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Gonadotropin induced transcriptional and epigenetic regulation of gene expression in ovarian granulosa cells

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ABSSTRACT

Mammalian pituitary gland secretes two gonadotropins (**GPNs**), follicle-stimulating hormone (**FSH**) and luteinizing hormone (**LH**) that act on the gonads to regulate gonadal development and functions. In females, FSH promotes the development of ovarian follicles, and LH induces oocyte maturation, induction of ovulation, and formation of corpus luteum. Oocytes do not express the GPN receptors, FSHR or LHCGR. GPN responses are accomplished by follicular somatic cells, theca cells (**TCs**) and granulosa cells (**GCs**) that surround the oocytes and express GPN receptors. GPN signaling regulates the expression of genes in TCs and GCs, which are essential for ovarian functions. We have studied the GPN-induced regulation of genes in GCs using immature rats. Four-week-old female rats were administered with exogenous GPNs, pregnant mare's serum GPN (**PMSG**), and human chorionic GPN (**hCG**), which act like long acting FSH and LH, respectively. GCs were isolated from the rat ovaries 48h after PMSG administration, 4hr after hCG administration following the PMSG-priming or without GPN administration. RNA-sequencing was performed to identify the differentially expressed genes in GCs following PMSG or PMSG and hCG administration. The majority (~87%) of the differentially expressed genes 48h after PMSG treatment were found to be downregulated indicating that gene repression plays a crucial role in FSH-induced follicle development. However, hCG treatment upregulated a group of the genes in GCs that was repressed by PMSG. Strikingly, hCG administration rarely impacted the expression of any GC-gene, which was not modulated by PMSG administration. This observation suggests that FSH-induced epigenetic and/or transcriptional regulation is essential for subsequent LH actions in GCs. We further investigated the mechanism of GPN-mediated gene regulation in GCs by methyl-sequencing of genomic DNA. Our results demonstrated that changes in genome-wide DNA methylation is an important epigenetic process that is vital for GPN-mediated gene regulation in GCs.

INTRODUCTION

Follicle stimulating hormone (**FSH**) and luteinizing hormone (**LH**) are the two gonadotropins (**GPNs**) that are secreted from the anterior pituitary gland and act on the ovarian follicles. GPNs play essential roles in ovarian follicle development, oocyte maturation, induction of ovulation, and formation of corpus luteum[1, 2]. While FSH stimulates ovarian follicle development, LH mediates the preovulatory oocyte maturation, induces ovulation and luteinization. Although GPN-stimulation of the ovarian follicles is essential for the induction of oocyte maturation, oocytes do not express the GPN receptors (**Fig. 1**). The somatic cells in ovarian follicles, granulosa cells (**GCs**), and theca cells (**TCs**) are the actual sites of GPN action. GCs surrounding the oocytes express both LH receptors (**LHCGRs**), and FSH receptors (**FSHRs**) but **TCs** that surround the GCs only express the LHCGR (**Fig. 1**).
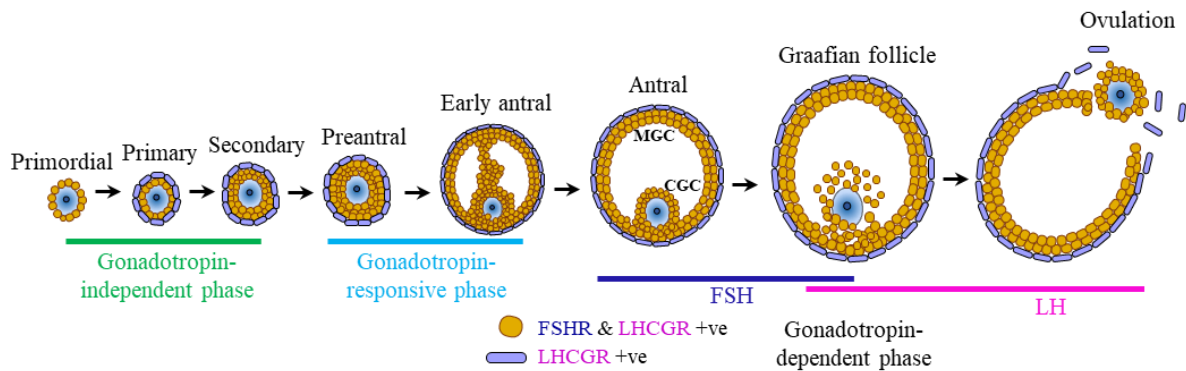
Fig. 1 The role of gonadotropins on ovarian folliculogenesis. FSH stimulates the development of small antral follicles to the Graafian stage, whereas LH mediates further preovulatory maturation of the oocytes, and induces ovulation as well as the luteal transformation of granulosa cells (GCs) following ovulation. Gonadotropin (GPN) responses are accomplished by the follicular somatic cells, GCs, and theca cells (TCs) because oocytes do not express the GPN receptors.

Ovarian steroidogenesis starts in the TCs, which is completed in the neighboring antral GCs. Cumulus GCs that are in contact with the oocytes, relays the response of GPN signaling to

oocytes. During the ovarian follicle development, once the somatic cells express GPN receptors, they become GPN responsive, but development of ovarian follicle beyond antral stage is completely dependent on GPNs. The GPN receptors LHCGR and FSHR are G-protein coupled receptors that primarily signal through cAMP-dependent mechanisms (**Fig. 2**). However, a crucial step in GPN signaling is mediated through activation or inhibition of transcriptional regulators that induces or represses genes in GCs and TCs.
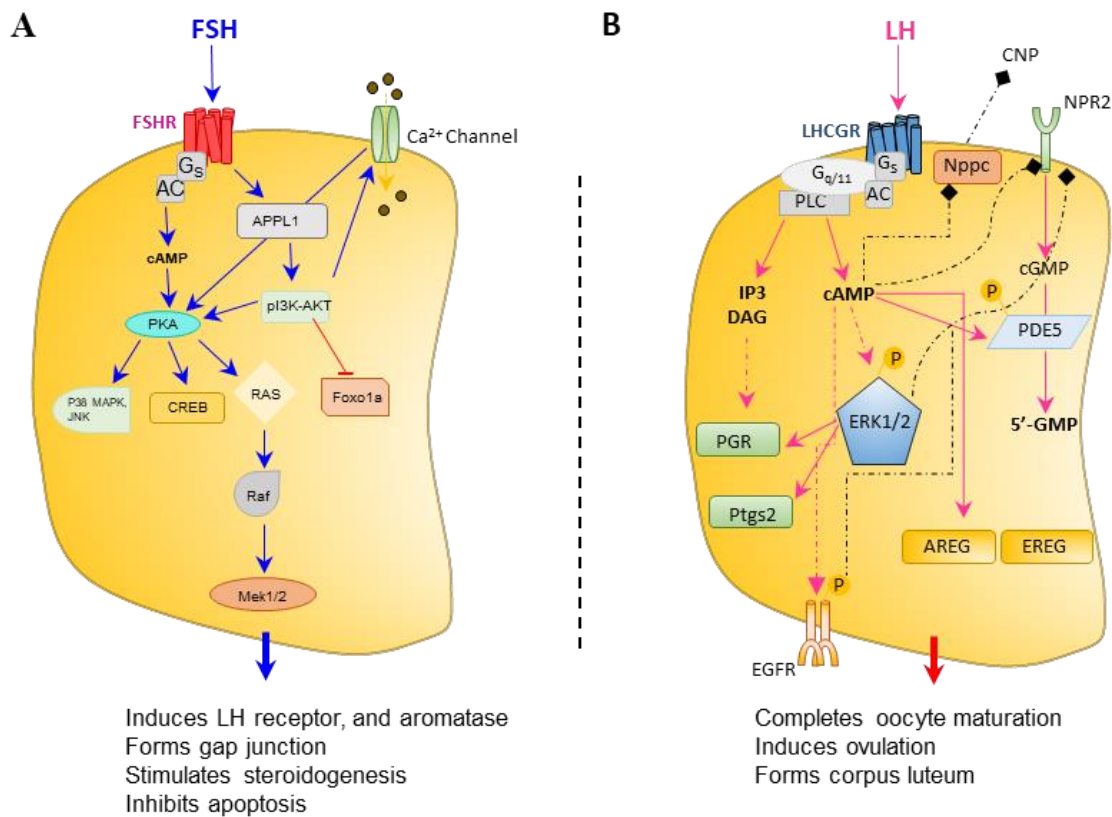
Fig. 2 FSH and LH signaling in granulosa cells. FSHR and LHCGR are G protein-coupled receptors (GPCRs) expressed on granulosa cells (GCs) that binds FSH (or PMSG) and LH (or hCG), respectively. **A**) Binding of FSH activates FSHR, which stimulates adenylyl cyclase and increase production of cAMP. Effectors of cAMP lead to upregulation of LHCGR, and CYP19A1 essential for follicle development. **B**) Following FSH (or PMSG) stimulation, LH signaling is dramatically increased in GCs. The binding of LH to LHCGR activates Gs, which increases cAMP levels within GCs. This leads to induction of LH responsive genes, which are essential for the final stages of oocyte maturation, and induction of ovulation.

In this study, we have determined the GPN-regulated changes in gene expression in GCs using an immature rat ovary model[3-6]. Four-week-old immature rats were exposed to exogenous GPNs, pregnant mare's serum GPN (PMSG that acts like FSH), and human chorionic GPN (hCG that acts like LH). GCs were isolated from the ovaries with or without GPN administration and used for RNA-sequencing and genome wide methyl sequencing analyses. We observed a large number (~87%) of the differentially expressed genes (**DEGs**) to become repressed following PMSG treatment and a group of the repressed genes were upregulated following hCG treatment. We observed that hCG administration rarely impacted the expression of GC-genes, which was not modulated by PMSG. We hypothesized that an epigenetic priming occurs in GCs following PMSG treatment, which is essential for hCG to regulate a gene. Accordingly, we performed methyl-seq analyses of genomic DNA purified from GCs following GPN treatment. Our results demonstrated that changes in genome-wide DNA methylation is an important epigenetic process for GPN induced gene regulation in GCS.

MATERIALS AND METHODS

Animal models. Immature Holtzman Sprague Dawley (HSD) female rats were included for studying GPN induced transcriptional and epigenetic changes in GCs. The rats had access to food and water ad libitum and housed in 12/12-hour dark/ light cycles throughout the study. Only the female rats were included in the study whereas a few of the male rats were used only for the breeding purpose. All procedures were performed in accordance with the protocols approved by the University of Kansas Medical Center Animal Care and Use Committee.

Gonadotropin treatment and ovulatory response. Four-week-old wildtype female HSD rats were administered GPNs (PMSG and hCG) to induce the development and maturation of ovarian follicles as described in our previous publications[6-9]. Briefly, 30 IU of PMSG (BioVendor LLC, Asheville, NC) dissolved in 300 µl of normal saline was intraperitoneally injected to the immature female rats (**Fig. 3A**).
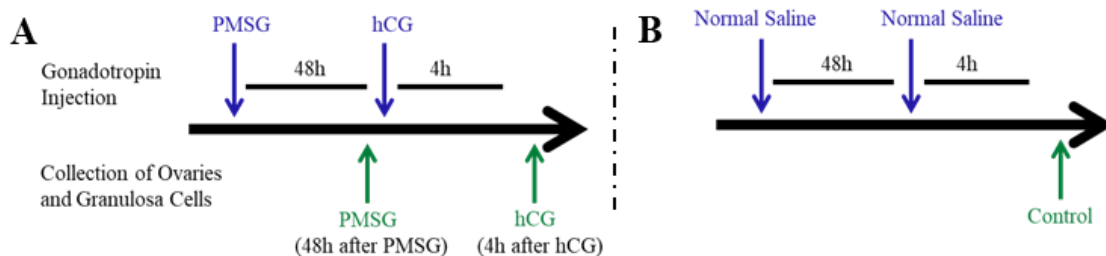
Fig. 3 Timeline of gonadotropin administration and collection of rat ovaries. Four-week-old wildtype female rats were injected with gonadotropins (PMSG and hCG) as shown in the schematic diagram (**A**). Ovaries were collected 48h after PMSG injection (PMSG group), and 4h after hCG administration to PMSG primed rats (**A**). Age-matched wildtype female rats were also administered normal saline instead of PMSG or hCG in the control group (**B**). Ovaries were washed in M199 media and immediately processed for granulosa cell isolation.

Forty-eight hours after the PMSG administration, one group of rats were euthanized to collect their ovaries and another group of rats were intraperitoneally injected with 30 IU of hCG (BioVendor LLC) dissolved in 300 µl of sterile normal saline. Age matched female rats were injected with 300 µl of normal saline and their ovaries were collected and used as control to assess the basal gene expression. Collected ovaries were either processed for histological examination or collection of GCs for molecular analyses.

Histological analyses. Ovaries were fixed in 10% Neutral Buffered Formalin containing ~4% formaldehyde (Epredia Netherlands B.V., DA Breda, Netherlands) for 24 hours. Following fixation, the ovaries were washed and stored in 70% ethanol at 2-8ºC refrigerator until subsequent processing. Fixed ovaries were dehydrated using an automated tissue processor (Leica ASP6025, Deer Park, IL) and embedded in paraffin on an automated tissue embedding station (HistoCore Arcadia, Deer Park, IL). Tissue blocks were sectioned at 5µm thickness using a microtome (Leica Rm2255, Deer Park, IL). Microscopic examination was performed on the ovary sections following hematoxylin and eosin (H&E) (Epredia Netherlands) staining, following standard procedure (Ref). H&E-stained slides were mounted using EcoMount media (BioCare Medical, Pacheco, CA).

Isolation of grnaulosa cells. Ovaries were washed in Media 199 (M199, Cytiva, Marlborough, MA) and kept in a 35mm noncoated sterile Petri dish immersed in cold M199. Cumulus oocyte complexes (COCs) were isolated from the large antral follicles by 25G needle puncture under stereomicroscopic observation as described previously[6-9]. Cumulus GCs were dissociated from the oocytes by using repeated pipetting, and both cumulus and mural GCs were filtrated through a 15µM cell strainer (PluriSelect USA, El Cajon, CA) to exclude the oocytes. GCs were washed twice with cold sterile phosphate buffered saline (PBS) (ThermoFisher Scientific),

pellets were snap frozen in liquid nitrogen and stored at -80ºC freezer until total RNAs or genomic DNAs were extracted.

Purification of total RNA from granulosa cells. Total RNA was extracted from GCs by using TRI Reagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instruction. Extracted RNA samples were frozen at the isopropanol mediated precipitation step in a -80ºC freezer until downstream uses. The last steps of total RNA purification and quantification were performed immediately before the uses including library preparation for RNA-sequencing and cDNA preparation RT-qPCR analyses.

RNA-sequencing of granulosa cells. Five hundred ng of total RNA was used for the RNA-seq library preparation. Libraries were prepared by using a TruSeq stranded mRNA sequencing library preparation kit (Illumina, San Diego, CA) following the manufacturer's instructions. Brief procedure of the library preparation have described in our previous publications[7]. RNA quality was assessed on an Agilent Bioanalyzer, and samples with RIN values over 9 were selected for mRNA-seq library preparation. Each library was prepared using RNA samples from 3 individual rats and 3 independent libraries were made for each experimental time point. The cDNA libraries were first evaluated for quality at the KUMC Genomics Core and then sequenced on an Illumina HiSeq X sequencer (Novogene Corporation, Sacramento, CA). All the RNA-seq data are available at SRA (**PRJNA551764**) (**Appendix I**).

Analyses of RNA-sequencing data. RNA-sequencing data were demultiplexed, trimmed, aligned, and analyzed using CLC Genomics Workbench 23.0.3 (Qiagen Bioinformatics, Germantown, MD) as described previously[6-8, 10]. Trimming was performed to remove low quality reads, and good quality reads were aligned with the *Rattus norvegicus* genome (Rnor_6.0) using the default parameters: (a) maximum number of allowable mismatches was 2; (b) minimum length and similarity fraction was set at 0.8; and (c) minimum number of hits per read was 10. Gene

expression values are shown as TPM (Transcripts per million base pairs). The threshold *p*-value was determined according to the false discovery rate (FDR). DEGs were selected if the absolute fold change in expression was ≥ 2 with an FDR p-value of ≤0.05. RNA-seq results were validated by RT-qPCR analyses of selected genes.

   cDNA preparation and RT-qPCR analyses. One µg of total RNA was reverse transcribed to cDNAs by using Applied Biosystem's High-Capacity cDNA Reverse Transcription Kit reagent (ThermoFisher Scientific, Waltham, MA) following the manufacturer's instruction. Real-time RT-qPCR amplification of cDNAs was carried out in a 20 µl reaction mixture containing Applied Biosystems Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). Amplification and fluorescence detection of RT-qPCR were carried out on Applied Biosystems StepOne Real Time PCR System (ThermoFisher Scientific). The ΔΔCT method was used for relative quantification of target mRNA normalized to 18S RNA as described in our previous publications[6-9]. All PCR primers were designed using Primer 3[11] and the sequences are shown in **Appendix II**.

   Purification of genomic DNA from granulosa cells. Genomic DNA from was purified from the frozen granulosa cells by using a DNeasy Blood and Tissue Kit (Qiagen) following the manufactures protocol. Total RNA was removed from the purified DNA by using RNAse A (ThermoFisher Scientific) treatment. RNase free genomic DNA samples were quantified and frozen at -80ºC freezer until the methyl-seq library preparation.

   Preparation of methyl-seq libraries and sequencing. Approximately 200 ng of genomic DNA was used for each methyl-seq library preparation. Four independent libraries (**PRJNA974823**) (**Appendix III**). For each group (PMSG, hCG and control), there were four replicates of methyl-seq library. Each replicate contained genomic DNA extracted three ovary samples and combined to form 200 ng of genomic DNA. DNA was fragmented to ~300 bp length using a Q-Sonica 8 controlled temperature sonicator (Newtown, CT). Sonicated internal control

DNAs, 0.5 ng unmethylated lambda and 0.5 ng methylated pUC19, were spiked into each replicate of the library. These DNA fragments were treated with bisulfite using a NEBNext Enzymatic Methyl-seq Kit (New England Biolabs, Ipswich, MA) to construct the libraries. Premade libraries were on an Illumina NovaSeq 6000 platform (Illumina) at the Novogene Corporation (UC Davis Sequencing Center, Sacramento, CA). Methyl-seq was performed as 150 bp paired-end sequencing at Novogene and the sequencing data were analyzed in our lab at the University of Kansas Medical Center.

Analysis of methyl-sequencing data. Bisulfite-sequencing data were demultiplexed, trimmed, aligned, and analyzed using CLC Genomics Workbench 23.0.3 (Qiagen Bioinformatics, Germantown, MD). Clean reads were obtained by trimming and low-quality reads were removed. Good quality reads were aligned with the Rattus norvegicus genome (mRATBN7.2) using the default parameters: (a) maximum number of allowable mismatches was 2; and (b) minimum length and similarity fraction was set at 0.8. Bisulfite-treated methyl-seq data were aligned to the rat reference genome with default parameters. We focused only on CpG methylation, which is important for mammalian gene regulation. Differential methylation was recognized if the changes (hypomethylation or hypermethylation) in CpG methylation involved at least 10 cytosines, occurred in all four replicates in the same study group, exhibited at least 2-fold difference, and p-values were less than 0.05.

Statistical analyses. Gene expression analyses were performed on at least 6 individual rats. The experimental results are expressed as mean $\pm$ SE. Statistical comparisons between two means were determined with Student's t-test while comparisons among multiple means were evaluated with ANOVA and the significance of mean differences was determined by Duncan post hoc test, with $p \leq 0.05$ considered a significant level of difference. All statistical calculations were done with SPSS 22 (IBM, Armonk, NY).

RESULTS

Ovarian responses to exogenous gonadotropins. Four-week-old females were treated with GPNs, and ovarian weights and histological changes were examined after H&E staining. Treatment of PMSG stimulated the ovarian weight significantly, which was increased further with hCG treatment (**Fig. 4A**). Histological examination showed development of large antral follicles after PMSG treatment. After hCG treatment, the number of large antral follicles as well as the sizes of growing follicles increased remarkably (**Fig. 4A-D**).
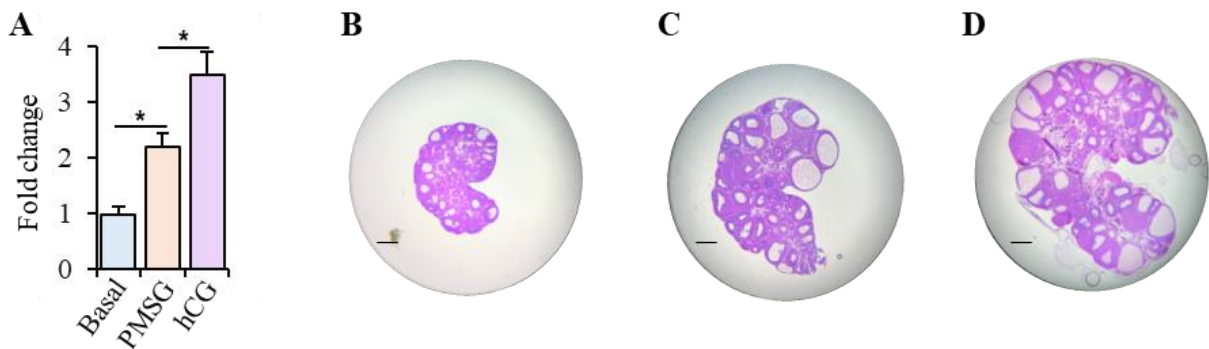

Fig. 4 Ovarian responses to exogenous gonadotropins. Four-week-old female rats were injected with 30IU PMSG. 48h after PMSG injection, rats were administered with 30IU hCG. The rats were euthanized after gonadotropin (GPN), or saline treatments as described in **Fig. 3**. Ovaries were weighed, normalized to body weight, and the results were expressed as GPN induced fold changes in weight (**A**). Ovaries were also fixed in neutral buffered formalin and processed for histological examination after H& E staining (**B-D**). Control ovaries without GPN treatment contained a few of small antral follicles (**B**), whereas PMSG stimulated the development of small antral follicles to large antral stage (**C**), and administration of hCG resulted in the maturation of antral follicles to the preovulatory stage (**D**).

Gonadotropin induced transcriptomic changes in granulosa cells. Either only PMSG (48h after PMSG) administration or hCG (4h after hCG) administration 48h after PMSG treatment

resulted in a large number of DEGs while significant increase or decrease was considered at least more than 2 folds (FDR p value <0.05). We identified a total of 2385 genes were differentially expressed in GCs after 48h PMSG administration (**Fig. 5A**). The majority (2079 out of 2385 or ~87%) of those genes were downregulated (**Fig. 5A**). In hCG 4h group, 3061 genes were differentially expressed in GCs, of which ~40% were upregulated (**Fig. 5A**). Hierarchical clustering performed on the DEGs also represent the data among the replicates of control and gonadotropin treated samples (**Fig. 5 A, B**).
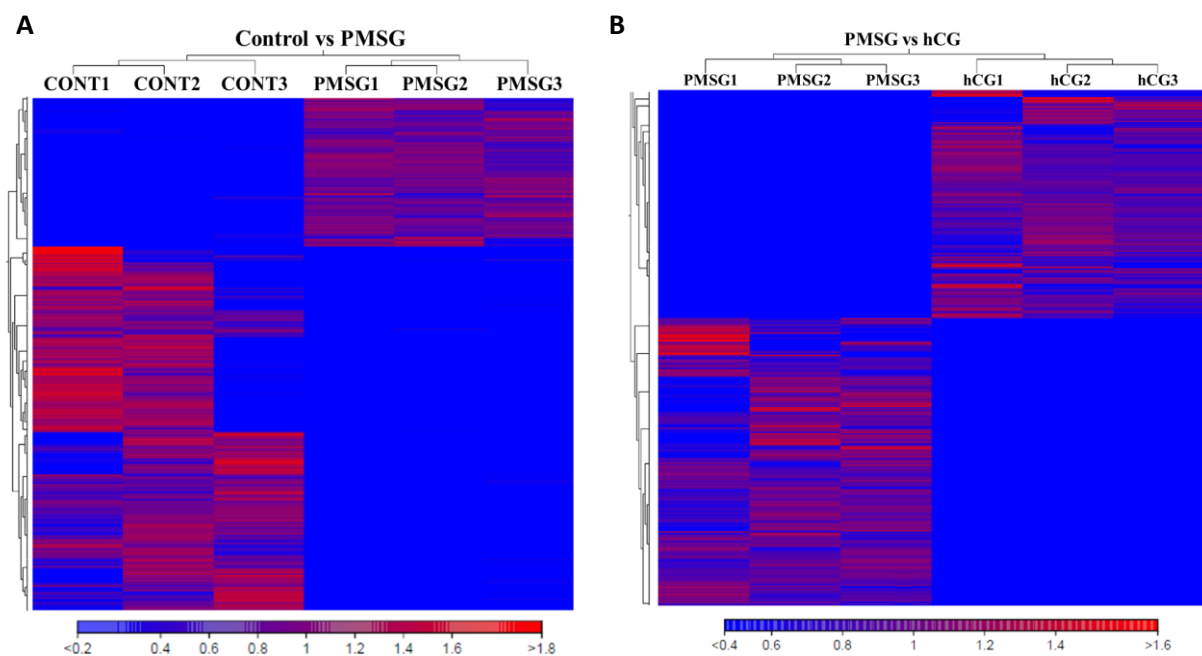
Fig. 5 RNA-sequencing of granulosa cells collected from gonadotropin-treated rat ovaries. RNA-seq data were analyzed by using CLC Genomics Workbench. Hierarchical clustering was performed on the differentially expressed genes (DEGs) among the replicates of control and gonadotropin treated samples; Control vs. PMSG 48h group (**A**) and PMSG vs. hCG 4h group (**B**). A large number of genes were downregulated in the PMSG group (**A**), which was partially reversed after hCG treatment (**B**). However, a group of granulosa cell genes that was initially upregulated by PMSG, was downregulated by hCG treatment (**B**).

Volcano plots representing the comparison of DEGs between Control vs. PMSG group and PMSG vs. hCG group also indicate that a large number of genes were repressed after PMSG treatment (**Fig. 6A**), whereas a group of those repressed genes were upregulated 4h after hCG treatment (**Fig. 6B**).
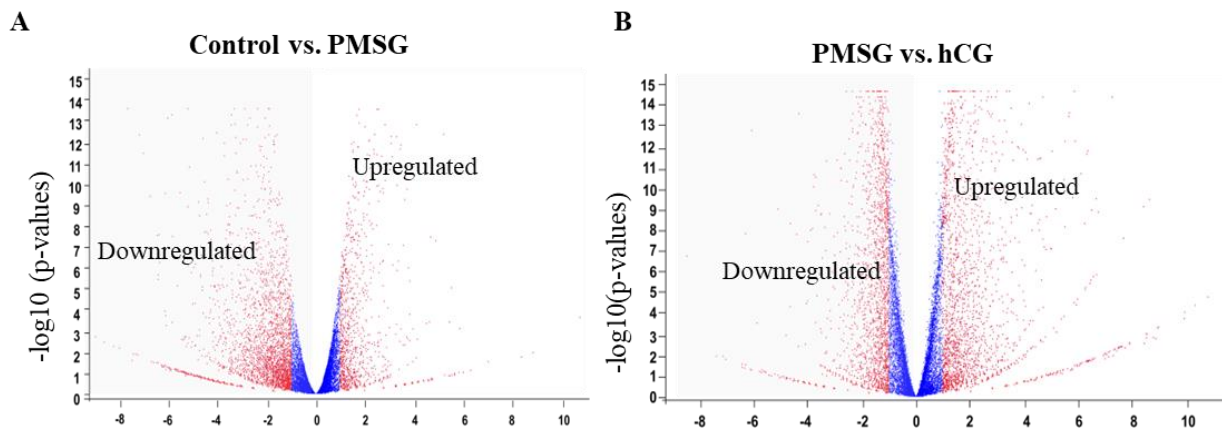

A Control vs. PMSG

B PMSG vs. hCG

Fig. 6 Volcano plots representing changes in granulosa cell transcriptome after gonadotropin treatment. Volcano plots representing the comparison of differentially expressed genes between Control vs. PMSG group (**A**) and PMSG vs. hCG group (**B**). The data distributions indicate that a large number of genes were repressed after PMSG treatment, whereas a portion of those repressed genes were upregulated 4h after hCG treatment.

Gonadotropin-induced differentially expressed genes in granulosa cells. We observed that a group of FSH responsive genes including *Lhcgr, Cyp11a1, Cyp19a1, Gata4,* and *Npr2* were markedly upregulated in PMSG group of GCs. Similarly, a group of known hCG responsive genes like *Pgr, Runx2, Egfr, Ptgs2* and *Adamts1* were also significantly upregulated in GCs. Expression of these genes were further validated by RT-qPCR analyses (**Fig. 7**).
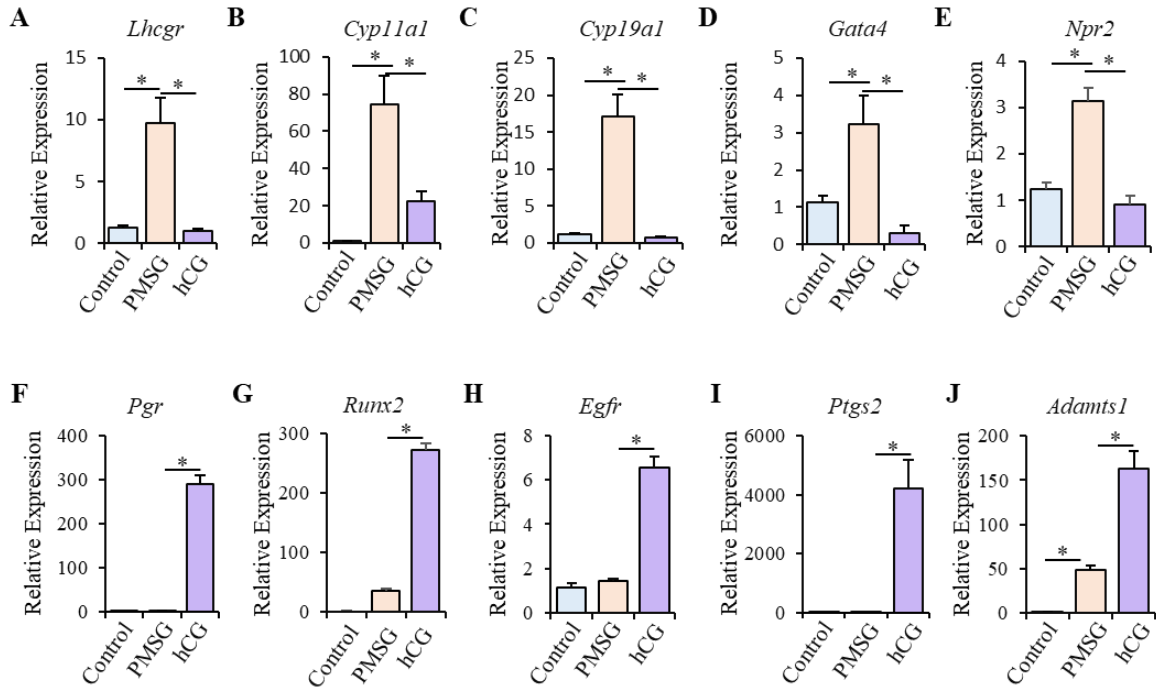
Fig. 7 Gonadotropin-induced differentially expressed genes in granulosa cells. Expression of gonadotropin induced genes granulosa cells (GCs) were also validated by RT-qPCR analyses. We observed that PMSG treatment significantly increased the expression *Lhcgr, Cyp11a1, Cyp19a1, Gata4,* and *Npr2* expression in GCs compared to that of control ovaries (**A-E**). However, expression of these genes were markedly reduced after hCG treatment (**A-E**). In contrast, treatment of hCG following PMSG priming, increased the expression of *Pgr, Runx2, Egfr, Ptgs2* and *Adamts1* (**F-J**). These data also represent the reproducibility of our RNA-seq data.

Distinct patterns of gene expression in granulosa cells in response to gonadotropins. Of the total 2385 differentially regulated genes in GCs, PMSG downregulated 2079 (87%) genes, whereas 303 (13%) genes were upregulated. Among the 2079 genes downregulated by PMSG treatment, 995 (48%) were upregulated and 279 (13%) were downregulated and 805 (39%) did not show any significant changes with subsequent hCG treatment. Among the 303 genes upregulated by PMSG, 214 (71%) were downregulated, 37 (12%) were upregulated and 52 (17%) remained

unchanged. However, irrespective of PMSG response, 1035 (43%) genes were upregulated, 494 (21%) genes were downregulated and 857 (36%) by hCG treatment.
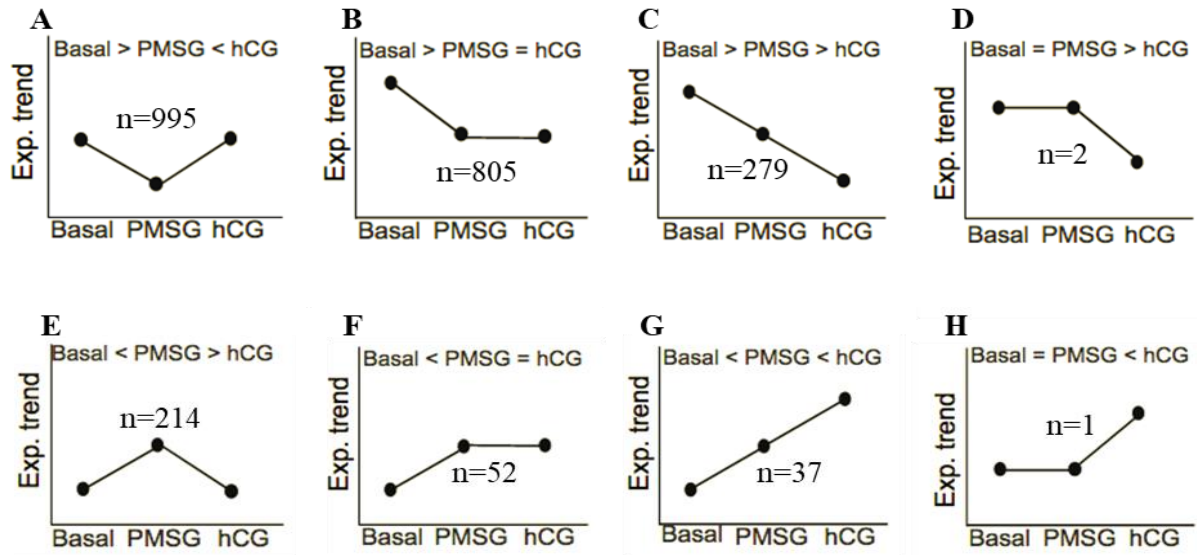

Fig. 8 Distinct patterns of gene expression in granulosa cells in response to gonadotropins. We observed that after PMSG treatment, 87% of the DEGs (2,079 out of 2,385) were downregulated compared to their basal levels (**A-C**). While PMSG treatment predominantly repressed the granulosa cell genes, approximately 60% of the DEGs were upregulated 4h after hCG treatment (**A,G,H**). Only 3 out of 2385 differentially expressed genes showed hCG-induced changes in the absence of PMSG effects (**D, H**). (We considered more $\geq$ 2-fold changes, and FDR *p-value* $\leq$ 0.05 to define a significant change in gene expression).

We observed that majority of the genes had a low level of expression without GPN administration. A group of genes were upregulated by PMSG treatment, however, they showed three different patterns following hCG treatment (**Fig. 8 A-C**). While some of the PMSG induced genes were further upregulated by hCG (**Fig. 8 A**), others either remained unchanged (**Fig. 8 B**) or downregulated after hCG treatment (**Fig. 8 C**). We also identified genes, which were downregulated by PMSG treatment, but then showed variable responses with hCG treatment (**Fig.**

8 D-F). Some of the genes that were downregulated by PMSG treatment were further downregulated by hCG (**Fig. 8D**). However, others either remained unchanged (**Fig. 8E**) or upregulated by hCG treatment (**Fig. 8F**).

Gonadotropin-induced differential methylation of granulosa cell genomic DNA. We further investigated the mechanism of GPN-induced gene repression or gene induction in GCs by analyzing the methyl-sequencing of genomic DNA (**Fig. 9A, 9B**). Analyses of differentially methylated CpG sites showed both increased and decreased methylation in all chromosomes. In the PMSG only group, extent of methylation was more than that was observed after hCG administration in corresponding chromosomes (**Fig. 9A, 9B**). As expected, the relative extent of demethylation was more after hCG treatment of PMSG primed group (**Fig. 9A, 9B**).
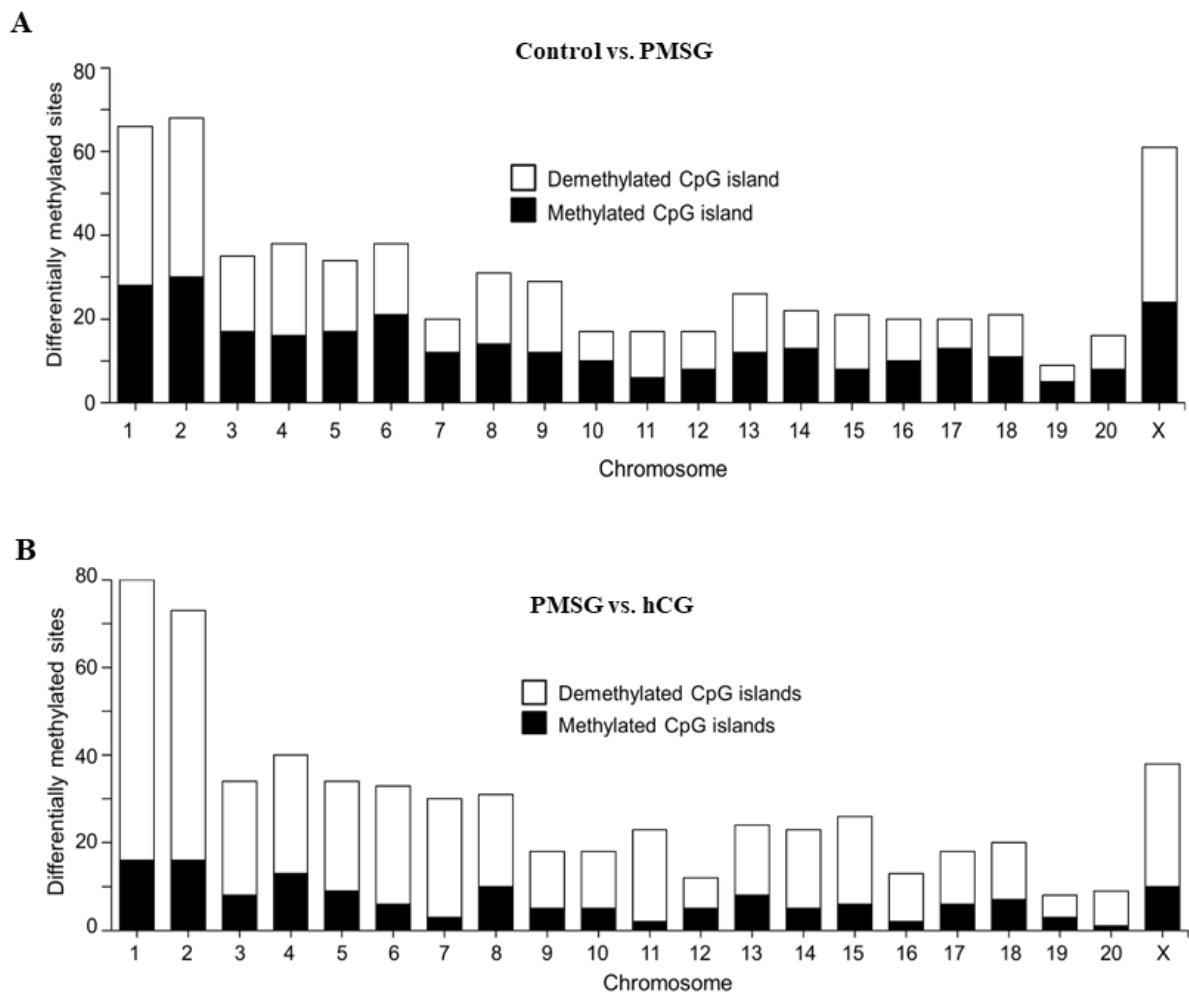
Fig. 9 Analyses of gonadotropin-induced differential methylation in granulosa cells. Differentially methylated CpG sites in granulosa cell genomic DNA were detected after PMSG treatment, and hCG treatment of PMSG primed rats. Differentially methylated sites were recognized that contained ≥ 10 CpGs over a stretch of ≥ 100bp genomic DNA, with the presence methylation or demethylation in $\geq 80\%$ sites of all samples (n=4) and showing at least 2-fold changes (p$\leq$0.05). Predominant repression of gene expression after PMSG treatment correlated well with the relatively more genomewide methylated sites in corresponding chromosomes compared to that after hCG treatment (**A, B**). On the other hand, relative hypomethylation in corresponding chromosomes were more prominent after hCG treatment compared to PMSG only group (**B, A**).

DISCUSSION

In this study, we have studied the transcriptional and epigenetic regulation of gene expression in rat ovarian GCs in response to GPNs. Granulosa cells express both FSHR and LHCGR and they respond to both GPNs when administered from exogenous source. While both mural and cumulus GCs constitutively express the FSHR and LHCGR, FSH signaling markedly increase the expression of LHCGR in mural GCs. However, in this study we have pooled the cumulus or mural GCs together to analyze the GPN responses. Immature rats were chosen for the GPN-induced synchronized development ovarian follicles because they are highly responsive to exogenous GPNs before the establishment of their own GPN secretion. The absence of corpus lutea in ovary also make it convenient for follicle puncture and isolation of GCs. We observed a clear response after injecting exogenous PMSG, which acts like FSH and exogenous hCG, which acts like LH in the rats (**Fig. 4**). This approach has been used in many previous studies for GPN response.

GPN-regulated gene expression in GCs was determined using the genomewide transcriptome analysis with or without exogenous gonadotropin administration. A large number of genes were differentially expressed in response to PMSG (2385) or PMSG followed by hCG (3061) administration. We observed that PMSG administration markedly upregulated including *Lhcgr*, *Cyp11a1*, *Cyp19a1*, *Gata 4* and *Npr2*, which are involved in follicle development (**Fig. 7**). In addition, administration upregulated *Pgr*, *Runx2*, *Egfr*, *Ptgs2*, and *Adamts1*, which are involved in oocyte maturation, and ovulation (**Fig.7**). These RNA-seq data were confirmed by RT-qPCR analyses and the findings are in line with previous studies on gonadotropin regulated gene expression in GCs, which validates our RNA-sequencing data.

GPNs act on the ovarian follicles to carry out the two major functions of ovary-steroidogenesis and oogenesis[1]. Thus, it is expected that GPN treatment would upregulate the

genes required for steroidogenesis and oogenesis. It might also repress the expression of genes that would inhibit these ovarian functions. Remarkably, ~ 87% of the DEGs in GCs were significantly downregulated 48h after PMSG treatment suggesting that gene repression may be the predominant mechanism in PMSG/FSH signaling. However, such an extensive downregulation of genes were not observed among the 4h post hCG DEGs, which indicated that hCG administration upregulates a group of genes that were repressed by PMSG. These RNA-seq data are clearly demonstrated in the heatmaps and Volcano plots (**Fig. 5, 6**).

PMSG binds to and activates FSHR in rat ovarian follicles, which is a GPCR that activates protein kinase A[1, 2]. However, an optimal ovarian response to PMSG administration in immature female rats requires 48 to 52 hrs[12, 13]. This observation indicates that PMSG induced changes in gene expression is unlikely to be executed by a protein kinase mediated activation or deactivation of transcriptional regulators. While PMSG signaling repressed the majority of genes in GCs, hCG effects were different from that of PMSG. More importantly, hCG administration rarely impacted the expression of a GC gene that was not modulated by PMSG administration suggesting that hCG response may require a PMSG-primed epigenetic change (**Fig. 8**). Collectively, we our findings suggest that PMSG response can be dependent on a genome-wide changes in epigenetic marks like methylation of regulatory DNA sequences in GCs, that repress the genes.

Analyses of methyl-sequencing data demonstrated that GPN stimulation altered the genomewide methylation of genomic DNA in GCs. While the extent of genomewide methylation was more following PMSG administration, the extent of demethylation was more in the hCG group (**Fig. 9A, 9B**). These results are in line with our RNA-sequencing data, where more genes were downregulated after PMSG treatment. Increased demethylation of chromosomal DNA also correlated with the upregulation of gene expression after hCG treatment.

While PMSG or hCG following PMSG administration decreased methylation (hypomethylation) in some chromosomal loci, GPNs also increased methylation (hypermethylation) in other chromosomal loci. It has been shown that hypomethylation or hypermethylation may directly or indirectly regulate the expression of genes. While increased methylation of the regulatory DNA may directly repress the expression of certain genes. However, hypomethylation of regulatory sequences of transcriptional repressors may decrease the expression of other genes indirectly. Further analyses of the methyl-seq data would provide further information regarding the nearby gene. Annotated genes could be correlated with the DEGs detected in RNA-Seq analyses.

Although transcriptional regulation of GPN-induced gene expression have been reported in many studies, epigenetic changes resulting from GPN administration remain unexplored. A previous study has highlighted genomewide hypomethylation in mouse ovary following PMSG treatment[14]. However, other modes of epigenetic changes including alteration of histone marks can also modify the chromatin conformation and impact GPN-induced gene regulation in granulosa cells. Therefore, future studies are required to determine the histone marks to correlate with changes in genomewide DNA methylation and to understand the regulation of gene expression in GPN stimulated GCs.

FUTURE DIRECTION

CpG-islands are associated with gene promoters. Regional methylation of CpG-islands can regulate gene promoters on the 5' end, 3' end, or both. Our ongoing studies are directed towards, annotating the differentially methylated sites for nearby gene information. The annotated genes will be correlated with the differentially expressed genes as identified in RNA-Seq analyses.

Histone modifications are also common epigenetic mechanism of gene regulation. Studies have demonstrated an intricate relationships between DNA methylation and histone modification in regulation of gene expression during embryonic development. Therefore, in addition to Methyl-Seq analyses, GCs will be examined for histone marks of active or repressed chromatins by using ChIP seq analyses. ChIP-seq analyses of H3K27me3 and H3K4me3 will be employed to identify the heterochromatin regions, whereas ChIP-seq analyses of H3K27ac and H3K9me3 will be used for identifying the active chromatin regions.

ACKNOWLEDGEMENTS

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APPENDIX I: LIST OF RNA-SEQ DATA SETS IN SRA

Genotype	Group	Study ID	Sample ID
Wildtype	Control	PRJNA551764	SAMN12165660
Wildtype	Control	PRJNA551764	SAMN12165661
Wildtype	Control	PRJNA551764	SAMN12165662
Wildtype	PMSG	PRJNA551764	SAMN12165663
Wildtype	PMSG	PRJNA551764	SAMN12165664
Wildtype	PMSG	PRJNA551764	SAMN12165665
Wildtype	hCG 4h	PRJNA551764	SAMN12165666
Wildtype	hCG 4h	PRJNA551764	SAMN12165667
Wildtype	hCG 4h	PRJNA551764	SAMN12165668

APPENDIX II: LIST OF RT-QPCR PRIMERS

Gene	Reference mRNA	Primer 5'-3'	Size (bp)
Lhcgr	NM_012978.1	Forward: ATGCTTTCCAAGGGATGAAT Reverse: GAGATTAGAGTCGTCCCATT	103bp
Cyp11a1	NM_017286.3	Forward: CCCCTGACTCCATCAAGAAC Reverse: CCCATGGCAGATTCTTGTGG	200bp
Cyp19a1	NM_017085.2	Forward: CCCATGGCAGATTCTTGTGG Reverse: TGATGCCATTCTCGTGCATG	216bp
Gata4	NM_144730.1	Forward: CTCCTACTCCAGCCCCTACC Reverse: GCCGGTTGATACCATTCATCT	287bp
Npr2	NM_053838.1	Forward: CCTTGATGTCTTTGGGGAGA Reverse: GACTTGGGCATAGAGCAGGA	280bp
Pgr	NM_022847.1	Forward: AAGTCCCTTTTGCTCCACCT Reverse: GTAAACTGGGAACCCGTCCT	125bp
Runx2	NM_001278483.1	Forward: CGACAGCCCCAACTTCCTGT Reverse: CTTGGGGAGGATTTGTGAAG	254bp
Egfr	NM_031507.1	Forward: TTAGCAACAACCCCATCCTC Reverse: TTCTGGCAGTTCTCCTCTCC	178bp
Ptgs2	NM_017232.3	Forward: CCACTTCAAGGGAGTCTGGA Reverse: GAAGGGCCCTGGTGTAGTAG	179bp
Adamts1	NM_024400.2	Forward: TTCCACATCCTGAGGCGAAG Reverse: GCTGGACACAAATCGCTTCT	202bp

APPENDIX III: LIST OF METHYL-SEQ DATA SETS IN SRA

Genotype	Group	Study ID	Sample ID
Wildtype	Control	PRJNA974823	SAMN35298758
Wildtype	Control	PRJNA974823	SAMN35298759
Wildtype	Control	PRJNA974823	SAMN35298760
Wildtype	Control	PRJNA974823	SAMN35298761
Wildtype	PMSG	PRJNA974823	SAMN35298762
Wildtype	PMSG	PRJNA974823	SAMN35298763
Wildtype	PMSG	PRJNA974823	SAMN35298764
Wildtype	PMSG	PRJNA974823	SAMN35298765
Wildtype	hCG 4h	PRJNA974823	SAMN35298766
Wildtype	hCG 4h	PRJNA974823	SAMN35298767
Wildtype	hCG 4h	PRJNA974823	SAMN35298768
Wildtype	hCG 4h	PRJNA974823	SAMN35298769