during gestational days 4 to 21, alters testis weights, histology, increased testicular inflammation, and oxidative stress in the adult offspring. Moreover, sperm DNA damage and DNA methylation were also increased in both BPA and BPS-exposed adult offspring (178). Exposure to BPA and its analogs bisphenol B (BPB), bisphenol F (BPF), and bisphenol S (BPS) during gestation resulted in decreased testosterone secretion, sperm production, and histological changes in the reproductive tissues of adult SD male rats (179). BPA exposure during pregnancy and lactation can also affect

TABLE 1 Epidemiology and clinical evidence of bisphenol exposure on fetal and neonatal growth outcome.

EDCs	Subjects	Sample types	Assays	Key findings	Ref.
BPA and BPA-glucuronide	Mother-infant pairs (n=60)	Maternal serum and cord blood	Bisphenol and steroid hormone	-Positive relationship between cord blood estradiol, BPA and maternal BPA levels. -Cord blood testosterone from male infants showed a negative correlation with maternal BPA	(159)
BPA	Overweight (n=26) and age-match normal weight pregnant women (n=32)	Placenta	GLUT1 and GLUT4 in placental explants exposed to BPA (1 nM and 1 μM)	-↑ GLUT1 expression in normal weight -↓ GLUT1 expression in overweight -GLUT4 expression lower in the overweight explants	(22)
BPA	Mother- child pairs in third trimester (n=788) and neonate (n=366)	Urine	BPA exposure & fetal and neonatal growth outcome	-Negative correlation between BPA exposure and intrauterine linear growth -Positive association with volume growth during childhood	(160)
BPA and phthalates	Mother-child pairs (n=488)	Urine	Prenatal BPA and phthalate exposure, fetal growth, and birth outcomes at 12, 20, and 34 weeks of pregnancy	-Prenatal phthalate metabolite (MBzP) exposure positively associated with femur length at 20-34 weeks and birth weight among boys. -Mono-n-butyl phthalate (MnBP) negatively associated with head circumference at 12-20 weeks.	(161)
BPA	Pregnant women (n=219)	Urine	BPA exposure, fetal growth, and birth outcomes	-Women with creatinine-normalized BPA>4.22 μg/g had lower growth rates for fetal weight and head circumference than women with lower creatinine-normalized BPA	(162)
BPA	Mother-new born pairs (n=97)	Maternal and umbilical cord blood	BPA exposure and birth outcomes	-Elevated risks of low birth weight, short gestation, and altered secretion of leptin and adiponectin observed in male newborns	(163)
BPA, BPS, and BPF	Pregnant woman before delivery (n=1197)	Urine	Urine bisphenol and fetal growth parameters	-Maternal urinary BPA and BPF negatively related to birth length and positively associated with ponderal index. -BPS exposure associated with short gestational age only in girls.	(164)
BPA, BPS, and BPF	Pregnant women third trimester (n=322)	Urine	Urine bisphenol and fetal growth parameters	-Gender difference in the association of maternal urinary BPA concentrations and fetal head circumference. -Maternal urinary BPF showed an inverse and positive associations with abdominal circumference and femur length respectively	(165)
BPB, BPF, BPS, TBBPA (tetrabromobisphenol A)	Mother-infant pairs (n=2023)	Serum	Serum bisphenol and birth size	-Serum BPA and TBBPA negatively correlated with birth weight -Higher BPF was associated with decreasing birth weight and ponderal index.	(166)
BPA, BPS and BPF	Pregnant women (n=845)	Urine	Urine bisphenol and size at birth	-Urinary BPF and BPS positively associated with lower birth weight, birth length, or ponderal index	(167)
BPA, BPS and BPF	Pregnant women (n=1379)	Urine	Urine bisphenol and birth outcomes	-BPS exposure associated with larger fetal head circumference, higher weight and lower risk for small size for gestation age	(168)
BPA, BPS and BPF	Twin-pregnant women (n=289)	Urine	Urine bisphenol and birth outcomes differences in twins	-Urinary BPA positively associated with the within-pair birth weight and birth length differences across pregnancy trimesters	(169)
BPA	Pregnant women (n=120)	Urine and serum	Urinary bisphenol, serum β-hCG and anthropometry	-No significant association existed between BPA and β-hCG with birth outcomes	(170)
BPS	Pregnant women (n=985)	Urine	Urinary BPS and birth outcomes	-Higher maternal urinary BPS associated with increased gestational age	(171)

"↑" and "↓" indicate up and down-regulated respectively.

epididymal sperm count and sperm motility (180). CD-1 mice exposed to bisphenols, dysregulated serum estradiol-17β, testosterone, expression of steroidogenic enzymes, increased mRNA levels of DNA methyltransferases, and histone methyltransferases in F3 adult testis (181). Similar results were found with BPA and its analogs on female reproductive functions in mice. Early life exposure in mammals affects early oogenesis and follicle formation in the fetal ovary (182, 183). Bisphenol exposure in the F0 generation accelerated the onset of puberty and exhibited abnormal estrous cyclicity in F3 females (184). Gestation and lactation-specific BPA exposure decreased sex hormones and reduced the number of tertiary ovarian follicles in the female offspring (185). BPA exposure affected placentation and exhibited preeclampsia-like phenotype in pregnant mice (21). Exposure to BPA was also associated with a risk of preterm birth (186).

Growing evidence suggests that BPA and its substitute BPS have distinct action modes in different tissues. e.g., *in-utero* exposure to BPA altered endogenous long-chain fatty acid metabolism in the testis of adult offspring, thereby affecting sperm maturation and quality. However, no changes were observed in the testis of BPS-exposed offspring (187). The bisphenols interfere developmental programming of the reproductive system in the offspring (178) since fetuses do not equip with enzymatic machinery to catabolize these compounds (2). Thus, *in-utero* exposure to BPA during development can program the fetus for endocrine-related diseases in later life.

6 Endocrine-disrupting chemicals and endocrine risk of pre-pregnancy

Female infertility is a rapidly evolving endocrine disorder in pre-menopausal women, with an estimated prevalence of 5%-18% globally (188). Often it remained underdiagnosed and became one of the leading causes of female infertility. The risk of failed pregnancy can be associated with metabolic, reproductive, and endocrine features such as anovulation, hyperandrogenism, arrested folliculogenesis, infertility, polycystic ovaries, type 2 diabetes, and obesity (189). Dysregulated hypothalamic-pituitary-ovary (HPO) axis, followed by hyperandrogenism, increased serum luteinizing hormone, and metabolic disturbance, contribute to the complex etiology of PCOS (190). Despite studies, the pathophysiological cycle of hyperandrogenism, hyperinsulinemia, and anovulation in PCOS remains unclear. Studies pointed out that exposure to common household plastics containing chemicals could associate with risks of PCOs in humans.

BPA levels were significantly elevated in women with PCOs (191). Similarly, adolescent girls with PCOS had markedly increased serum BPA than controls, and BPA was correlated considerably with androgen (192). Thus, BPA may play a potential role in the etiology of PCOS, specifically a significant contributor to ovarian hyperandrogenism observed in women with PCOS. In addition to hormonal changes, BPA can exert its effects through epigenetic modifications. BPA can change DNA methylation or alter histone modifications without altering the nucleotide sequence of the genes. BPA altered stress-related promoter DNA methylation in placental cells (27). *In-utero* BPA exposure decreases the CpG islands in the promoter and intronic region of the HOXA10 gene, which is involved in uterine organogenesis (193). Prenatal or neonatal exposure to BPA causes an increase in levels of androgen-hyperandrogenism, one of the hallmarks of PCOS. Hyperandrogenism promotes epigenetic modifications of histone deacetylase 3 (HDAC3), peroxisome proliferator-activated receptor gamma 1 (PPARG1), and nuclear corepressor 1 (NCOR1) genes in granulosa cells of PCOS women (194). BPA exposure could dysregulate endocrine balance by changing the expression of several genes, such as CYP11A1, GnRH, AdipoQ ESR1, and StAR, whose disruption is commonly observed in the pathogenesis of PCOS (195).

The endocrine disruptor BPA can mimic estrogen to interact with its receptor. BPA exposure modulated steroidogenic enzymes' expression in the ovary, lowering aromatase expression and estrogen production in granulosa cells. BPA is reported to stimulate androgen synthesis in ovarian theca-interstitial cells. Moreover, BPA concentration was significantly higher in PCOS patients' follicular fluids. BPS is structurally similar to BPA, where sulphonyl groups substitute phenolic groups. *In utero*, BPS exposure also disrupted female reproductive development by changing the estrogen-responsive gene expression in the uterus and ovary (196). Whether exposure to bisphenols is causally related to developing PCOs risk is not established yet. However, BPA could be a causal factor for PCOS because it mimics the hormone estrogen's function (197). BPA (500 μg/kg/day) exposure causes irreversible alteration in the hypothalamic-pituitary-gonadal axis in rats, leading to anovulation and infertility (198).

Subcutaneously injected with BPA from postnatal days 1 to 10 resulted in the development of the PCOS morphology after 4 to 5 months. Serum testosterone and estradiol were elevated, with an irregular GnRH pulsatility (199). In a similar study, SD female offspring perinatally exposed to 1.2 mg/kg bw/day BPA from gD 6 till lactation exhibited increased body weight, altered patterns of estrous cyclicity, and decreased plasma LH levels (200). Prenatal exposure to BPA in CF-1 mice significantly reduced the days between the vaginal opening and the first oestrus (201). Similarly, ICR mice prenatally exposed to BPA induce early vaginal opening (202). Neonatal SD rats were subcutaneously exposed to BPA, which caused multiple cystic follicles forming in the ovary (203). Similarly, an increase in cystic ovaries and pathologies of the oviduct and uterus were observed in mice (204). BPA-exposed ovaries showed a decreased antral & corpus luteum and increased atretic & cystic follicles (205). Maternal BPA exposure increases antral follicles' size, elevates the incidence of ovarian cysts, and changes ovarian follicular dynamics (206).

BPA exposure affects steroidogenesis in the ovary by reduced E2 production and decreased aromatase (CYP19A1) expression in human granulosa cells (207). Prenatal exposure to BPA caused alterations in fetal ovarian steroidogenesis by increasing Cyp19 and 5α-reductase expression and miRNA expression of folliculogenesis in sheep (208). Moreover, it was shown that patients with PCOS have decreased mRNA expression of aromatase (CYP19A1) compared to the non-PCOS group. Hyperandrogenism in PCOS is due to reduced aromatase activity (209). Bisphenol A exposure

can also modulate hormone levels. Lower E2 levels and higher testosterone were observed in all the BPA-treated groups compared to the control in rat ovarian theca-interstitial and granulosa cells (210). On the contrary, LH and E2 significantly increase in the BPA-treated Wistar rats (211).

BPA is reported to modulate the HPO axis and elevate serum LH. Still, their low-level exposure *in utero* to develop a PCOS-like phenotype in the offspring must be reported. Despite the broader presence of direct hormone-regulated animal models in studying human PCOS, the pathogenesis and treatment still need to be determined. Thus, investigating the PCOS-like phenotype in animals without direct hormonal intervention is expected to determine the underlying mechanism for better understanding the pathogenesis of PCOS. Moreover, evidence from animal models may clarify the causal roles of EDC exposure in determining the PCOS risk factors.

7 Maternal phthalate exposure, endocrine effects on fetal growth and reproductive development

In the food processing, pharmaceutical, and medical industries, Phthalates are used as plasticizers in daily use products such as cosmetics, personal care, detergents, toys, household products, solvents, sealants, and paints. Men and women are differentially exposed to cosmetics. A recent study of participants (n=9218) investigated the exposure to EDCs based on occupation and gender differences in personal care product use. Higher urinary concentration of EDCs (BPA and mono-n-butyl phthalate, MnBP) was observed in women compared to men since the latter were exposed to phthalates-containing cosmetics more frequently than men (212). Women are suspected of exposure to various EDCs, including abundant phthalates in cosmetics. Women can carry and transmit this risk exposure to a new life. Numerous forms of phthalate exist in our environment, in which DEHP (Di 2-ethyl hexyl phthalate) is prominently present in food items. Samples originating from meats, fats, and dairy products may contain DHEP at concentrations (≥300 μg/kg) and significantly contribute to exposure in epidemiological studies. The molecular weight of DEHP is 390.56 g/mol. The first step in the metabolism of DEHP is the hydrolysis of DEHP to mono (2-ethylhexyl) phthalate (MEHP) catalyzed by unspecific lipases. Upon oral exposure, phthalates are metabolized quickly into their monoester metabolites. These phthalate metabolites have been detected in urine, maternal blood, amniotic fluid, and cord blood. In mice, MEHP is metabolized into a wide range of secondary metabolites (diacids and keto acids), excreted as glucuronide conjugates in human urine, and unconjugated metabolites in rat urine. A single oral dose clinical study found that about 71% of the DEHP delivery was excreted after 24 h, and 4% was excreted in the following 20h (213). European Food Safety Authority (EFSA) reported that a 50 μg/kg/day dose could lead to testicular toxicity. *In utero*, DEHP exposure diminishes mineralocorticoid receptor expression in adult rat Leydig cells, affecting aldosterone-induced androgen formation and decreasing testosterone production. *In-utero* exposure to DEHP (100, 300, and 750 mg/kg/day) results in a 50% decrease in testosterone and aldosterone without changing corticosterone concentrations in male rats. DEHP was reported to enhance estrogenic activity at concentrations of 1.50 ppm *in vitro* and *in vivo*, suggesting its role in the transactivation of ER.

DEHP exposure decreased steroidogenic acute regulatory protein expression in pregnant mice and lowered fetal testicular 17α-hydroxylase and cytochrome P450 17A1 levels. Early trimester DEHP exposure is inversely associated with the anogenital distance of male newborns but not girls, indicating gender-specific effects on male genital development (214). In addition, the concentration of DEHP increases the chances of limb malformation in both litters and fetuses (215). The defects were specific to hind limbs and represented a spectrum of polydactyly phenotypes. In addition to the known effects of phthalate on fetal testicular functions, unexpected limb malformations and high rates of fetal death following exposure to 250 mg/kg DEHP was noted. However, further studies are needed to identify the mechanism responsible for DEHP-induced limb malformations and to determine the potential relevance of this finding concerning humans.

Due to their lipophilic nature, phthalates can act through nuclear membrane receptors and involves epigenetic modifications by altering the gene expression (216). Several animal studies and epidemiological data have been raised to define the risks of phthalates and their metabolites. Pregnant mice exposed to DEHP-induced fetal IUGR (217) lowered placental weight and reduced blood sinusoid area in the placental labyrinth layer in a stage-specific manner (218). Gestational DEHP exposure alters the expression of steroidogenic enzymes and circulatory steroid hormone levels in pregnant females (219). High doses of DEHP caused IUGR and induced fetal malformations (220). DEHP exposure also damaged DNA in the trophoblast (221) and altered the placental receptor expression (222).

Early exposure to phthalates can lead to metabolic, and reproductive diseases in later life. Gestational and lactation exposure to phthalates causes hyperglycemia, impaired glucose, and insulin tolerances, and altered insulin transduction pathways in adult male offspring (223). Gestational DEHP exposure results in impaired β-cell function by downregulating genes involved in their development and function by the increase in the global DNA methylation (224, 225). Recent data reported that maternal urinary phthalate metabolites are associated with DNA methylation profiling of the first-trimester placenta. The results showed 282 differentially methylated regions (DMRs) corresponding to 245 unique genes in the early human placenta for high compared to low total phthalate exposure. The phthalate metabolites in urine were related to changes in miRNA expression of the human placenta, where miR-142-3p, miR15a-5p, and miR-185 expression were associated between phthalate exposure and protein serine/threonine kinase activity (226). Phthalate exposure altered the first-trimester placental transcriptome and methylome connected with the placental growth factors (227). Maternal exposure to phthalates seems to promote the transgenerational inheritance of adult-onset disease risk factors in subsequent generations. Exposure of DHEP to F0 generation activated the

PI3K/Akt/mTOR signaling in the germ cells of the adult mice testis in F1 and F2 generations (228). Prenatal DEHP exposure during gonads differentiation displayed symptoms similar to the human testicular dysgenesis syndrome in adult male offspring mice, which was causally associated with promoter silencing of genes involved in seminal vesicle secretory protein and antigens (229). Early and postnatal lactational DEHP exposure decreased testis weights and disturbed testis architecture by altering seminiferous tubule diameter and germinal epithelium height in adult offspring (230). Emerging epidemiological and clinical data on phthalates exposure as measured by their metabolites indicated pregnancy risks and skewed growth & development of the newborns. Maternal phthalates exposure was associated with fetal and neonatal growth parameters, including anogenital index, gestational age, birth length, birth weight, head circumference, and others (Table 2).

8 *In utero* phthalates exposure and testicular functions

Several in vivo studies show that phthalate exposure, irrespective of exposure stages can have long-term programming consequences in developing reproductive dysfunctions. Sprague Dawley dams were exposed to dibutyl phthalate (DBP) (2550 mg/kg) by oral gavage from gestational day 12 for 10 days, lowered the serum testosterone levels, caused fetal Leydig cell aggregation and lowered Lhcgr, Star, Insl3, and Nr5a1 levels in the male fetuses (243). Diisobutyl phthalate (DiBP) has structural and application properties similar to DBP and is used as a substitute for DBP. DiBP exposure to pregnant Wistar rats significantly reduced the male offspring's anogenital distance and testicular production. The histology data showed Leydig cell hyperplasia, Sertoli cell vacuolization, and central location of gonocyte (244). In utero exposure to dipentyl phthalate (DPeP) from gD 14 to 21 decreased male offspring's serum testosterone and luteinizing hormone levels and increased the fetal Leydig cells by increasing proliferation (245). Gestational di-(2-ethylhexyl) phthalate (DEHP) exposure to SD rats significantly reduced serum testosterone levels, reduced fetal Leydig cell size, induced abnormal aggregation of these cells, and altered MDA levels in pup's testis (246). Another synthetic phthalate plasticizer diisodecyl phthalate (DIDP), decreased serum testosterone, and induced abnormal aggregation of fetal Leydig cells. However, no effect on Leydig and Sertoli cell numbers was observed in the male pups (247). Diisopentyl phthalate (DiPeP) lowered mRNA levels of key steroidogenic proteins like Star, Cyp11a1, Cyp17a1, Ar, Esr1, Gper, and Insl3 in testis of prenatally exposed rats (248). In-utero exposure to diisononyl phthalate (DiNP) caused a reduction in fetal testosterone and Insl3 levels in male rats (249). Similar outcomes were observed with the exposure to unique phthalates such as bis (2-butoxyethyl) phthalate (BBOP) which contains oxygen atoms in the carbon backbone (250). In-vivo studies were conducted with mixtures of different phthalates to study their combined effect on testicular testosterone production. Co-administration of DBP and DEHP in pregnant SD rats reduced fetal testosterone levels in addition to reproductive malformations such as epididymal agenesis and reduced androgen-dependent organ weights (251). Similar findings were observed when co-administration of five phthalate esters i.e., benzylbutyl phthalate (BBP), di(n)butyl phthalate (DBP), diethylhexyl phthalate (DEHP), diisobutyl phthalate (DiBP) and dipentyl phthalate (DPP) in dams inhibited fetal testosterone production in the SD rats (252). In addition to testosterone reductions, phthalates impair sperm quality and induce inflammation of testicular Sertoli cells (253, 254). All these in vivo data indicate that phthalates lead to testicular dysgenesis syndrome irrespective of the type of the phthalate exposed (Figure 3).

9 Modulation of early development by epigenetic-DNA methylation: effects of endocrine disruptors

Epigenetic control of early development is susceptible to the factors associated with environmental and lifestyle practices since a set of genomic imprinters is programmed to execute their functions for early development. The window of fetoplacental development is plastic, capable of remodeling, and sensitive, during which changes in the maternal milieu by internal or external factors might significantly impact developmental programming. Epigenetic mechanisms involve multiple ways, including DNA methylation, histone modifications and microRNAs, and others. A dysregulated epigenetic-DNA methylation results in a change in fetal outcomes by regulating placental methylome and function (255). Maternal nutrition, stress, physical activity, hormones, and endocrine disruptors can modulate epigenetic control of fetal development during gestation. For example, maternal intake of alpha-linolenic acid during pregnancy and lactation causes changes in DNA methylation of the offspring's liver (256), and maternal omega-3 deficiency increased 5-methylcytosine content in the mice's placenta, indicating placenta's sensitivity to epigenetic changes during development (257). Thus, a successful pregnancy requires controlled epigenetic modifications that lead to genome stability during developmental events, including organ formation from gamete production and fertilization to fetoplacental development and fetus outcome (258).

Unlike peptide hormones, steroid hormones are lipophilic that can cross cell membranes and signal through nuclear receptors, acting on transcription factors to turn on or off gene expression. Estrogen, a steroid hormone, binds to either membrane estrogen receptors (ER) or nuclear ERs. Endocrine disruptors like BPs are also known as synthetic estrogen. Activating a non-genomic pathway by binding estrogen to membrane ER causes activation of the PI3K-AKT signaling and downstream activation of gene transcription. Nuclear ER provides DNA-dependent regulation of gene expression mediated by binding of ER with coactivators or corepressors, which encode enzymes with histone acetyltransferase activity (259). RIZ1 is an ER coactivator, which represses transcription by methylation of lysine 9 of histone 3. Knockout of RIZ1 in female mice exhibit a decreased response to female sex hormones, reduced vaginal epithelial thickening in response to

TABLE 2 Phthalates exposure, pregnancy risks and growth & development of the newborns: epidemiological and clinical data.

Subjects	Sample types	Parameters[#]	Key findings	Ref.
Pregnant women (n=463)	Urine and blood	Phthalate metabolites (12) and maternal hormones	-MnBP and ∑DEHP positively associated with TSH, while MEP and MnBP inversely associated with free thyroxine and total triiodothyronine	(231)
Mother-infant pairs (n=65)	Maternal blood and cord blood	Phthalate metabolites (10) and birth outcomes	-MBzP, MMP, MiBP, and ∑DEHP in maternal blood inversely correlated with the anogenital index (AGI) of male infants. -MOP, MMP, MiBP, MnBP, and MBzP wepositively correlated with the AGI of female infants. Cord blood levels of MnBP, mono-(2-ethyl-5-oxohexyl)-phthalate, MEHP, and ∑DEHP inversely associated with head circumference.	(149)
Pregnant women (n=165)	Urine	Phthalate metabolites (11) and birth outcomes	-Phthalate exposure inversely associated with shorter pregnancy and a decreased head circumference	(232)
Pregnant women (n=149)	Urine	Phthalate esters (9), PAHs and birth outcomes	-No effect of phthalate metabolite and PAHs on birth outcomes	(233)
Mother- new born pairs (n=72)	Urine, serum	Phthalate metabolites, birth outcomes and maternal hormones	-Positive correlation between neonatal anthropometric parameters (gestational age, birth length, birth weight, head circumference) and maternal concentration of phthalate metabolites	(234)
Pregnant women (n=121)	Urine	Phthalate metabolites (4) and anthropometry	-Association of maternal MBzP and MEHHP with the birth weight of female newborns. -MBP and MBzP negative associated with the head circumference in male and female newborns, respectively	(235)
Pregnant women (n=158)	Amniotic fluid	Phthalate metabolites (4) and anthropometry	-No association between phthalate metabolites and newborns anthropometric parameters.	(236)
Pregnant women (n=3474)	Urine	Phthalate metabolites (7) and birth weights	-MMP and MEP exposure during pregnancy associated with a decreased birth weight of infants	(237)
Pregnant women (n=3474)	Urine	Phthalate metabolites (7) and GDM	-MMP, MEP, MnBP, MBzP, and MEHHP exposure positively correlated with the fasting blood glucose in the third trimester, while MEHP and MEOHP exposure negatively correlated	(238)
Pregnant women (n=434)	Urine	Phthalate metabolites (19) and hormones	-Positive associations between phthalate metabolites, maternal estrogens, and testosterone	(239)
Pregnant women (n=299)	Urine	Phthalate metabolites (19) and gestational age-specific z-scores (GWGz)	-Sums of metabolites of ΣDEHP, ΣDiNCH, and ΣDEHTP had consistent inverse associations with GWGz	(240)
Pregnant women (n=677)	Urine and serum	Phthalate metabolites (19) and nine serum hormones	-Maternal testosterone positively associated with MHBP, and inversely associated with MEP. -Maternal CRH inversely associated with MCNP, MCPP, MECPP, MEHHP, and MEOHP.	(241)
Mother-infant pairs (n=553)	Urine and cord blood	Urinary phthalate metabolites and cord blood glucocorticoids	-MBzP in the first trimester associated with a higher cortisol/cortisone ratio. -MECPP and MEOHP measured in the third trimester were correlated with decreased cortisone.	(242)

[#] The number of phthalate metabolites measured are indicated within bracket. Elaboration of abbreviated phthalate metabolites are followed as : Mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP); mono-2-ethyl-5-carboxypentyl phthalate (MECPP); mono-3-carboxypropyl phthalate (MCPP); mono carboxyisononyl phthalate (MCNP); mono hydroxybutyl phthalate (MHBP); di(isononyl) cyclohexane-1,2-dicarboxylate (ΣDiNCH); di(2-ethylhexyl) terephthalate (ΣDEHTP); Mono-isobutyl phthalate (MiBP); mono-oxo-iso-nonyl phthalate (MOiNP); mono-(2-ethyl-5-hydroxylhexyl) phthalate (MEOHP); mono-methyl phthalate (MMP); mono-ethyl phthalate (MEP); mono-n-butyl phthalate (MnBP); mono-n-octyl phthalate (MOP); mono-benzyl phthalate (MBzP); and the metabolite of di-2-ethylhexyl phthalate (DEHP), which were mono (2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono-(2-ethyl-5-carboxypentyl) phthalate.

estrogen, and reduced litter sizes compared with their wild-type (260).

Although the role of epigenetics in successful pregnancy is well known, endocrine regulation of epigenetic processes is complex, and less data is available on the endocrine regulation of DNA methylation and histone modifications in response to endocrine disruptors like BPA. The sensitive window of early embryonic and placental development stages is more vulnerable as significant imprinting genes control these events. *In utero*, BPA exposure significantly altered the methylation levels of differentially methylated regions (DMR) imprinting control region and Igf2 DMR (261). Placental trophoblast cells respond to various exogenous agents by activating stress pathways, and epigenetic controls often regulate some of these responses. Data showed that alteration in DNA methylation profile in the first-trimester trophoblast genomic DNA correlated with CpG methylation of

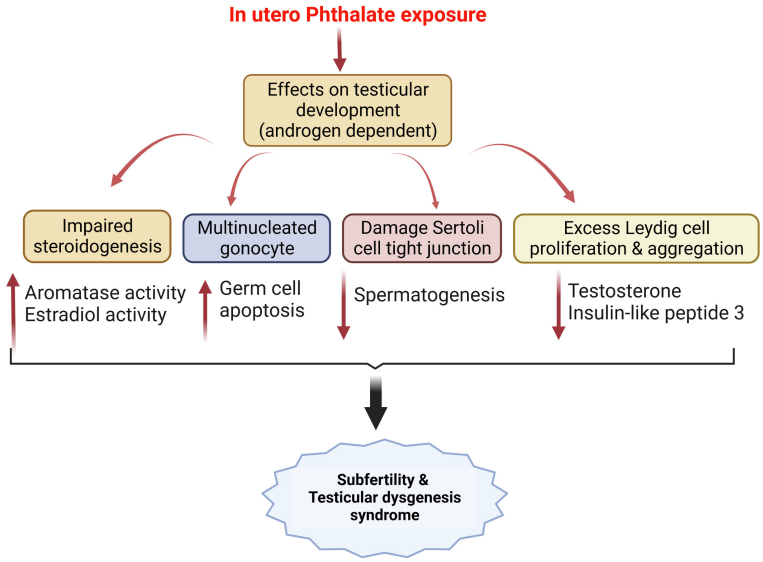

FIGURE 3
In utero phthalate exposure and its effects on testicular development modulates male fertility and results in testicular dysgenesis syndrome, as reported by several pre-clinical studies mentioned in the text.

gene promoters associated with stress responses, including oxidative stress, inflammation, DNA damage, apoptosis, hypoxia, and the unfolded protein response (27).

Furthermore, the percentage of promoter methylation was lowered by BPA exposure compared with those of control cells. Normally, DNA methylation of gene promoters represses gene transcription to those that encode stress and toxicity pathways. However, stress response genes were de-repressed due to BPA exposure. In normal physiology, the trophoblast cortisol could protect the fetus by methylating genomic DNA to repress gene transcription of stress and toxicity mediators (262), which was unmethylated by the presence of BPA, indicating that activation of these pathways in response to BPA exposure by epigenetic modification. Recently we observed that gestational BPs exposure significantly raised plasma cortisol, body weight, and adiposity in 90d offspring (unpublished data). Thus, placental programming in DNA methylation due to BPA exposure could make the offspring susceptible to stress and anxiety behavior later in life.

BPA modulates gene expression through epigenetic modifications, such as DNA methylations, by either DNA hyper or hypo-methylation. The maternal exposure to BPA shifted the coat color of yellow Agouti mouse offspring toward yellow by decreasing CpG (cytosine-guanine dinucleotide) methylation in an intra-cisternal retrotransposon upstream of the Agouti gene (263). *In-utero* BPA exposure in pregnant CD-1 mice affects the expression of the homeobox (Hoxa10) gene by decreasing the promoter DNA methylation of CpG sites of the Hoxa10 gene in the reproductive tract of mice (193). In a recent study, the association between prenatal BPA exposure and DNA methylation in the placenta found that BPA causes differential hypo/hypermethylation of CpG sites in the BPA-exposed groups (264). Maternal BPA exposure caused promoter hypermethylation of three genes (CAPS2, TNFRSF25, and HKR1) in the cord blood (265). Perinatal BPA exposure results in abnormal DNA methylation in hepatic tissue, which precedes with the development of insulin resistance, indicating fetal reprogramming via epigenetic changes (266). Developmental exposure to BPA predisposed the offspring to progress steatohepatitis when fed a high-fat diet due to dysregulated gene expression in the liver (267). BPA exposure *in utero* induced preadipocyte differentiation by DNA hypermethylation of the CPT1A gene, indicating that BPA exposure induced obesity in the offspring by accumulating free fatty acids (268). Prenatal exposure to BPA altered the MEST promoter methylation, leading to increased risks of childhood obesity until about 6 years of age (269).

Further study confirmed that hypomethylation of the MEST promoter, a gene code for α/β hydrolase, is associated with obesity in mice exposed to BPA prenatally. Apart from being sex-specific, the tissue-specificity of prenatal exposure to BPA was also associated with the global methylation of long-interspersed nuclear element-1, a repetitive DNA element expressed in the liver (270). Maternal BPA exposure during gestation and lactation affected glucose homeostasis in the F2 offspring where hepatic glucokinase gene promoter was completely methylated in all CpG sites compared with unmethylated sites in controls of the exposed liver tissue, indicating BPA's control on the metabolic fate of the offspring via epigenetic programming of metabolic gene expression (271). Furthermore, BPA-exposed mice exhibited preeclampsia-like features, including hypertension, disrupted angiogenesis biomarkers in circulation, and placenta's involvement in the reprogramming of DNA methylation of WNT2/beta-catenin gene, indicating that exposure window of placental development is the most critical for the epigenetic targets, thereby, a key determinant for the progression of placental-disease preeclampsia (272).

Epigenetic changes are sensitive to environmental factors, including exposure to EDCs like phthalate. Phthalates can cross

the placental barrier, and their metabolites have been detected in the placenta. During pregnancy, the placenta produces and releases diverse miRNAs into the maternal circulation, which can be modulated due to the impact of phthalate exposure. Studies have indicated that phthalates and their metabolites can alter miRNA profiles in placental tissue, suggesting a potential link between phthalate exposure, miRNA dysregulation, and adverse pregnancy outcomes. Urinary levels of 13 phthalates in pregnant women with uncomplicated term dichorionic, diamniotic twin pregnancies and the miRNA profile of placental EVs (EV-miRNAs) circulating in maternal blood were measured. The expression of miR-518e in the maternal blood was highest among women with high mono-benzyl phthalate Field urinary levels (273). A study on 179 women found associations between first-trimester phthalate levels and miRNA expression in the placenta. miR-142-3p, miR15a-5p, and miR-185 expressions were found to be associated with urine phthalate levels (226). Mono-(2-ethylhexyl) phthalate (MEHP) induces oxidative stress-responsive miR-17-5p, miR-155-5p, and miR-126-3p in HTR8/SVneo in a dose- and time-dependent manner by altering the expression of genes involved in oxidative stress (274). A clinical study conducted on 202 pregnant women between 22 and 29 gestational weeks measured 11 phthalate metabolites in the spot urine samples and global DNA methylation in the placental tissue of the fetal side at delivery. Mono-benzyl phthalate concentration was inversely associated with the placental methylation of Alu repeats.

Moreover, all phthalate biomarkers except for mono-carboxy-iso-octyl phthalate and mono (2-ethyl-5-hydroxyhexyl) (MEHHP) phthalate were associated with at least one differentially methylated region (275). A case-control study examined the associations of prenatal phthalate exposure, infant growth, and global DNA methylation in human placenta 119 subjects [55 fetal growth restriction (FGR) cases and 64 normal controls]. Concentrations of mono (2-ethyl-5-hydroxyhexyl) phthalate, mono (2-ethyl-5-oxohexyl) phthalate (MEOHP), and sum DEHP (molar sum of MEHP, MEHHP, and MEOHP) were significantly higher in FGR cases than those in normal controls. Placental LINE-1 methylation was positively associated with fetal birth weight and negatively associated with urinary phthalate metabolites concentrations (MEHHP and mean DEHP) (276). Another study conducted in 181 mother-newborn pairs (80 fetal growth restriction newborns, 101 normal newborns) measured third-trimester urinary phthalate metabolite concentrations and placental DNA methylation levels of IGF2 and found that urinary concentrations of MEHHP and MEOHP were inversely associated significantly with placental IGF2 DNA methylation (277). A recent study measured the effect of maternal phthalate exposure on the human placental DNA methylome, transcriptome and identified 39 genes with significantly altered methylation and gene expression in the high phthalate exposure group, with most of these relationships were inversely correlated (29 out of 39) (227).

Moreover, placental imprinting was assessed at birth in placental samples in 179 subjects and found a significant decrease in H19 methylation, which was associated with high levels of the sum of phthalate metabolites and metabolites of low molecular weight (LMW) phthalates. Σ phthalate and LMW phthalate concentrations were inversely associated with IGF2 differentially methylated regions methylation values (278). Placental exposure to phthalates may result in aberrant miRNA expression. Pregnant SD rats exposed to DEHP promoted the placental miR-155-5p expression, the cAMP/PKA inactivation, lipid metabolism and altered placental histopathology. Moreover, the knockdown of miR-155-5p abrogated DEHP-induced proliferative, migrative, and invasive inhibition in HTR8/SVneo cells (279). Phthalates (MEHP) have been reported to induce apoptosis by inducing miR-16, which alters the BCL-2/BAX ratio in the first trimer placental trophoblast HTR-8/SVneo cells (280).

Available data indicate that gestational phthalate exposure could modulate the placental gene expression related to its function or altered response to the fetus due to aberrant expression of miRNAs in the placenta.

10 Conclusion

EDCs exposure can significantly affect fetoplacental growth and pregnancy outcomes via different mechanisms. Several EDCs are estrogen-mimicking steroids, and those can quickly diffuse into the nucleus across the cell membrane and modulate the transcription of genes. These endocrine disruptors can mediate their effects by interfering endocrine function of the hypothalamic-pituitary glands, changing the secretion of gonadotropin-releasing hormone in the hypothalamus, promoting the proliferation of the pituitary, premature puberty, and resulting in infertility risks in both men and women. The epigenetic fingerprint of steroidogenesis and organogenesis in the uterus, follicles, implant, and placenta could risk the gonadal programming of the offspring. The most abundant EDCs include BPs, which report obesogenic effects, disrupting regular metabolic activity, making the body prone to overweight, and obesity. Being overweight and obese state impedes a successful pregnancy. In addition, prenatal BPs exposure may influence postnatal fetal HPA responsiveness due to alterations in cortisol levels and HSD11β activities.

Several epidemiological studies pointed out maternal exposure to BPs and Phthalates is associated with adverse outcomes on fetal growth and development, its life-long impact in the endocrine-linked growth process, and risks of reproductive organ deformities. However, data correlating of these EDC exposure with the early stage of puberty and sexual maturity and its follow-up with pre-pregnancy endocrine regulation is required for renewing better strategies in preventing EDCs-exposure induced harmful effects on pregnancy outcomes.

Author contributions

SB: writing, revision, and approval of the final version. SV: writing. AD: revision and approval of the last version. All authors have approved the final version for submission.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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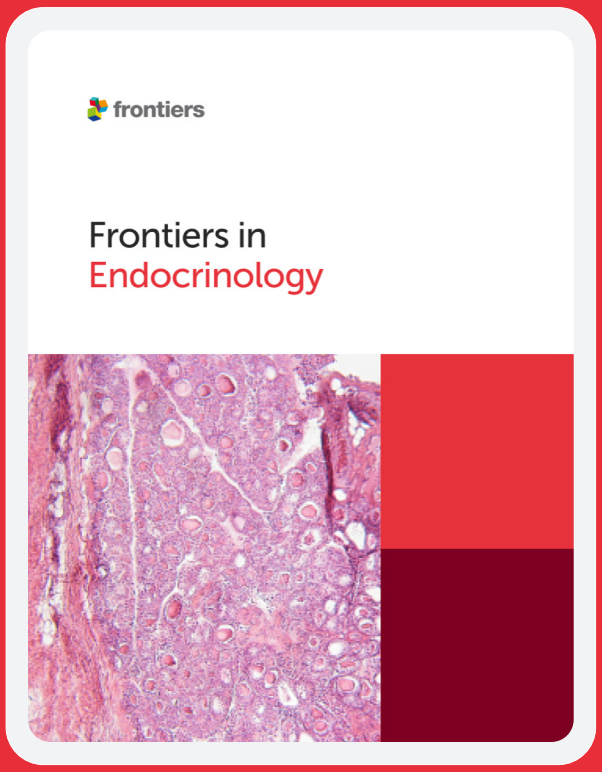

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