



Major Effect of Oxidative Stress on the Male, but Not Female, SP-A1 Type II Cell miRNome

George T. Noutsios^{1†}, Nithyananda Thorenoor¹, Xuesheng Zhang¹, David S. Phelps¹, Todd M. Umstead¹, Faryal Durrani¹ and Joanna Floros^{1,2*}

¹ Center for Host Defense, Inflammation, and Lung Disease (CHILD) Research, Department of Pediatrics, College of Medicine, Pennsylvania State University, Hershey, PA, United States, ² Department of Obstetrics and Gynecology, College of Medicine, Pennsylvania State University, Hershey, PA, United States

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*Correspondence:

Joanna Floros
jfloros@pennstatehealth.psu.edu

†Present Address:

George T. Noutsios,
School of Mathematical and Natural
Sciences, Arizona State University,
Glendale, AZ, United States

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Pulmonary surfactant protein A (SP-A) plays an important role in surfactant metabolism and lung innate immunity. In humans there are two proteins, SP-A1 and SP-A2, encoded by *SFTPA1* and *SFTPA2*, respectively, which are produced by the alveolar type II cells (T2C). We sought to investigate the differential influence of SP-A1 and SP-A2 in T2C miRNome under oxidative stress (OxS). SP-A knock out (KO) and hTG male and female mice expressing SP-A1 or SP-A2 as well as gonadectomized (Gx) mice were exposed to O₃-induced oxidative stress (OxS) or filtered air (FA). Expression of miRNAs and mRNAs was measured in the T2C of experimental animals. (a) In SP-A1 males after normalizing to KO males, significant changes were observed in the miRNome in terms of sex-OxS effects, with 24 miRNAs being differentially expressed under OxS. (b) The mRNA targets of the dysregulated miRNAs included *Ago2*, *Ddx20*, *Plcg2*, *Irs1*, *Elf2*, *Jak2*, *Map2k4*, *Bcl2*, *Ccnd1*, and *Vhl*. We validated the expression levels of these transcripts, and observed that the mRNA levels of all of these targets were unaffected in SP-A1 T2C but six of these were significantly upregulated in the KO (except *Bcl2* that was downregulated). (c) Gonadectomy had a major effect on the expression of miRNAs and in three of the mRNA targets (*Irs1*, *Bcl2*, and *Vhl*). *Ccnd1* was upregulated in KO regardless of Gx. (d) The targets of the significantly changed miRNAs are involved in several pathways including MAPK signaling pathway, cell cycle, anti-apoptosis, and other. In conclusion, in response to OxS, SP-A1 and male hormones appear to have a major effect in the T2C miRNome.

Keywords: alveolar epithelium, surfactant protein A, ozone, sex differences, MAPK

INTRODUCTION

Ambient ozone (O₃)-induced oxidative stress (OxS) is one of the major environmental factors contributing to the occurrence and development of upper and lower airway disease, including chronic rhinosinusitis (CRS) (1), asthma, and chronic obstructive lung disease (COPD) (2, 3). In the distal lung, the alveolar epithelial cells provide the first line of defense against environmental pathogens such as O₃, pollutants, bacteria, viruses, and allergens by producing a number of protective factors (4). In addition to secreting pulmonary surfactant to reduce the alveolar surface tension, they produce chemokines and cytokines that regulate alveolar inflammatory responses as well as proteinases and proteinase inhibitors (5). Upon environmental stress such as that of

OxS, O₃ increases the production of reactive oxygen species (ROS) (6) that disrupt the alveolar epithelial cell barrier function by the dissociation of the tight junctions of alveolar epithelium (7), thereby allowing the entrance of opportunistic bacteria such as *Pseudomonas aeruginosa* to infect the alveolar epithelium. Exposure to O₃ also reduces pulmonary surfactant secretion (8). The alveolar epithelium initiates a self-repair process by recruitment, proliferation, and differentiation of new epithelial cells to maintain the structural and functional traits that are required to maintain a normal respiratory function (9).

The alveolar epithelium is comprised of two different cells types, the alveolar type I cells (T1C) and the type II cells (T2C) that are in close proximity with the alveolar macrophages (AM) that reside in the alveolar space. The T1C are large, flat cells with a thin attenuated cytoplasm that line 90% of the alveolar surface. This distinct shape enables them to facilitate O₂/CO₂ gas exchange by minimizing the diffusion distance between the alveolar surface and the blood (10). The T2C cover ~10% of the alveolar surface and their main function is to produce and secrete pulmonary surfactant, a phospholipid and protein mixture, which lowers the surface tension in the alveolus during the respiration process. T2C possess unique secretory organelles, called lamellar bodies, which contain surfactant lipids and surfactant proteins A (SP-A), SP-B, and SP-C. Also, T2C play a very important role in the epithelium repair process after lung injury and are considered the progenitor cells of the alveolar epithelium. Upon epithelial damage T2C proliferate as new T2C and they differentiate into T1C repairing the scarred epithelial surface (9, 11, 12).

SP-A is the most abundant protein in pulmonary surfactant and has both surfactant-related functions and innate immunity functions (13–17). SP-A knock-out mouse studies have revealed important host defense functions of SP-A, where KO mice are more vulnerable to bacterial infections compared to the mice that express SP-A (18–22). Also, SP-A has been shown to have regulatory effects on the proteome, function, cell shape, and activation state of AM (21, 23–26). OxS stress increases the production of reactive oxygen species (ROS) (6) and these in turn damage the alveolar epithelium (27), oxidize SP-A, and compromise innate immune functions (23, 28–33). In humans however, unlike in rodents, there are two different genes, *SFTPA1* and *SFTPA2*, encoding SP-A1 and SP-A2 proteins, respectively. We have previously shown that SP-A1 and SP-A2 differentially affect the proteomic expression in AM (34, 35), the AM function (36–38), surfactant secretion (39), structure of surfactant monolayers (40, 41) and more recently we have

shown that SP-A1 and SP-A2 differentially regulate the AM miRNome and antioxidant pathways in the AM (42), lung function mechanics (43), and survival after *K. pneumoniae* infection (44). MicroRNAs (miRNAs) have also been shown to differentially affect SP-A1 and SP-A2 expression (45), and also contribute to the maintenance of the T2C phenotype (46).

In the present study, using humanized transgenic (hTG) mice, where each expresses either SP-A1 or SP-A2, we sought to investigate the differential influence of SP-A1 and SP-A2 on the T2C miRNome under the effect of OxS. We found that the T2C miRNome is regulated in response to OxS and that O₃ exposure has a major effect on the male SP-A1 miRNome. We also show that sex hormones play a role in T2C miRNome under the studied conditions.

METHODS

Oxidative Stress Animal Model

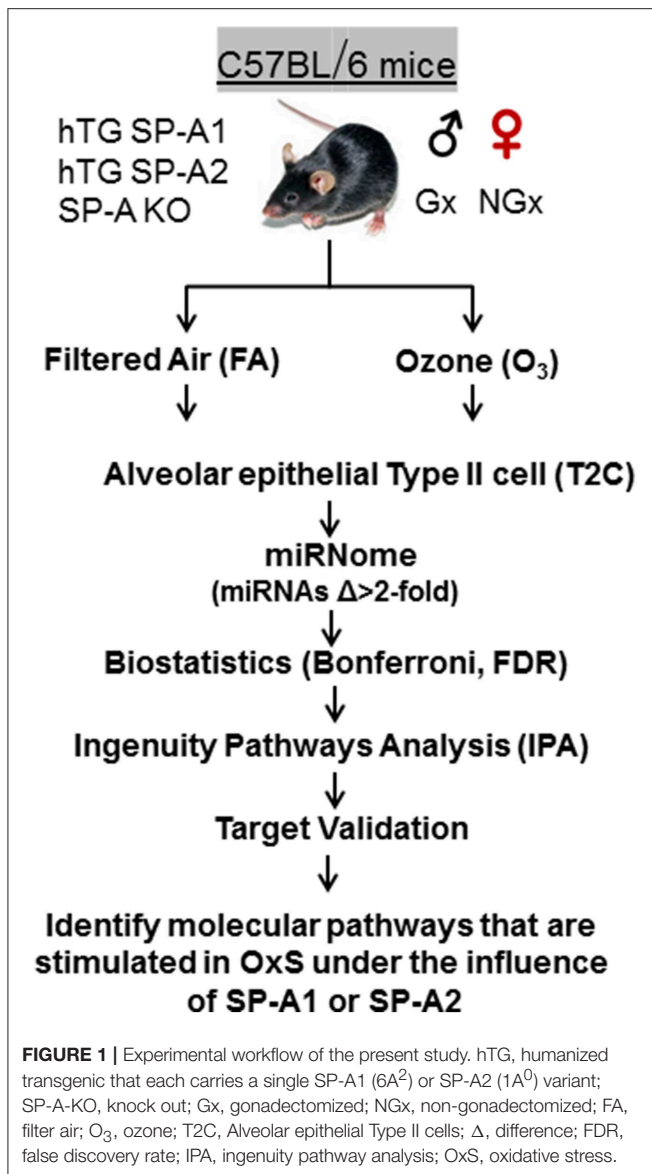
Twelve weeks old humanized transgenic (hTG) C57BL/6 mice (males and females) each carrying human SP-A1 (6A²), SP-A2 (1A⁰) (47), as well as SP-A knock-out (KO) were used in the present study. Females were synchronized for 7 days as described previously (42) to stimulate estrus. A total of $n = 52$ mice (36 for miRNA study, 16 for gonadectomy analysis). Protocols involving animal procedures were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine.

Animals were exposed to 2 ppm ozone (O₃) or filtered air (FA) (control) at 25°C as described previously (42, 48). We used 3 mice per group. i.e., 3 males, 3 females, 3 SP-A KO, 3 hTG SP-A2, 3 hTG SP-A1, 3 for O₃, and FA exposure ($n = 36$). All O₃ and FA exposures were conducted in parallel as described (49). Mice were sacrificed 4 h post exposure. Each animal was analyzed individually, and we did not pool any samples. Summary of the experimental workflow is depicted in **Figure 1**.

Mouse Alveolar Type II Cells Isolation

Mouse type II cells were isolated based on a modified method that was described previously (50). Briefly, mice were anesthetized with intraperitoneal injection of 87.5 mg/kg ketamine and 12.5 mg/kg xylazine and exsanguinated by cutting the inferior vena cava. Cardiac perfusion of the lung was performed with 10 mL of normal saline solution followed by endotracheal intubation and infusion of the lungs with 3 mL solution of 50 U/ml dispase II (Sigma-Aldrich, St. Louis, MO) in HBSS 1x and sealed with 0.5 mL of 1% solution of low melting agarose (Sigma-Aldrich). The lungs were removed from the thoracic cavity and lung lobes digested in 15 mL tube containing 2 mL of dispase II for 45 min at 37°C with constant shaking at 150 rpm. Digested lungs were dissected and homogenized in 7 mL of complete DMEM solution, supplemented with 10 μL DNase I (5,000 Kunitz U/ml Sigma-Aldrich). The lung epithelial cells were filtered through a 100 and 40 μm strainer, passed through 20 μm- nylon mesh, cells were collected by centrifugation at 130 xg for 8 min, and resuspended in 10 mL of DMEM/25 mM HEPES/10% FBS/1x AB/AM. Negative selection of T2C was performed by incubating the cell suspensions in 10-cm cultured dishes coated with 42

Abbreviations: *Ago2*, argonaute 2; AM, alveolar macrophages; *Bcl2*, B-cell lymphoma 2; *Cnd1*, cyclin D1; COPD, chronic obstructive lung disease; CRS, chronic rhinosinusitis; *Ddx20*, dead-box helicase 20; eIF2, eukaryotic initiation factor 2; FA, filtered air; Gx, gonadectomized; FDR, false discovery rate; hTG, humanized transgenic; *Irs1*, Insulin Receptor Substrate 1; *Jak2*, Janus Kinase 2; KO, SP-A knock-out; *Map2k4*, Mitogen-Activated Protein Kinase 4; *MAPK*, mitogen-activated protein kinases; miRNAs, microRNAs; NGx, non-gonadectomized; O₃, ozone; OxS, oxidative stress; *Plcg2*, Phospholipase C Gamma 2; ROS, reactive oxygen species; *SFTPA1*, gene encoding SP-A1; *SFTPA2*, gene encoding SP-A2; SP-A, surfactant protein A; SP-B, surfactant protein B; SP-C, surfactant protein C; SP-D, surfactant protein D; T1C, alveolar type I cells; T2C, alveolar type II cells; *Vhl*, Von Hippel-Lindau protein.



μ g anti-mouse CD45 (targeting hematopoietic cells) and 16 μ g anti-mouse CD16/32 (BD Pharmingen, San Jose, CA) (targeting alveolar macrophages) at 37°C, 10% CO₂ for 2 h. Non-attached cells were centrifuged, washed with 1x PBS (Gibco, Waltham, MA) and counted. A fraction was used to prepare cytopspins, cells were stained, and a differential cell count was performed. T2C purity was 95% as assessed by Papanikolaou staining. The remaining T2C pellet was resuspended in 500 μ L solution of DMEM supplemented with 40% fetal bovine serum (Gibco) and 10% DMSO (Sigma Aldrich, St. Louis, MO) and T2C were cryopreserved in liquid nitrogen until further use.

Gonadectomy and Ozone Exposure

Male and female SP-A1 and KO mice were gonadectomized (Gx) and exposed to O₃ (2 ppm) for 3 h and were sacrificed 4 h post OxS as described (51). The differentially expressed miRNAs from

Gx samples were identified by RNA sequencing as described previously (42, 52). The miRNAs identified from Gx mice were selected for analysis and changes in miRNA expression in SP-A1 mice were calculated by normalizing to KO as described previously (42). Samples from 16 animals (8 males and 8 females for SP-A1 and KO) were individually analyzed.

Isolation of miRNAs, qRT-PCR, and Statistical Analysis

Total RNA from the isolated mouse T2C was prepared using QIAzol Lysis Reagent (Qiagen, Valencia, CA) and the miRNA-enriched fraction was purified and used to generate cDNA, and then served as a template for real-time qPCR. Expression profiles of the 372 most abundantly expressed and best-characterized miRNAs in miRBase were then studied as described previously (42). The expression of miRNAs from FA and O₃ exposed T2C samples from SP-A1, SP-A2, and KO mice were analyzed as described previously (42). The variability across the 3 samples was assessed by *p*-values ($p < 0.0166$) and miRNAs with significantly changed levels were studied further ($p < 0.0166$). Bonferroni correction applied for sex, treatment, and genotype variability. The miRNA:gene target interactions were identified and reported in a format which enables direct transfer of results to genomic databases cataloging validated miRNA-target interactions as described previously (53, 54).

Gene Expression Analysis

To assess the expression of levels of *Ago2*, *Ddx20*, *Plcg2*, *Irs1*, *Elf2*, *Bcl2*, *Jak2*, *Map2k4*, *Bcl2*, *Ccnd1*, and *Vhl* genes at mRNA level in the male non-gonadectomized (NGx) and gonadectomized (Gx) KO and SP-A1 T2C, we performed qRT-PCR as described previously (42). The specific RT2 qPCR Primer assay was purchased from Qiagen. Cell samples were obtained from 3 separate animals/treatment (FA and O₃), and each sample was analyzed in triplicate/animal and quantified relative to *Gapdh* mRNA expression.

RESULTS

SP-A1 and SP-A2 Differentially Regulate the T2C miRNome

The expression levels of the hTG SP-A1 and SP-A2 T2C miRNomes were determined in males and females that were exposed to FA or O₃ and compared to the corresponding KO T2C. The miRNome levels are presented as volcano plots to show the fold change regulation differences between levels of miRNAs in hTG and KO mice, as well as their statistical significance (Figures 2, 3).

After FA exposure, which serves as control, in our experimental model, we observed in Figure 2A, a very tightly packed cluster of data points with few data points exceeding the cutoff for significance (Bonferroni corrected $p < 0.0166$), indicating that there are only a few differences between FA-exposed SP-A1 males and KO males. In Figure 2B, when the same comparison is made with female mice, we observed a very similar pattern to that of males, with only two miRNAs to exceed the significance threshold. Following O₃ exposure the SP-A1

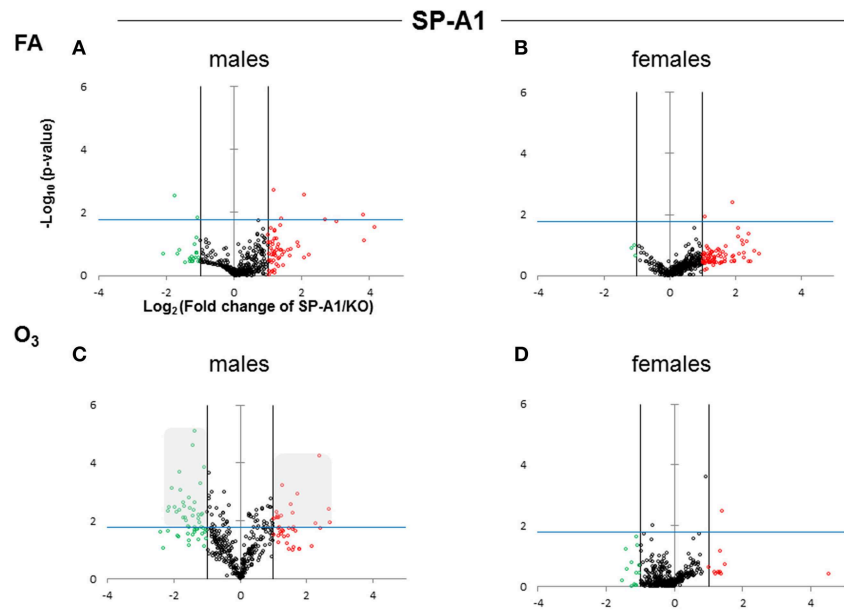


FIGURE 2 | Volcano plots indicating the statistical significance of SP-A1 T2C miRNome expression levels compared to SP-A KO under FA or O₃ exposure for males and females. The x-axis plots the log₂ of the fold-changes, while the y-axis plots the $-\log_{10}$ of their *p*-values based on *t*-test of the replicate raw Ct data (section Materials and Methods). Each plot has three vertical lines. The middle vertical line that is graded corresponds to zero changes. The lines on either side represent ≥ 2 -fold differences. Dots in the volcano plots above the blue horizontal line identify fold-changes with statistical significance of at least the Bonferroni corrected $p < 0.0166$. The red and green dots represent miRNAs that were upregulated ≥ 2 fold and downregulated ≥ 2 fold, respectively, compared to KO. Black dots signify miRNAs that were regulated < 2 -fold times (i.e., $x \leq 2$, x is the fold change). **(A)** Male SP-A1 mice compared to KO exposed to FA; **(B)** Female SP-A1 mice compared to KO exposed to FA; **(C)** Male SP-A1 mice compared to KO after O₃ exposure; **(D)** Female SP-A1 mice compared to KO after O₃ exposure. The shaded gray area in **(C)** shows the differences in the miRNAs that are highly and significantly regulated.

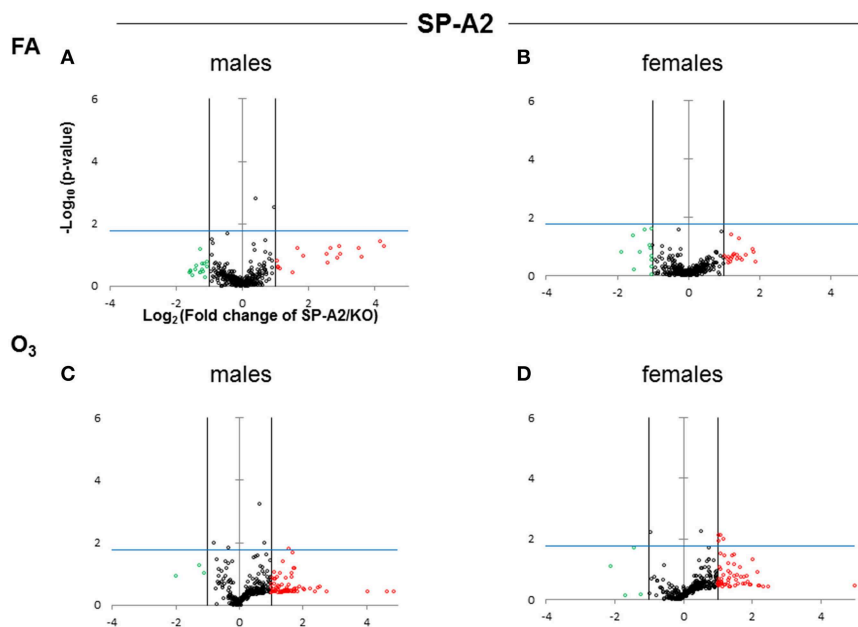


FIGURE 3 | Volcano plots (as described in **Figure 2**) indicating the statistical significance of SP-A2 T2C miRNome expression levels compared to SP-A KO under FA or O₃ exposure for males and females. **(A)** Male SP-A2 mice compared to KO exposed to FA; **(B)** Female SP-A2 mice compared to KO after FA exposure; **(C)** Male SP-A2 mice compared to KO after O₃ exposure; **(D)** Female SP-A2 mice compared to KO after O₃ exposure.

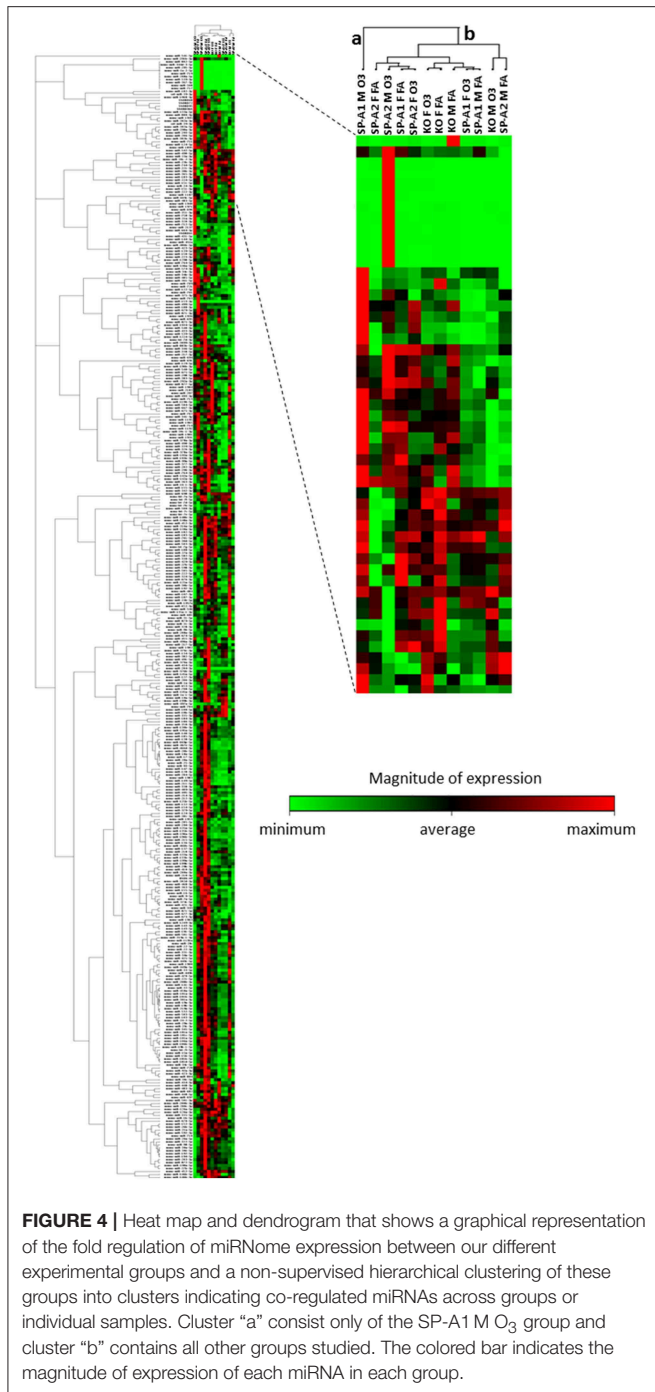


FIGURE 4 | Heat map and dendrogram that shows a graphical representation of the fold regulation of miRNome expression between our different experimental groups and a non-supervised hierarchical clustering of these groups into clusters indicating co-regulated miRNAs across groups or individual samples. Cluster “a” consist only of the SP-A1 M O₃ group and cluster “b” contains all other groups studied. The colored bar indicates the magnitude of expression of each miRNA in each group.

male mice (**Figure 2C**) show a very different picture. There are many more differences (both in magnitude and in significance) between O₃ exposed SP-A1 and KO males. SP-A1 females on the other hand show minimal changes compared to females KO (**Figures 2C,D**, respectively).

When we compared the SP-A2 hTG males and females after FA and OxS we do not see any robust differences as those seen with the SP-A1 male hTG (**Figures 3A–D**). It is immediately obvious that the pattern seen after OxS for the

SP-A1 males is unique (shaded area of **Figure 2C** compared to the rest of the panels of **Figure 2** and all panels of **Figure 3**) in the volcano plots analysis. This indicates that in response to OxS the male T2C miRNome of the SP-A1 mice is more responsive compared to the rest of the hTGs and exhibits a higher number of changed miRNAs that reach the Bonferroni corrected significance threshold $p < 0.0166$ (compare shaded area).

Oxidative Stress Has a Major Effect on the Male T2C miRNome

We performed a non-supervised hierarchical clustering of our entire dataset to display a heat map with a dendrogram indicating co-regulated genes across groups or individual samples (**Figure 4**). We found two distinct clusters a and b, with clade a being the one of SP-A1 male T2C miRNome, while the rest of our experimental animals clustered together in group b. A two-way ANOVA test for sex and treatment effects showed that the F-stat for the SP-A1 mice regarding the sex effect is $F = 161.91$ with $F \text{ crit} = 3.84$ and $p = 2.79 \times 10^{-5}$. The F-stat for the interaction between the two factors (sex and treatment) was $F = 16.69$ with $F \text{ crit} = 3.84$ and $p = 4.63 \times 10^{-5}$. The same analysis for the SP-A2 mice miRNome did not show that sex, treatment, or the combination of these two factors were significantly different (F-stat for SP-A2 mice was $F = 1.24$ lower than the $F \text{ crit} = 3.84$ and p -value not significant $p = 0.265$). These data show that in the T2C miRNome, there is a difference between sexes in response to O₃ exposure as a function of SP-A variants.

mRNA Targets of the Male SP-A1 T2C miRNome Associate With Cell Cycle, Apoptosis, and MAPK Pathway

To better understand and integrate the T2C miRNome data, we performed Ingenuity Pathway Analysis (IPA) for the T2C miRNAs whose expression was significantly altered by OxS. Only miRNAs that were shown to pass the corrected Bonferroni $p < 0.0166$ and a false discovery rate (FDR)-adjusted $q < 0.05$ were used to ensure that sex, treatment, gene, and array variability do not lead to false discoveries. Fifty-four miRNAs met the above criteria and were used for IPA. This analysis identified several mRNA transcripts, the expression of which could be affected by approximately half of the miRNAs selected for IPA. The targets identified include the following transcripts *Ago2*, *Ddx20*, *Plcg2*, *Irs1*, *Elf2*, *Jak2*, *Map2k4*, *Bcl2*, *Ccnd1*, and *Vhl* mRNAs. The levels of miRNAs that targeted the above molecules and were significantly changed in SP-A1 males in response to OxS are shown on **Table 1**.

Next, we performed qRT-PCR to assess the expression levels of *Ago2*, *Ddx20*, *Plcg2*, *Irs1*, *Elf2*, *Jak2*, *Map2k4*, *Bcl2*, *Ccnd1*, and *Vhl* genes in the male KO and SP-A1 T2C (**Figure 5**). To our surprise, we observed that in response to OxS the levels of *Ago2*, *Elf2*, *Jak2*, *Map2k4*, *Ccnd1*, and *Vhl* were significantly upregulated in the KO T2C but remained unaffected in the SP-A1 T2C. Also, the *Bcl2* gene was significantly downregulated only in the KO T2C. The above molecules are involved in several pathways including mitogen activated protein kinases (MAPK) signaling pathway, cell cycle, and apoptosis.

TABLE 1 | Levels and statistical significance of the male SP-A1 T2C miRNAs in OxS and shown by IPA to be directly associated with genes *Ago2*, *Ddx20*, *Plcg2*, *Irs1*, *Eif2*, *Bcl2*, *Jak2*, *Map2k4*, *Bcl2*, *Ccnd1*, and *Vhl*.

Mature miRNA ID	Fold regulation	p-value	FDR (q-value)	Target gene(s)	PMID	Validation experiments*
miR-124-3p	-2.6902	0.006674	0.038944405	<i>Ago2</i> , <i>Ccnd1</i> , <i>Eif2</i>	27577603	HITS-CLIP
miR-135a-5p	-2.9089	0.001569	0.037656	<i>Jak2</i>	30854107	Luciferase
miR-141-3p	-3.3007	0.007623	0.038944405	<i>Map2k4</i>	28454307	WB, IHC, qRT-PCR
miR-143-3p	-3.4508	0.000843	0.029428364	<i>Bcl2</i>	29581736	WB
miR-143-5p	-2.5534	0.00237	0.038944405	<i>Bcl2</i>	20878132	qRT-PCR
miR-148a-3p	-2.193	0.00597	0.038944405	<i>Ppara</i> and indirectly <i>Bcl2</i>	26001136	Luciferase
miR-153-3p	-4.1674	0.000733	0.0281472	<i>Bcl2</i>	30537994	Luciferase
miR-190a-5p	-3.1766	0.003205	0.038944405	<i>Ddx20</i>		<i>in silico</i> report
miR-19b-3p	-3.5226	0.004329	0.038944405	<i>Ccnd1</i>	29455644	WB
miR-204-3p	2.2073	0.007978	0.038944405	N/A		
miR-208a-3p	-3.7777	0.004875	0.038944405	<i>Ddx20</i>		<i>in silico</i> report
miR-20b-5p	-2.2419	0.00638	0.038944405	Indirect effect on <i>Bcl2</i>	30816540	Luciferase
miR-219a-5p	-16.1578	0.000011	0.002112	<i>Plcg2</i>	20956612	qRT-PCR
miR-223-3p	6.3634	0.003918	0.038944405	<i>Irs1</i>	29286159	qRT-PCR
miR-26a-5p	-2.3634	0.006167	0.038944405	Indirect effect on <i>Htr1a</i>	30766477	Luciferase
miR-29b-3p	-2.6192	0.004297	0.038944405	<i>Ago2</i>		IP
miR-301a-3p	-3.0775	0.010054	0.045186977	Indirect effect in <i>Ago2</i>	28332581	qRT-PCR
miR-302a-3p	2.2073	0.007978	0.038944405	Indirect effect in <i>Cod1</i>	28510621	Luciferase
miR-302a-5p	2.2073	0.007978	0.038944405	Indirect effect in <i>Cod1</i>	28510621	Luciferase
miR-34b-3p	2.1409	0.004946	0.038944405	N/A		
miR-499-5p	-2.6842	0.000025	0.0032	<i>Sox6</i>	31076992	qRT-PCR
miR-539-3p	2.2073	0.007978	0.038944405	<i>Ntrk3</i>	21143953	Luciferase
miR-708-5p	-2.3546	0.008364	0.039182049	<i>Vhl</i>	21852381	qRT-PCR
miR-758-3p	3.3301	0.001181	0.032393143	Indirectly <i>Bcl2</i>	31138034	qRT-PCR

PMID, unique identifier number used in PubMed for miRNA; -, indicates downregulation; FDR, false discovery rate; HITS-CLIP, high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation; WB, western blot; IHC, immunohistochemistry; qRT-PCR, quantitative reverse transcription polymerase chain reaction; IP, immunoprecipitation; N/A, non-applied; *, last column shows the validation techniques used for each miRNA as noted in the PMID.

Effect of Gonadectomy and OxS on the Expression of miRNAs in SP-A1 Male and Female Mice

To study the effect of sex hormones on the expression of miRNAs after OxS, we performed miRNA expression analysis in T2C from gonadectomized (Gx) SP-A1 and KO male and female mice and compared it with that of non-gonadectomized (NGx) mice after O₃ exposure.

For this analysis, we used 120 miRNAs that were identified in both NGx (males and females) and Gx (males and females) groups. Of these, in the NGx (males vs. females) group, 89 miRNAs had their levels significantly changed (fold change ≥ 2) after FA exposure (Figure 6A). In the Gx group (male vs. female) compared to the corresponding NGx (male vs. female) group, expression of 9 miRNAs (10.1%) was significantly increased (fold change ≥ 2), and expression of 61 miRNAs (68.53%) was significantly decreased (fold change ≥ 2) (Figure 6A). In response to OxS, the level of 56 miRNAs was significantly altered (≥ 2 fold) in NGx (male vs. female) groups (Figure 6B). Following, comparison of the Gx group (male vs. female) to the corresponding NGx (male vs. female) group, the expression of 5 miRNAs (8.9%) was significantly increased (≥ 2 fold), and

the expression levels of 33 (58.9%) miRNAs was significantly decreased (≥ 2 fold) (Figure 6B). Of the 89 (Figure 6A) and 56 (Figure 6B) miRNAs differentially expressed in Gx males vs. females after FA and O₃ exposure, 24 miRNAs (26.96%) are specific to FA exposure and 16 miRNAs (17.97%) are specific to O₃ exposure (Figure 6C). Moreover, a one-way ANOVA pertaining to the gonadectomy effect on the miRNA expression showed a significant difference with F stat = 120.5 with F crit = 3.88 and $p = 5.95 \times 10^{-23}$ (see Supplementary File 1) indicating that sex hormones play a role.

Furthermore, we monitored the expression levels of the target genes discussed above after gonadectomy in both SP-A1 and KO mice (Figure 7). We found that Gx had a major effect in three genes (*Irs1*, *Bcl2*, *Vhl*) in KO mice. *Irs1* was upregulated in Gx KO only. No significant change was observed for IRS1 in the NGx KO (Figure 4). *Bcl2* and *Vhl* showed decrease and increase expression, respectively, compared to control *Gapdh* mRNA (Figure 7); both of these had shown the reverse in NGx (Figure 5). Of interest, *Ccnd1* was upregulated in KO regardless of Gx. These data indicate that sex hormones play an important role in the observed miRNA sex differences by affecting regulation of miRNAs, as most of the miRNAs that were increased in NGx were decreased in Gx.

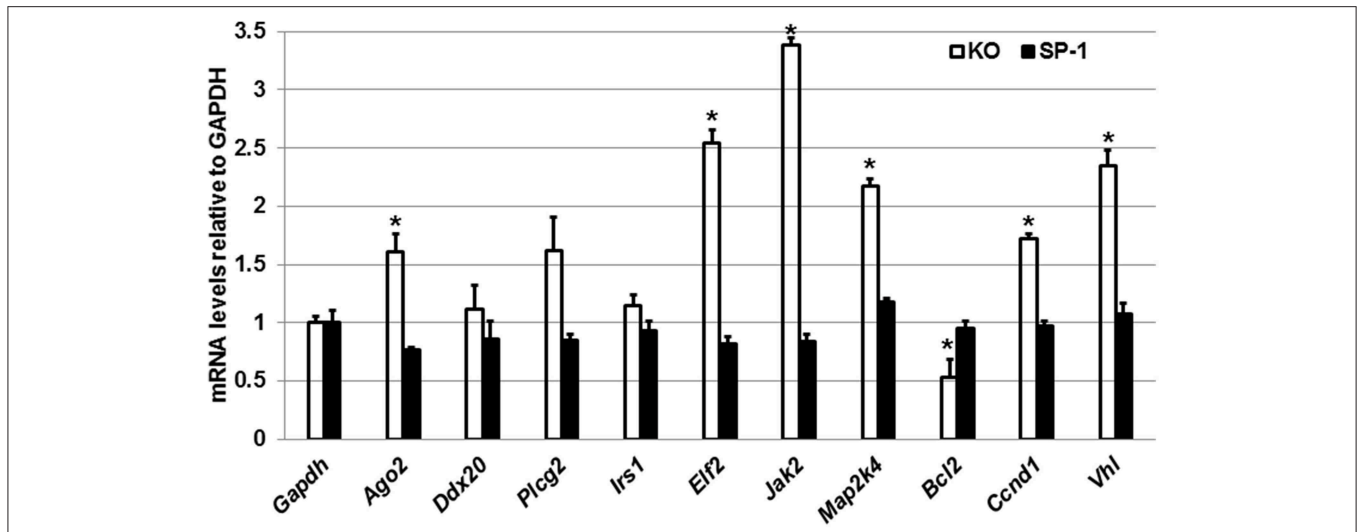


FIGURE 5 | Effect of OxS in the T2C transcriptome of males. mRNA levels of *Gapdh*, *Ago2*, *Ddx20*, *Plcg2*, *Irs1*, *Elf2*, *Bcl2*, *Jak2*, *Map2k4*, *Bcl2*, *Ccnd1*, and *Vhl* genes shown to be targeted by the T2C miRNome were measured in KO and SP-A1 male mice 4 h post O₃ exposure. mRNA levels were measured by qRT-PCR and normalized to *Gapdh*. In KO the *Jak2*, *Elf2*, *Vhl*, *Map2k4*, *Ccnd1*, and *Ago2* were significantly upregulated by 3.4-, 2.5-, 2.3-, 2.2-, 1.7-, and 1.7- fold ($p < 0.05$), respectively, while the *Bcl2* was significantly downregulated by 0.5-fold. The levels of those mRNAs did not change in the SP-A1 mice. *means $p < 0.05$.

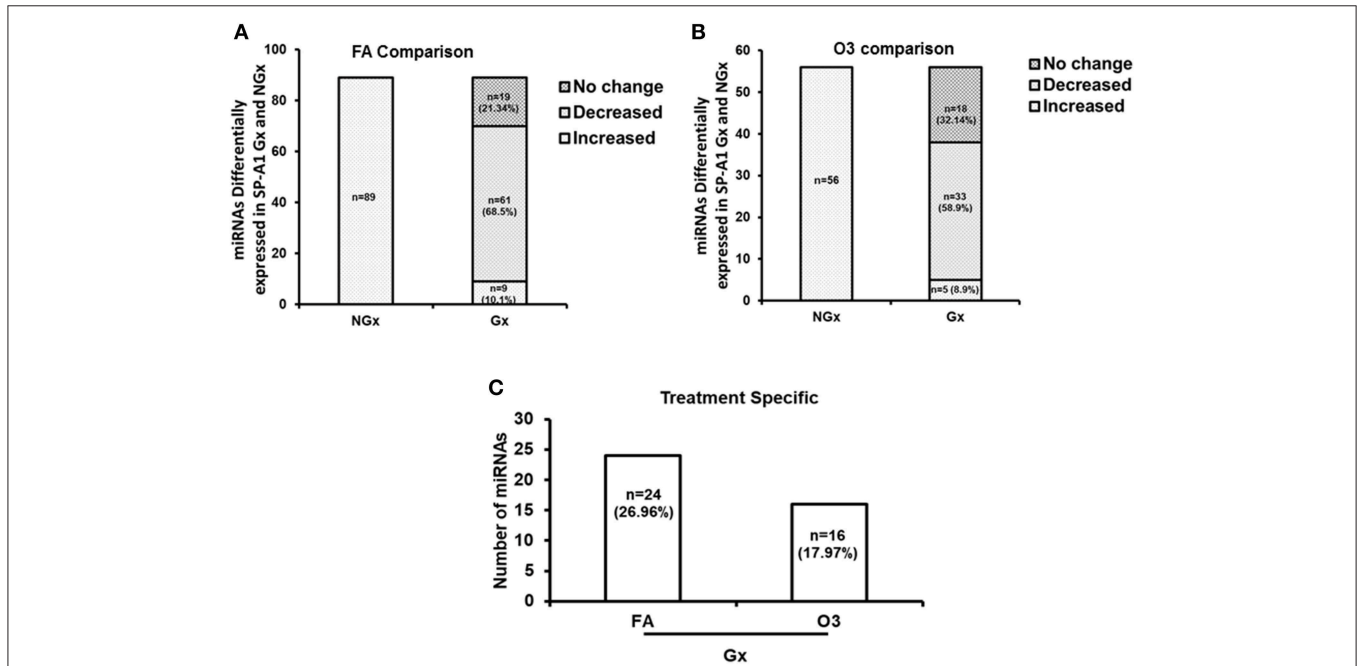


FIGURE 6 | The effect of gonadectomy and OxS on T2C miRNA expression profiles of SP-A1 mice. **(A)** The differentially expressed miRNAs in SP-A1 non-gonadectomized (NGx) and gonadectomized (Gx) mice were identified after normalizing to corresponding NGx and Gx KO. NGx shows the miRNAs ($n = 89$) that changed significantly (≥ 2 -fold) in FA when males were compared to females. Gx depicts the comparison of Gx values (male vs. female) to NGx (male vs. female). Out of the same 89 miRNAs (found to have their levels increased in NGx), 9 miRNAs (10.1%) showed a significant increase (≥ 2 -fold) and 61 miRNAs (68.53%) showed a significant decrease (≥ 2 -fold). **(B)** NGx shows the miRNAs ($n = 56$) that changed significantly (≥ 2 -fold) in OxS when males were compared to females. Gx depicts the comparison of Gx values (male vs. female) to NGx (male vs. female). Out of the same 56 miRNAs (found to have their levels increased in NGx), 5 miRNAs (8.9%) showed a significant increase (≥ 2 -fold) and 33 miRNAs (58.9%) showed a significant decrease (≥ 2 -fold). **(C)** Depicts the comparison of the 89 and 56 differentially expressed miRNAs identified between Gx males and females. Out of 89 miRNAs studied, 24 miRNAs (26.96%) are significantly increased in FA (≥ 2 -fold), and 16 miRNAs (17.97%) are significantly increased in OxS (≥ 2 -fold).

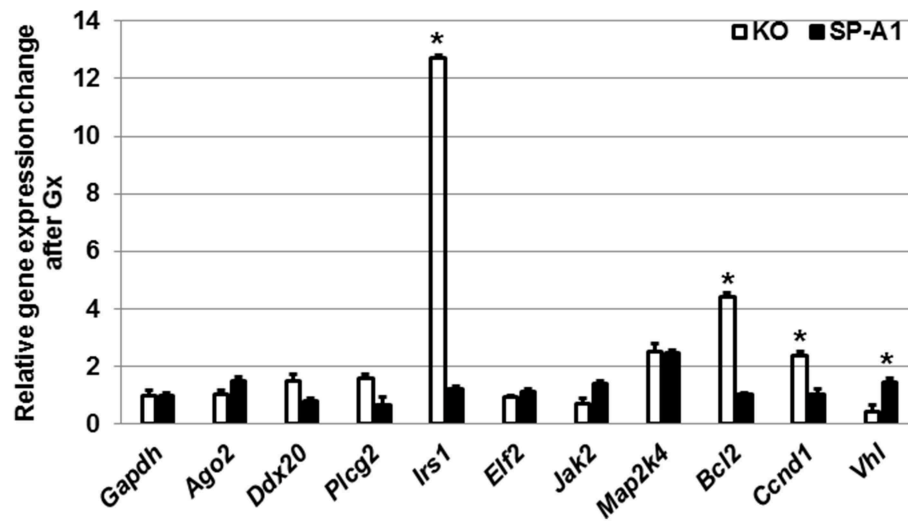


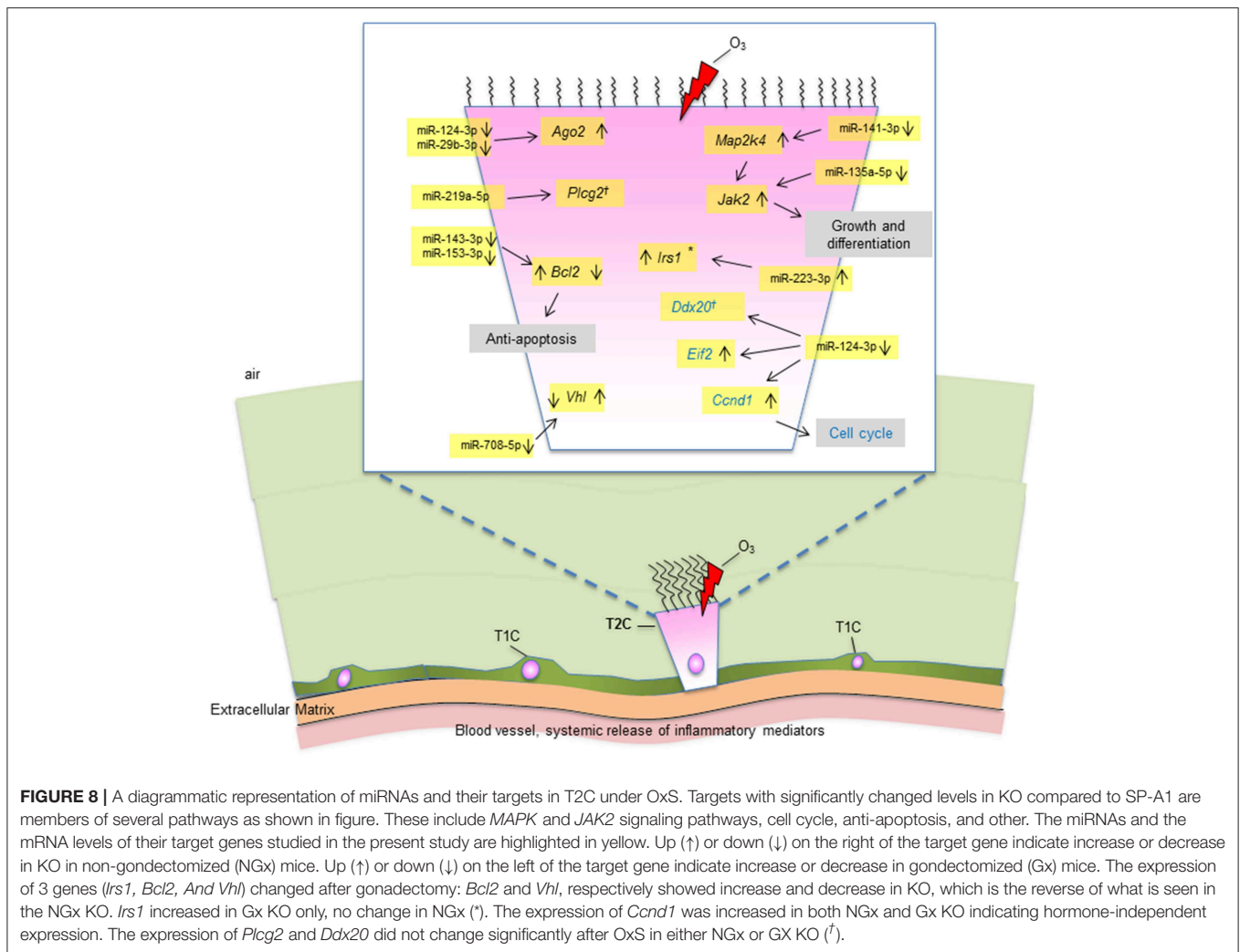
FIGURE 7 | The effect of gonadectomy and OxS on genes that are targeted by the T2C miRNome. Genes were measured by qRT-PCR in KO and SP-A1 gonadectomized (Gx) male mice 4 h post O₃ exposure and normalized to housekeeping gene *Gapdh*. The graph shows the relative expression changes of mRNAs Gx mice compared to non-gonadectomized. In the Gx KO the *Irs1*, *Bcl2*, and *Ccnd1* expression levels were increased 13-, 4.5-, and 2.8-fold ($p < 0.05$), respectively. In the Gx SP-A1 the *Vhl* gene was significantly increased in SP-A1 by 1.8-fold. *means $p < 0.05$.

DISCUSSION

We have previously shown a differential effect of SP-A1 and SP-A2 proteins on AM function (31, 37, 38), AM proteome (34, 35) and AM miRNome (42) as well as sex differences after OxS. In the present study, we investigated the effect of OxS on mouse T2C under the influence of either SP-A1 or SP-A2 and compared it to KO mice as well as studied the role of sex hormones in the T2C miRNome of the SP-A1 hTG mice. We found significant changes after O₃ exposure in SP-A1 males but not in the other animals. When a non-supervised hierarchical clustering analysis on the entire dataset was performed, we observed that the O₃-exposed SP-A1 male miRNome clustered separately from the rest of the experimental animals showing that OxS has a major effect on the male SP-A1 T2C miRNome. Also, a two-way ANOVA analysis showed that there is an interaction between the male sex hormones and the SP-A1 gene under the effect of OxS. Gonadectomy had a major effect on the expression of the T2C miRNome compared to non-gonadectomized mice. Our miRNome analysis in the T2C that was subjected to OxS was based on strong validation methodologies. Ingenuity Pathway Analysis (IPA), pairs miRNAs/mRNA targets based not only on the gold standard *in silico* predicted algorithms (miRBase, miRTarBase, miRWalk, Targetscan, etc.) but also on experimental data from the published literature. With IPA we showed that miRNAs that were changed significantly >2-fold in male SP-A1 T2C mice, targeted genes that are involved in the MAPK signaling pathway, cell cycle, and anti-apoptosis. We monitored/validated experimentally via qRT-PCR which of the predicted mRNA targets are responding to the effect of OxS. Gene expression analysis of the target mRNAs of interest surprisingly showed that the OxS affected predominantly the KO mouse while the SP-A1 mouse showed no significant shifts in the expression

levels of the same genes. Gonadectomy of male SP-A1 and KO mice prior to O₃ exposure led to significant changes in the expression levels of three genes (*Irs1*, *Bcl2*, and *Vhl*) in KO, whereas the expression of *Ccnd1* remained increased in KO regardless of Gx. These data indicate that sex differences are in part attributable to circulating gonadal hormones (51), which are believed to influence the innate immune responses. The specific roles of these hormones and the underlying mechanisms of regulation remain yet to be explored.

The mRNA targets of the SP-A1 T2C miRNAs that were changed significantly in the non-gonadectomized males under OxS included genes being involved in the MAPK signaling, apoptosis, and cell cycle. Although the activation of the MAPK pathway by OxS has been described before in other systems and tissues (55–57), in the present study our data show that there are miRNAs that may regulate genes of the MAPK signaling pathways in the respiratory alveolar epithelial T2C. The validation experiments on the mRNA targets showed that the mRNAs of interest (that were targeted by significantly changed miRNAs, **Figure 2C**) are particularly responsive in the male KO but not responsive in SP-A1 except the *Bcl2* which showed increased levels in SP-A1. The increase of *Bcl2* in SP-A1 indicates that the SP-A1 T2C may be protected against apoptosis. Previously, we have shown that AM of SP-A2 hTG mice were also protected from apoptosis under OxS (42), indicating a differential effect of the two SP-A genes, *SFTPA1* and *SFTPA2*, in T2C and AM, respectively, with regards to apoptosis. However, the fact that most of the target genes in SP-A1 remained unaffected after OxS is puzzling. We speculate that this may in part be due to: (1) The time point used (4 h after OxS) for study is not optimal to assess mRNA levels of target genes in SP-A1; (2) the target genes may recover from OxS faster in SP-A1 than in KO; (3) SP-A1 may protect other molecules from harmful effects of OxS



by being more readily oxidized by scavenging ROS (48); (4) SP-A1 may protect the T2C by affecting functions modulated by molecules that are not regulated by miRNAs. An example of this may be its role in surfactant structural organization (41) and potentially lung function. Another interesting observation was the significant upregulation of Von Hippel-Lindau (*Vhl*) mRNA, targeted by miR-708-5p (58), in the gonadectomized (Gx) SP-A1 T2C when the experimental male mice were exposed to O₃. It is known that *Vhl* is part of a degradation complex that removes damaged or unnecessary proteins and helps maintain the normal functions of cells (59). In particular, this degradation complex is known to degrade proteins when oxygen levels are lower than normal, such as hypoxia (60) and possibly in OxS. The increase in *Vhl* in Gx SP-A1 mice (but not in NGx) indicates that sex hormones may play a role in its expression in SP-A1 mice.

The increase of several targets in KO but not in SP-A1 may point to deficiencies in KO and that these increases may reflect a more active gene regulation to overcome the effects of OxS. These include, the mitogen-Activated Protein Kinase 4 (*Map2k4*) and Janus Kinase 2 (*Jak2*), which are targeted by miR-141-3p (61) and miR-135a-5p (62) and shown

here to change by >2-fold; both targets are involved in the MAPK signaling. These could transmit the OxS distress signal across the cell membrane to the DNA in the nucleus triggering a number of different functions such as apoptosis, cell differentiation and proliferation. The argonaute 2 (*Ago2*) mRNA, which is targeted by miR-124-3p and miR-29b-3p (63), was increased significantly in the KO. *Ago2* is an important enzyme in the biogenesis of miRNAs that plays a role in the formation of the RNA-induced silencing complex (64). Of interest, cyclin D1 (*Ccnd1*) was upregulated in Gx and NGx KO males indicating independence of circulating hormones, and possibly a dependence on the presence or absence of SP-A, no change in *Ccnd1* was observed in SP-A1 T2C either in Gx or NGx males. The eukaryotic initiation factor 2 (*eIF2*) was upregulated in NGx KO but not in the Gx KO mice indicating a role of hormones in its expression. These mRNAs (*Ccnd1* and *eIF2*) are targeted by miR-124-3p. This molecule is known to regulate global and specific mRNA translation in response to stress-related signals (such as OxS) (65).

Collectively, our data show that OxS has a major effect on the male SP-A1 T2C miRNome. The targets of the significant

miRNAs are implicated in several pathways that include the MAPK signaling pathway (*Mapk*, *Jak2*, *Irs1*), cell cycle (*Ccnd1*), anti-apoptosis (*Bcl2*), protein degradation (*Vhl*), and other. These observations diagrammatically are depicted in **Figure 8**. The limitations of the present study are that we studied a single time point and we did not look at the protein levels of the targeted mRNAs. Our publication on the alveolar macrophages (AM) miRNome showed that in response to OxS, it was SP-A2 that had a major effect on the male AM with pro-inflammatory, anti-apoptotic, and anti-oxidant pathways playing a role (42). The two studies together indicate that there is a differential role of SP-A1 and SP-A2 in the alveolar cells. The SP-A1 findings in the present study are consistent with our previous observations where SP-A1 is shown to play a role in the surfactant structural organization (41), and may also play an important role on the integrity and function of T2C. SP-A2 on the other hand has been shown to not only affect the AM male miRNome but also exhibit a higher innate immune activity (31, 36, 38).

We conclude that dysregulation of either SP-A1 or SP-A2 may affect the innate immunity and/or surfactant structure and potentially lung function. Both processes are essential for normal lung function and derangement of the regulation of either gene may be a problem in pulmonary diseases, including OxS.

ETHICS STATEMENT

All protocols used in this study were evaluated and approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee and conformed to the guidelines of the National Institutes of Health on the care and use of laboratory animals.

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AUTHOR CONTRIBUTIONS

GN performed experiments, run statistics, analyzed and synthesized the data, contributed to the manuscript writing. NT performed experiments, analyzed and synthesized the data, contributed to the manuscript writing. XZ and TU performed maintenance and breeding of mouse lines, exposed mice to experimental conditions (FA & O₃) and isolated alveolar type II cells. FD performed all gonadectomy experiments. DP contributed to data analysis and synthesis and manuscript writing. JF designed and provided oversight to the entire project, involved in data analysis, integration, and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.01514/full#supplementary-material>

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Conflict of Interest Statement: 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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