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Wnt Signalling in Regenerative Dentistry

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Teeth are complex structures where a soft dental pulp tissue is enriched with nerves, vasculature and connective tissue and encased by the cushioning effect of dentin and the protection of a hard enamel in the crown and cementum in the root. Injuries such as trauma or caries can jeopardise these layers of protection and result in pulp exposure, inflammation and infection. Provision of most suitable materials for tooth repair upon injury has been the motivation of dentistry for many decades. Wnt signalling, an evolutionarily conserved pathway, plays key roles during pre- and post-natal development of many organs including the tooth. Mutations in the components of this pathway gives rise to various types of developmental tooth anomalies. Wnt signalling is also fundamental in the response of odontoblasts to injury and repair processes. The complexity of tooth structure has resulted in diverse studies looking at specific compartments or cell types of this organ. This review looks at the current advances in the field of tooth development and regeneration. The objective of the present review is to provide an updated vision on dental biomaterials research, focusing on their biological properties and interactions to act as evidence for their potential use in vital pulp treatment procedures. We discuss the outstanding questions and future directions to make this knowledge more translatable to the clinics.

Keywords: tooth development, regeneration, adult stem cells, reparative dentinogenesis, reactionary dentin

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WNT SIGNALLING PATHWAY

Wnt signalling pathway is critical during various stages of embryogenesis, tissue homeostasis and wound repair where it controls regulation of cell proliferation, differentiation, polarisation, and apoptosis. Here, a brief introduction of Wnt signalling pathway is followed by its role during tooth and periodontal tissue development, homeostasis, and regeneration. Particularly, significance of Wnt signalling is reviewed in dental pulp stem cells and odontoblasts. We also look at factors affecting odontoblast's function and regulation of epigenetics in dental pulp cells by Wnt signalling pathway. We finally look at how new advances in biomedicine and technology utilises the new knowledge in tissue regeneration and how that can be applied in dentistry.

Wnt signalling pathway consists of 19 cysteine rich protein ligands and the receptor complex which comprises of 10 seven-pass transmembrane receptors called Frizzled (Fzd), and LDL receptor-related proteins 5 and 6 (LRP5 and LRP6) which mediate the signalling. Upon binding of the Wnt ligand to the extracellular cysteine rich domain of Fzd, signal is transduced to a cytoplasmic phosphoprotein called Dishevelled (Dsh/Dvl). Wnt signalling pathway can be canonical or non-canonical (1). In canonical Wnt signalling, binding of ligands to receptors and coreceptors results in the formation

of Wnt-Fz-LRP complex and recruitment of Dsh/Dvl. This results in recruitment of the Axin complex to the receptor, inhibition of Axin-mediated β-catenin phosphorylation and subsequently stabilisation of β-catenin. This results in translocation of β-catenin into nuclei to form the TCF/LEF complex and activate Wnt target genes. In the absence of Wnt, cytoplasmic β-catenin is degraded by a β-catenin destruction complex. This complex includes Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α) (2).

Non-canonical Wnt signalling consists of Wnt-PCP and Wnt-Ca^{2+} pathway (3). This pathway signals through Fzds, ROR2, RYK or through Fzds with ROR, or RYK as co-receptor and results in activation of downstream effectors such as calcium/Calmodulin dependent protein kinase II, mobilisation of Ca^{2+}, heterotrimeric G proteins and multiple small GTPases (4). Non Canonical Wnt signalling is involved in the maintenance of stem cells, regulating cell polarity, directional cell movement, promoting invasion, and inhibiting the canonical Wnt//β-catenin signalling cascade (5) (**Figure 1**).

WNT SIGNALLING DURING TOOTH DEVELOPMENT

Teeth are epithelial appendages and develop through reciprocal interaction between surface epithelium and the underlying neural crest derived mesenchyme in the developing maxillary and mandibular arches. Critical roles of various signalling pathways such as Wnt, Shh, FGF and Eda in this process has been well-established. The very specific location of tooth initiation is determined by these signalling pathways (6). Wnt/β-catenin signalling is expressed at early stages of tooth development and in the epithelial signalling centres that regulate budding and crown morphogenesis (6–9). Active β-catenin signalling is also expressed in the underlying mesenchyme and is required for epithelial morphogenesis and the induction of odontogenic fate (6, 8, 10). Similarly, late stages of tooth development and formation of the crown and roots requires a well-orchestrated network of different signalling pathways which also includes Wnt (11). It has recently been shown that Wnt inhibitor, NOTUM, is required for normal development of molar root. Ablation of Runx2, which is expressed in subpopulation of root progenitors results in down regulation of NOTUM upregulation of Wnt signalling and subsequently disruption of odontoblastic differentiation and altered root morphology (12).

Non canonical Wnt signalling pathway has also been shown to regulate root development through Receptor tyrosine kinase (RTK)-like orphan receptor 2 (Ror2) which is of the non-canonical Wnt receptors. Ror2 is expressed in dental mesenchyme and its loss results in disruption of proliferation and differentiation of mesenchymal cells and subsequently alteration in molar root size. Cdc42 required for cell cycle progression has been identified as a potential downstream mediator of Ror2 signalling in root formation (13). These findings partially explain how disruption in Wnt signalling during different stages results in various forms of developmental defects ranging from tooth agenesis to odontomas (14–17).

WNT SIGNALLING IN TOOTH HOMEOSTASIS

With the many significant roles that Wnt pathway plays during different stages of tooth development, it is no surprise for it to be implicated in tooth homeostasis. In fact, odontoblasts are responsive to endogenous Wnt signals and maintain their Wnt responsiveness throughout their lifetime. The regenerative capacity of multiple mammalian tissues depends on Wnt/β-catenin signalling pathway and its activation. This has been extensively shown in murine teeth where shallow tooth damage usually results in activation of odontoblasts and formation of reactionary dentin that protects the pulp. Severe tooth damage, however, leads to odontoblast death and subsequent activation of resident dental pulp stem cells, their proliferation and differentiation into new odontoblast-like cells. These cells are then recruited to the site of damage to form reparative dentin (18, 19). This repair process is accompanied by increased Axin2 expression which results in differentiation of Axin2 expressing cells into odontoblasts-like cells. These cells are produced in the event of trauma or injury and secrete reparative dentin, a process finely tuned by the autocrine Wnt signals produced by Axin2 expressing cells (20). In addition, proliferation and apoptosis in dental pulp stem cells is regulated by Wnt signalling. Treatment of human dental pulp stem cells with liposome-reconstituted form of Wnt3A (L-WNT3A) results in increased Wnt response, enhanced mitotic activity and reduced apoptosis. Similarly, treatment of injured teeth with L-WNT3A preserves pulp vitality after acute exposure and results in elevated Wnt response and subsequently dentin regeneration (21). Various studies have demonstrated the significance of Wnt signalling pathway in tooth regeneration. Similarly, Lithium ions and Lithium Chloride (LiCl) that activate the Wnt pathway can induce tubular dentin formation. *In vivo* application of LiCl in rat molars results in higher β-catenin and a complete tertiary dentin (22). A number of small molecules that inhibit glycogen synthase kinase 3 (GSK3), a key enzyme in Wnt signalling pathway, have been shown to promote activation of Wnt pathway *in vitro* and dentin repair in mice and rats with experimental pulp exposures (23–26). Treatment of dental pulp exposures with Semaphorin 3A, promotes formation of an odontoblastic layer, dentin tubules, and predentin (27). Presence of Wnt/β-catenin signalling in dental pulp cells does not necessarily translate into promotion of odontoblast differentiation and dentin regeneration. For example, overexpression of Wnt10a significantly increases the proliferation of DPSCs, but decreases the expression of odontoblast differentiation-related genes, such as Dentin Sialophosphoprotein (DSPP), Dentin Matrix Acidic Phosphoprotein 1 (DMP1), Alkaline Phosphatase (ALP), and Collagen type 1 alpha 1 chain (COL1A1), suggesting that overexpression of Wnt10a may negatively regulate the differentiation of DPSCs into odontoblast (28, 29). Another factor that can decrease dentin regeneration is the sympathetic

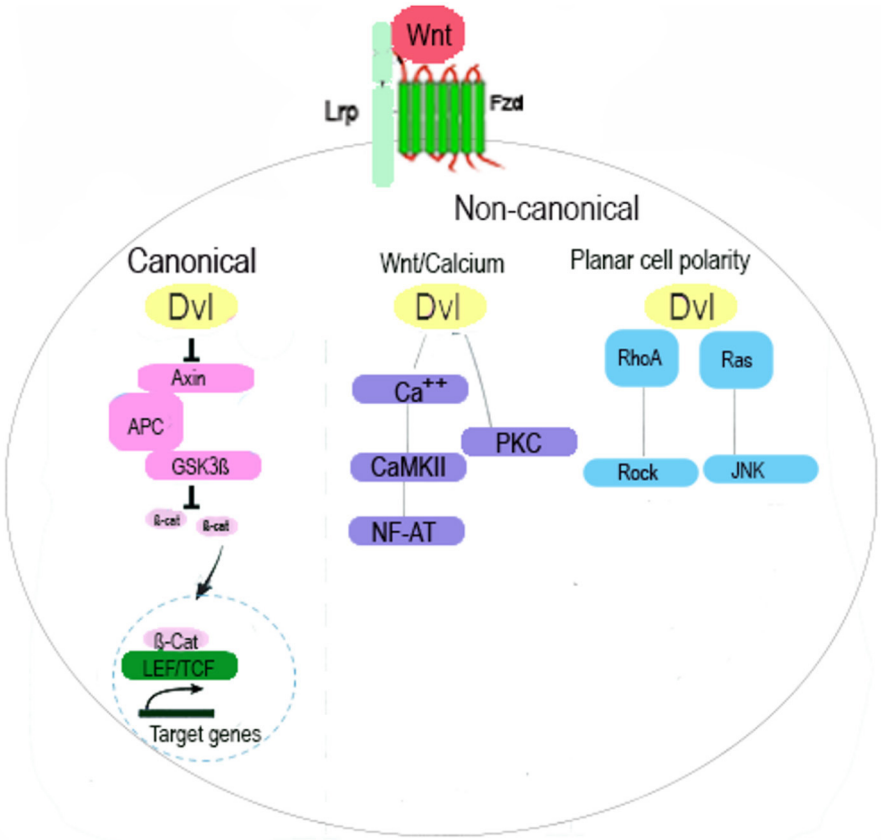

FIGURE 1 | Schematic Illustration of Wnt Signalling Pathway. The canonical Wnt pathway, acts through Fzd and LRP5/6 receptors is mediated by β-catenin and its translocation to nucleus, activation of TCF/LEF transcription factor and regulation of gene expression. Non-canonical Wnt pathway signals through Fzd and/or ROR1/ROR2/RYK receptors and is transduced by the WNT/PCP (planar cell polarity), WNT/RTK (receptor tyrosine kinase) and WNT/Ca^{2+} signalling cascades. In the Wnt/Ca^{2+} pathway, binding of Wnt to Fzd activates DVL which then activates calcium-binding proteins including protein kinase C (PKC) and calmodulin-dependent kinase II (CamKII) and subsequently transcription factor NFAT. In the Wnt/PCP pathway, signal is mediated by GTPases RhoA and Ras and activates RhoA-Rho-associated kinase (ROCK) axis or JNK and subsequently regulation the cytoskeleton.

nervous system. Beta-2 adrenergic receptors are located in the odontoblastic layer of dental pulp in rat molars and treatment of experimental cavities with the sympatholytic beta antagonist, propranolol, results in higher tertiary dentin formation than control groups (30). Other signalling pathways such as TGF-β and BMP are also involved in formation of reparative dentin by regulation. For example, BMP-2 regulates differentiation of pulp cells to odontoblasts whilst TGF-β stimulates odontoblast differentiation and mineralisation (31–33).

DENTAL PULP STEM CELLS

The close anatomical and functional relationship between dentin and pulp results in formation of a dentin-pulp complex. Pulp cells contribute to the turnover of extracellular matrix and play a crucial role in the recovery of tooth damage (34, 35). Dental pulp stem cells (DPSC) demonstrate great proliferation and have great potential for a range of applications in stem cell research and regenerative medicine thanks to their ability to differentiate into various cell lineages *in vitro*. As an example, when subject to loading, they express tendon makers such as Scleraxis, Tenascin-C, and Collagens (36). DPSCs have recently been shown to have higher odontogenic potential than other sources of stem cells in the tooth [stem cells from the apical papilla < DPSC, periodontal stem cells (PDLSC)]. This capacity can be enhanced by supplementing cultures with 17ß-estradiol (37). When cultured on different surfaces such as plastic, hydroxyapatite and β-tricalcium phosphate, DPSCs show higher proliferation capacity and greatest osteogenic potential when compared to cells isolated from adipose tissue, and bone marrow (38).

There is an increasing interest in better understanding the capacity of DPSC differentiation into odontoblasts to explore avenues that enhance this process. Induced pluripotent stem cells have been shown to induce pulp-like tissue with the presence of tubular dentin *in vivo* (39). A group of unique multipotent stem cells have been identified from mouse dental papilla

through three-dimensional spheroid culture. These cells ae called multipotent dental pulp regenerative stem cells and demonstrate osteogenic/odontogenic differentiation capabilities and are able to form dentin and neurovascular-like structure (40). Exogenous factors such as nitric oxide are shown to directly induce odontogenic capacity of DPSCs in rats (41). Platelet-rich plasma can promote proliferation and odontogenic differentiation of Neural Crest Stem cells derived from human dental apical papilla when used at the correct concentration (42). Further in-depth analysis of the mechanism of these processes and key singling pathways involved would be valuable. A recent study has shown that retinoid acid receptor-related orphan receptor α (RORα), is expressed in dental papilla cells and is upregulated during odontoblastic differentiation (43). RORα is also a receptor for melatonin and mediates the pro-odontogenic effect of melatonin suggesting a potential of their use in dentin regeneration.

IMPACT OF INFLAMMATION AND SENESCENCE ON ODONTOBLASTS

One of the factors affecting odontoblast function is senescence. Aged odontoblasts demonstrate decreased autophagic activity, accumulation of intracellular lipids, and loss of functionality (44). Aged DPSCs also demonstrate lower levels of cytoplasmic DMP1 in odontogenic differentiation, reduced contribution to mineralisation process, altered secretion of matrix metalloproteinases, and lower neurogenic differentiation potential (45–50). These changes may have an impact on formation of tertiary dentin at later stages of life. It is also worth exploring signalling pathways upstream and downstream of senescence in the tooth. Wnt signalling pathway can mediate senescence in bone marrow MSCs and its chronic activation can induce senescence in lung epithelial cells (51, 52). Regulation of senescence in odontoblasts can pave the way for new therapeutics in geriatric dentistry. Diet and metabolism have an impact on tooth homeostasis. Mice with Low Density Lipoprotein (LDL) receptor deficiency ($Ldlr^{-/-}$) and on high fat diet demonstrate narrower pulp, less elongated incisor, and disappearance of predentin in incisors (53). Treating experimental pulp exposures with leptin results in angiogenesis, odontogenic differentiation and mineralisation in rat (54). Interestingly, A drug commonly used for the treatment of hyperlipidaemia, Simvastatin, has been shown to promote odontogenic differentiation and formation of new dentin (55).

Odontoblasts are immunocompetent cells and respond to bacterial components at early stages. They produce a range of antibacterial substances such as defensins, nitric oxides, chemokines, and cytokines and contribute to a staged pulpal inflammatory response (56). This response is governed by the intensity of the inflammatory reaction. Detection of microbial pathogen in odontoblast is mediated by pattern recognition receptors (PRRs) such as Toll-like receptor and the nucleotide-binding oligomerisation domain (NOD) (57). Toll-like receptors (TLR) mediate signals from components of bacterial cell wall during inflammatory reactions (58). Interestingly, Toll-like receptors have a role during development of mouse tooth germs. Activation of TLR4 inhibits mineralisation of enamel and dentin suggesting that TLR4 may decrease the mineralisation of hard tissues and trigger the maturation of ameloblasts (59).

Resident inflammatory cells in a healthy pulp also detect their environment via immune-surveillance and challenge pathogenic bacteria. In fact, macrophage populations in dental pulp are critical for dental pulp stem cell activation and formation of reparative dentin (60). Wnt signalling may play an important role in regulation of tooth inflammation. An inflamed pulp tissue exhibits increased levels of some MMPs, such as MMP2 and 3 (50, 61, 62). Wnt signalling have been shown to induce MMP expression and subsequently affect transmigration of T cell (63). GSK3, is also a key mediator of pro-inflammatory cytokine production during bacterial infections through the TLR pathway and is a potential regulator of periodontal inflammation *in vitro* (64). These studies suggest potential regulation of inflammation in tooth by targeting Wnt signalling.

Treatment of murine teeth with Lipopolysaccharide which is an inflammatory stimulus and Simvastatin results in angiogenesis, repressed inflammatory mediators, and increased dentin regeneration. Here Simvastatin, acts by minimising inflammatory effects and increasing regenerative potential (55). Resolvin E1 (RvE1) is a dietary omega-3 polyunsaturated fatty-acid metabolite and effective in resolving inflammation and wound healing. Treatment of experimentally induced pulp injury in rats with RvE1, demonstrated pro-healing properties, reduced necrosis of damaged pulp, and promoted formation of reparative dentin (65).

One of the key requirements during tissue regeneration is angiogenesis vascular endothelial growth factor (VEGF) family are expressed in human dental pulp and exhibit autocrine and paracrine roles in local blood vessels and immune cells. VEGF is also the most potent angiogenic and vasculogenic factor in tertiary dentin formation with positive effect on proliferation, differentiation, mineralisation, neovascularizing, and formation of reparative dentin both *in vitro* and *in vivo* (66). Lineage tracing studies have shown that during reparative dentinogenesis, odontoblasts arise from perivascular cells expressing alpha Smooth Muscle Actin (aSMA) (33).

In a carious exposure, the unique effect of pathogens is added to cellular changes secondary to inflammation. A factor that needs to be considered in tooth regeneration. Interestingly, it has been shown that expression of VEGF is higher in teeth with caries (67). This finding can be utilised in development of natural avenues for treatment of carious teeth. Comparison of ultrastructural and chemical changes that take place in arrested carious lesions demonstrate extensive remineralisation with deposition of Mg containing Hydroxyapatite crystals (Mg-Hap) in tubules of caries-arrested dentin (68). This suggests natural process of remineralisation can contribute to caries treatment a potential that can be harvested in therapeutic approaches.

EPIGENETICS

Epigenetic modification serves an important role in cell differentiation. Epigenetics can operate at various levels, such as

interference with transcriptional and translational information through non-coding RNAs, modification of posttranslational histone cores and methylation of cytosine residues in DNA structure. Histone methylation is one of the most robust epigenetic marks and is essential for the regulation of multiple cellular processes. A recent study has shown that a short-term activation of Wnt signalling by WNT-3A induces a genomic DNA demethylation and increases histone acetylation and methylation in DPSCs, highlighting the regulation of the epigenetic barrier by Wnt signalling in DPSCs (69). Histone methyltransferases (HMTs) and histone demethylases (HDMs) are crucial for the osteogenic differentiation of human bone marrow and tooth. And histone demethylation may play an important role in reparative dentinogenesis. HDM KDM5A is an enzyme with significantly enhanced expression during cytodifferentiation in hDPCs undergoing odontogenic induction. Knocking down KDM5A in hDPCs results in greater alkaline phosphatase activity, mineral deposition and increased expression of odontogenic markers DMP1, DSPP, Zinc finger protein Osterix (OSX), and Osteocalcin (OCN) (70). DNA methylation regulates the inflammatory response of human odontoblasts in carious pulp. knocking down the DNA Methyltransferase 1 gene results in Lipoteichoic acid-induced inflammatory cytokines in human odontoblast-like cells (71). Acetylation governs differentiation and de-differentiation potential of DPSCs. A range of Histone deacetylases (HDACs) are expressed in dentin-pulp complex that regulate odontoblasts differentiation (72, 73). As an example, inhibition of HDAC4 and HDAC5, increases odontoblastic

	Wnt Pathway Component	Effect on tooth	Ref
1	Canonical Wnt pathway	Determination of specific tooth germ location during development / Induction of odontogenic fate , tooth budding and crown morphogenesis	Balic and Thesleff, 2015/Liu et al., 2008; Chen et al. 2009; Ahtiainen et al., 2016)
2	Non canonical Wnt pathway	Regulation of root development	Ma et al., 2021
3	Wnt3A	Regulation of proliferation and apoptosis in dental pulp stem cells / Regulation of epigenetics in dental pulp cells	Hunter et al., 2015 /Unbe-Etxebarria et al., 2020
4	Wnt10a	Proliferation of DPSC	Zhang et al., 2014; Lu et al., 2019
5	Axin2	Activation upon tooth injury and secretion of reparative dentin/ Contribution to cementoblasts in postnatal development and adult homeostasis	Babb *et al.*, 2017, Zhao et al., 2021
6	GSK3	Promotion of dentin repair upon inhibition /Potential regulator of periodontal inflmmation	Beurel et al., 2015; Neves et al., 2017; Zaugg et al., 2020/Wang, Brown and Martin, 2011
7	Wnt inhibitor, Notum	Molar root morphogenesis	Wen et al., 2020

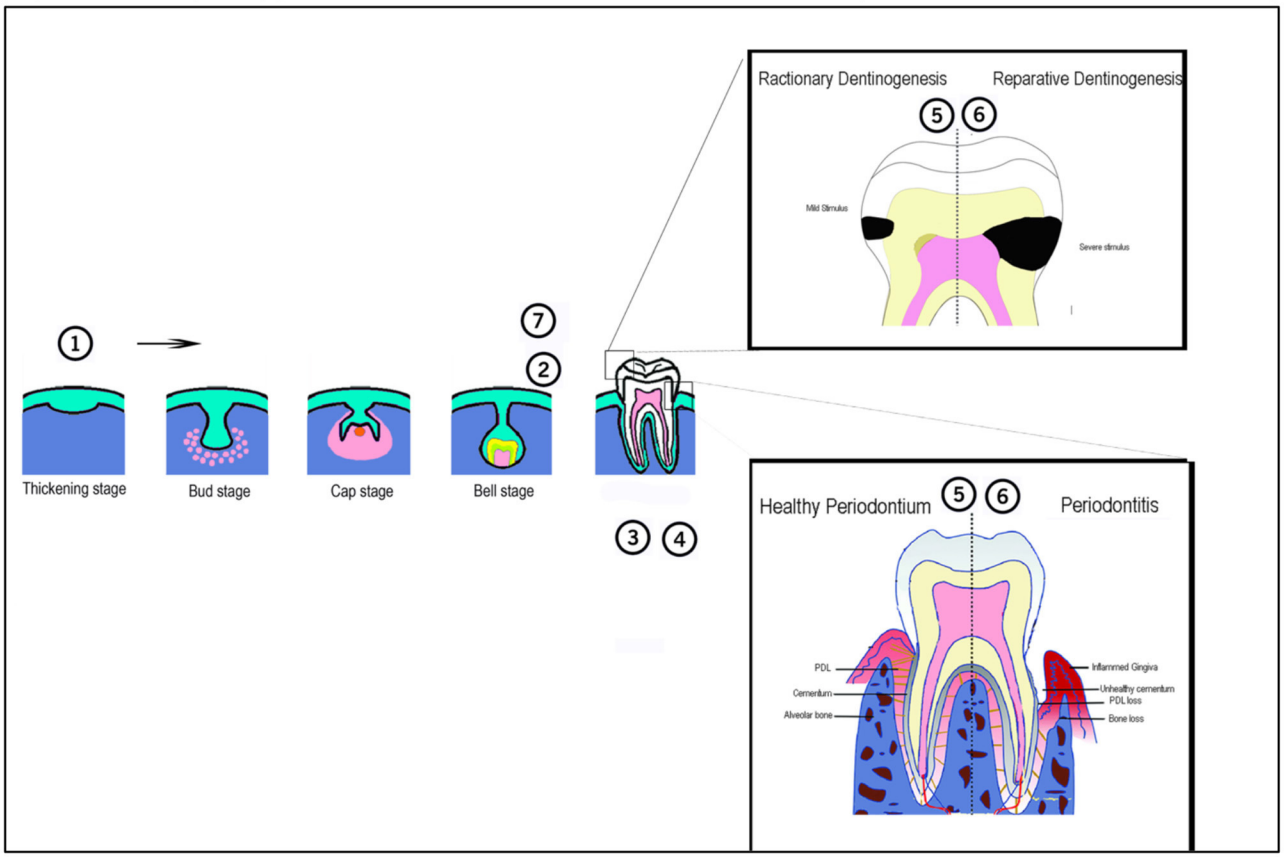

FIGURE 2 | Table and schematic, summarising different roles of Wnt pathway during development, homeostasis, and regeneration of tooth and periodontium. Numbers in the schematic corresponds to the number of pathway component in the table. 1: canonical Wnt pathway is involved in determination of specific tooth germ location during development and induction of odontogenic fate, tooth budding and crown morphogenesis. 2: Noncanonical Wnt pathway regulates root development. 3: Wnt3A regulates proliferation and apoptosis in dental pulp stem cells. It is also involved in the epigenetic regulation of DPSCs. 4: Wnt10 regulates proliferation of DPSCs. 5: Axin2+ cells are activated upon tooth injury and induce secretion of reparative dentin. In the periodontium these cells contribute to cementoblasts formation during postnatal development and adult homeostasis. 6; Inhibition of GSK3 promotes dentin repair. It is a potential regulator of periodontal inflammation. 7: Notum, Wnt inhibitor is involved in molar root morphogenesis. A part of this schematic was adapted from Xu et al. (79).

gene expression and promote the odontoblast induction (74). HDAC6 regulates the fusion of autophagosomes and lysosome during odontoblast differentiation. Decreased autophagy results in downregulation of odontoblastic differentiation capacity (75).

WNT SIGNALLING IN PERIODONTAL HOMEOSTASIS AND DISEASE

Periodontitis is a chronic inflammatory condition of tooth supporting tissues that results in loss of tissue attachment. This condition has also been linked to many systemic conditions such as diabetes, rheumatoid arthritis and cognitive impairment (76, 77).

GSK3 has been shown as a potential regulator of periodontal inflammation *in vitro* (64). Systemic administration of GSK3β inhibitors *in vivo* result in abrogation of bacterial-induced bone loss. A recent murine study has demonstrated that differentiation of cementoblasts producing the mineralised tissue in the root, contributes to maintenance of periodontal tissue attachment as well as its restoration in the event of periodontitis. Different populations of stem cells contribute to cementoblasts differentiation at various stages of life. Perivascular-derived cells expressing CD90 and perivascular-associated cells that express Axin2 contribute to cementoblasts during post-natal development. Whereas, during adult homeostasis, cementoblast are formed from responsive Axin2+ cells. Contribution of CD90 expressing cells to cementoblast differentiation occurs only upon induction of periodontitis (78). These findings have great clinical implication and emphasise the crucial role of Wnt signalling in homeostasis of tooth and its periodontium. These roles of Wnt signalling are summarised in table and schematic in the **Figure 2**.

The development of a new generation of dental therapies on biological-based approaches is now a major goal in regenerative dentistry. Our understanding of dental tissue regeneration is advancing but needs to be combined with a compatible and practical delivery system to be translated into clinical application. Advanced biomaterials and technology can be used to modulate tissue microenvironment and enhance the efficiency of regeneration process. This has been shown in the use of low level laser and magnetic fields to enhance DPCs differentiation as well as novel imaging techniques to visualise dental pulp using tissue clearing method (80–84). However, these advancements need to be tested for their accessibility and delivery. Development of suitable scaffolds that can stimulate and guide stem cell differentiation in dental tissues is equally important and can serve as delivery vehicle.

ReDent (*Regeneration of Dentine*) allows delivery of small concentration of a novel GSK3 small molecule inhibitor drug via hydrogel into tooth cavity and results in rapid stimulation of resident DPSCs to proliferate and differentiate into odontoblast-like cells that produce reparative dentine (85). LithGlass is a novel glass ionomer formulation specifically designed to rapidly release lithium ions to stimulate odontoblast activity in non-exposed pulp lesions. The reactionary dentin produced restores original dentin thickness (86). Both ReDent and LithGlass represent simple, affordable solutions to improve dental care and hopefully form part of the new vanguard driving regenerative dentistry.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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