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A Role for Nuclear Factor Interleukin-3 (NFIL3), a Critical Transcriptional Repressor, in Down-Regulation of Periovulatory Gene Expression

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The LH surge triggers dramatic transcriptional changes in genes associated with ovulation and luteinization. The present study investigated the spatiotemporal expression of nuclear factor IL-3 (NFIL3), a transcriptional regulator of the basic leucine zipper transcription factor superfamily, and its potential role in the ovary during the periovulatory period. Immature female rats were injected with pregnant mare’s serum gonadotropin, treated with human chorionic gonadotropin (hCG), and ovaries or granulosa cells were collected at various times after hCG. Nfil3 mRNA was highly induced both in intact ovaries and granulosa cells after hCG treatment. In situ hybridization demonstrated that Nfil3 mRNA was highly induced in theca-interstitial cells at 4–8 h after hCG, localized to granulosa cells at 12 h, and decreased at 24 h. Overexpression of NFIL3 in granulosa cells inhibited the induction of prostaglandin-endoperoxide synthase 2 (Ptgs2), progesterone receptor (Pgr), epiregulin (Ereg), and amphiregulin (Areg) and down-regulated levels of prostaglandin E2. The inhibitory effect on Ptgs2 induction was reversed by NFIL3 small interfering RNA treatment. In theca-interstitial cells the expression of hydroxyprostaglandin dehydrogenase 15-(nicotinamide adenine dinucleotide) (Hpgd) was also inhibited by NFIL3 overexpression. Data from luciferase assays demonstrated that NFIL3 overexpression decreased the induction of the Ptgs2 and Areg promoter activity. EMSA and chromatin immunoprecipitation analyses indicated that NFIL3 binds to the promoter region containing the DNA-binding sites of cAMP response element binding protein and CCAAT enhancer binding protein-β. In summary, hCG induction of NFIL3 expression may modulate the process of ovulation and theca-interstitial and granulosa cell differentiation by regulating expression of PTGS2, PGR, AREG, EREG, and HPGD, potentially through interactions with cAMP response element binding protein and CCAAT enhancer binding protein-β on their target gene promoters. (Molecular Endocrinology 25: 445–459, 2011)

The nuclear factor IL-3 (NFIL3) is a member of the mammalian basic leucine zipper (bZIP) transcription factor superfamily. Members of this superfamily of transcription factors include the cAMP response element binding protein (CREB)/activating transcription factor, activator protein 1, CCAAT enhancer binding protein (C/EBP), nuclear factor (erythroid-derived 2), and proline and acidic residue rich (PAR) families. The bZIP factors share an amphipathic α-helical dimerization domain which is characterized by a leucine zipper region comprised of a heptad repeat of leucine residues. These transcription factors form either homodimers or heterodimers...
that bind specific DNA sequences to regulate gene transcription, which impacts a diverse array of processes throughout the body (1–4). NFIL3, also known as E4-binding protein 4 or NFIL3/ E4-binding protein 4, was originally identified as a transcriptional repressor based on its DNA-binding activity at the promoter of the gene encoding the adenovirus E4 protein (5). Subsequently a transcriptional activator that bound to the human IL-3 promoter, NFIL3, was identified and was shown to have similar consensus binding sequences to E4-binding protein 4 (6). NFIL3 has the most similarity to the PAR family, which includes hepatic leukemia factor, D-box binding protein, and thyrotroph embryonic factor, which is also known as vitellogenin gene binding protein. The PAR family of transcription factors is characterized by an extended basic domain and a 65-amino acid proline- and acidic residue-rich region along with a high degree of amino acid sequence similarity over their DNA-binding domains. NFIL3 contains an extended basic region resembling the PAR basic domain but lacks the PAR region (3). The similarities between the DNA-binding domains of the PAR factors and NFIL3 have led to the suggestion of competition for DNA-binding sites if these transcription factors are expressed together (7). Whereas the PAR factors have been generally characterized as transcriptional activators (5), NFIL3 is proposed to be a transcriptional repressor.

In addition to similarities with the PAR factors, the DNA-binding domain of NFIL3 is homologous to the DNA-binding domain of other members of the bZIP family of transcription factors. The consensus NFIL3-binding site sequence (EBPRE) has been determined to be homologous to the cAMP-responsive DNA element (CRE) and consensus CREBPβ binding sites (8). Recognition of the same cis-binding element by NFIL3, CREB, and CREBPβ points to potentially interesting interactions of these transcription factors for the regulation of identical target gene expression. In addition, some of these transcription factors can form heterodimers that may alter their binding to DNA and their resultant function (4). For example, a dominant negative of NFIL3 can heterodimerize with CREB and inhibit DNA binding (4).

The bZIP family of transcription factors regulates key genes that are crucial for ovulation. It is well documented that the midcycle surge of LH/FSH initiates a complex series of cellular and molecular events in periovulatory follicles leading to resumption of oocyte meiosis, breakdown of the follicle wall, and oocyte release, followed by subsequent luteinization of the postovulatory follicle. These processes require the precisely regulated expression of a complex, interacting network of genes in the periovulatory follicle, many of which are initiated by bZIP family members, such as CREB and CREBPβ, in response to LH/FSH stimulation. For example, CREB and CREBPβ regulate key genes associated with the ovulatory process such as prostaglandin-endoperoxide synthase 2 (PTGS2), progesterone receptor (PGR), ADAMTS1, cathespsin L (9), epiregulin (EREG), and amphiregulin (AREG) (10). The importance of the bZIP transcription factors and their downstream target genes in oocyte release is underscored from studies where mutation or deletion of many of these factors results in sterility or subfertility due to defects in oocyte release and/or luteinization (11–13). For example, CREBPβ mRNA and protein are rapidly induced in granulosa cells in vivo by human chorionic gonadotropin (hCG) (7, 12) and deletion of CREBPβ results in sterility in mice due to a lack of ovulation (12). Based upon previous findings that NFIL3 potentially recognizes the same cis-binding element as CREB and C/EBPβ (8) and may regulate common target gene expression by competing for the same DNA-binding sites (14), we postulated that NFIL3 may act as a transcriptional repressor for these LH-induced transcriptional activators. Such a postulate is supported by previous findings of transcriptional repression of inhibin (15). In these studies, Burkart et al. (15) reported that inducible cAMP early repressor acts as transcriptional repressor of CREB in the regulation of the inhibin α inhibin subunit gene.

There are limited or no reports of the PAR family members in the ovary. Based upon the paucity of data regarding the expression of the NFIL3 in the ovary and its potential role in regulating gene expression, the present study was undertaken to characterize the expression pattern of NFIL3 and its target genes during the periovulatory period in the rat ovary. We hypothesized that the LH surge induces the expression of NFIL3 and that its induction may play an important role in ovulation and luteinization of granulosa and theca cells through its action as a transcriptional repressor.

**Results**

**hCG stimulates NFIL3 expression in periovulatory rat ovaries and granulosa cells**

To determine the periovulatory changes in Nfil3 mRNA and protein, expression patterns were analyzed from ovaries collected at different times after hCG administration. The level of Nfil3 mRNA in whole ovaries increased approximately 8-fold at 4 h after hCG and remained elevated through 24 h (Fig. 1A). Results from Western blot analyses showed that the protein level of NFIL3 in intact ovaries was highly induced at 4 h and remained elevated at 24 h after hCG treatment (Fig. 1B). To determine the relative expression of Nfil3 in the
granulosa cell compartment, granulosa and granulosa-luteal cells were collected at various times after hCG (i.e., in vivo). The levels of mRNA for Nfil3 were highest at 12 h after hCG treatment in granulosa cells collected in vivo (Fig. 1C) before decreasing at 24 h after hCG treatment. Western blot results showed that NFIL3 expression in granulosa cells was markedly induced at 8 h and maintained through 24 h after hCG (Fig. 1D).

Nfil3 mRNA is localized to the theca-interstitial and granulosa cells

In situ hybridization was used to localize the expression of Nfil3 mRNA in the ovary. The results revealed that before hCG administration (i.e., 0 h) the expression of Nfil3 mRNA was low throughout the ovary (Fig. 2, A and B). Nfil3 was highly induced in the theca and interstitial cells at 4 h (Fig. 2, C and D) and 8 h after hCG injection (Fig. 2, panels E and F and panels K and L). At 12 h after hCG, there was a marked induction in the granulosa cell compartment (Fig. 2, G and H) and the expression of Nfil3 mRNA in the theca cells remained elevated at this time point. After ovulation, Nfil3 mRNA expression was still present in the forming corpus luteum (Fig. 2, I and J). No signal was detected on the sections that were hybridized with the sense probe of Nfil3 (data not shown).

Overexpression of NFIL3 in cultured granulosa cells regulates genes associated with ovulation

To explore the potential function of NFIL3, Nfil3 was overexpressed in granulosa cells, and microarray analysis was used to identify potential NFIL3-regulated genes. This was accomplished by comparing gene expression patterns between granulosa cells infected with control green fluorescent protein (GFP) adenovirus (negative control) and cells infected with Nfil3 expressing adenovirus vector. Granulosa cells isolated from pregnant mare’s serum gonadotropin (PMSG)-primed immature rats were infected with either adenovirus expressing GFP (Ad-GFP) or adenovirus expressing Nfil3 (Ad-Nfil3) for 24 h. Granulosa cells were then stimulated with forskolin (FSK) + phorbol 12-myristate 13-acetate (PMA) for 6 h. These respective protein kinase A and protein kinase C activators were used to induce gene expression in preovulatory granulosa cells by mimicking the action of an ovulatory dose of LH/hCG. Overexpression of NFIL3 in cells infected with Ad-NFIL3 was confirmed by real-time PCR (Fig. 3A) and Western blot analysis (Fig. 4A), which revealed a 130-fold increase in mRNA for NFIL3. To determine the genes regulated by Nfil3, total RNA from the Ad-GFP-infected cells with no treatment (control), Ad-GFP/FSK/PMA-treated cells and Ad-NFIL3/FSK/PMA-treated cells were analyzed using the Affymetrix Rat 230–2.0 oligonucleotide array sets. The genes down-regulated by more than 60% after Ad-NFIL3 treatment are shown in Table 1. The results revealed that overexpression of Nfil3 resulted in an inhibition of genes known to be important for ovulation including PGR, EREG, AREG, HPGD, and PTGS2. Real-time PCR results confirmed the microarray data that overexpression of NFIL3 decreased the expression of mRNA for Ptgs2 (Fig. 3B), Areg (Fig. 3C), Ereg (Fig. 3D), and Pgr (Fig. 3E). Hpgd mRNA levels were also significantly decreased by NFIL3 overexpression although its expression did not change after FSK +
PMA treatment (Fig. 3F). Ad-NFIL3 had no effect on expression of these genes without FSK + PMA treatment (Fig. 3).

**Reduction of PTGS2 expression and promoter reporter activity by NFIL3 overexpression in preovulatory granulosa cells**

Studies were initiated to examine NFIL3 regulation of *Ptgs2* because PTGS2, a rate-limiting enzyme in the biosynthesis of prostaglandins (PGs), has been shown to be a key factor for ovulation (16, 17), and this gene has been a focus of interest in our laboratory. *Ptgs2* mRNA demonstrates a transient expression pattern after the LH surge, which may be important for its physiological effect during the periovulatory period. Based upon our microarray data (Table 1), we hypothesized that NFIL3 may play a role in the down-regulation of *Ptgs2* mRNA in the ovary. To test this hypothesis, the pattern of PTGS2 expression and its regulation was examined in the presence or absence of NFIL3. NFIL3 protein was highly expressed in the Ad-NFIL3-infected granulosa cells (Fig. 4A) but was low in Ad-GFP-infected granulosa cells at 6 h after FSK + PMA treatment. Western blots showed that the induction of PTGS2 protein at 6 h after FSK + PMA treatment was reduced when NFIL3 was overexpressed (Fig. 4A). We next determined whether this reduction of *Ptgs2* expression was due to interaction of NFIL3 with the *Ptgs2* promoter. Two rat *Ptgs2* promoter reporter constructs (−673/+20 and −366/+20 bp) were transfected into pre-ovulatory granulosa cells for 4 h before adenovirus infection. After 24 h, granulosa cells were then stimulated with FSK + PMA for 6 h. FSK + PMA treatment increased the luciferase activities of the −673/+20 and −366/+20 bp reporter constructs compared with that of cells cultured without FSK + PMA (Ad-GFP; Fig. 4B). The transactivation of both of the *Ptgs2* promoter reporter constructs was reduced by NFIL3 overexpression (Fig. 4B). To determine the importance of the consensus C/EBP-binding site at −130 bp of the *Ptgs2* promoter, site-directed mutants were generated and transfected into cultured pre-ovulatory granulosa cells (Fig. 4B). Noticeably, the mutation of C/EBP binding sequence abolished the induction of *Ptgs2* promoter luciferase activity by FSK + PMA, which suggested that C/EBP is critical for the up-regulation by LH.

**Endogenous NFIL3 binds to the Ptgs2 promoter region in periovulatory granulosa cells**

The NFIL3-binding site is very similar to the consensus C/EBP-binding site (8). This observation has led to the proposal that interactions may exist between these two transcription factors to regulate identical target gene expression (14). To determine whether NFIL3 can compete with C/EBP for the C/EBP-binding site in the ovarian
Ptgs2 promoter in vivo, we performed chromatin immunoprecipitation (ChIP) assays using chromatin samples extracted from periovulatory granulosa cells (0, 6, and 12 h post-hCG). PCR analysis revealed that immunoprecipitation of endogenous NFIL3-enriched chromatin fragments contained the C/EBP/H9252-binding sequence in the promoter region at 12 h post-hCG compared with 0 and 6 h (Fig. 4C). The binding of C/EBP/H9252 increased at 6 h post-hCG and then decreased at 12 h, whereas NFIL3 binding increased between 6 and 12 h after hCG (Fig. 4C). These results indicate that endogenous NFIL3 protein associated with the region containing the C/EBP/H9252-binding site in the Ptgs2 promoter in periovulatory granulosa cells and that as NFIL3 binding increased, C/EBP/H9252 binding decreased.

EMSAs were used to confirm the interaction of NFIL3 protein and C/EBP/H9252 binding motifs in the Ptgs2 promoter. The 36-bp probe including the C/EBPβ-binding motif was incubated with periovulatory granulosa cell nuclear extracts from different time points. The bandshift demonstrated the interaction of a nuclear transcription factor(s) in periovulatory granulosa cells with the C/EBPβ-binding motif (Fig. 5). The binding was competed by an excess of unlabeled wild-type probe but not by an unlabeled mutant probe, indicating the interaction is specific for the C/EBPβ-binding motif. Addition of NFIL3 antibody to probe/extract mixtures led to the formation of supershift bands at 12 h, confirming that NFIL3 is indeed able to interact with the C/EBPβ-binding motif on the Ptgs2 promoter (Fig. 5). The data suggested that NFIL3 could bind to the C/EBPβ-binding motif at 12 h after hCG treatment. However, the addition of the NFIL3 antibody also resulted in an unexpected increase in the reaction product at the 12-h time point, which has been observed with the addition of other antibodies in EMSAs (Dr. Kaetzel, D. M., personal communication).

NFIL3 down-regulates prostaglandin E2 (PGE2) levels in granulosa cells

To investigate whether NFIL3 regulates the level of PGs in granulosa cells, PGE2 levels were measured in granulosa cells that were infected with either a control Ad-GFP or Ad-NFIL3. Cells were infected with Ad-GFP or Ad-NFIL3 for 24 h and were then either untreated or stimulated with FSK + PMA for 6 h. PGE2 levels increased approximately 10-fold after FSK + PMA stimulation (Fig. 6). Overexpression of NFIL3 resulted in a 35% decrease in the levels of PGE2 induced by FSK + PMA treatment in granulosa cells (Fig. 6).
Knockdown of Nfil3 expression by small interfering RNA (siRNA) increases Ptgs2 expression in granulosa cells

Studies were conducted to determine whether down-regulation of Nfil3 expression could up-regulate the expression of Ptgs2 mRNA. We first validated that the expression of Nfil3 mRNA increased in response to FSK + PMA treatment of granulosa cells in vitro. Real-time PCR analysis revealed that FSK + PMA induced Nfil3 mRNA expression in cultured granulosa cells at 6 h (Fig. 7A). Nfil3 mRNA reached the highest levels at 12 h and remained elevated at 24 h (Fig. 7A). To explore the impact of Nfil3 on expression of mRNA for Ptgs2, Nfil3 mRNA was reduced using a siRNA approach. Granulosa cells were exposed to Nfil3 siRNA or a scrambled RNA (negative control) treatment for 4 h and subsequently treated either with or without FSK + PMA for 12 h. Real-time PCR analysis revealed that Nfil3 siRNA reduced the FSK + PMA-stimulated expression of Nfil3 at 12 h (Fig. 7B). Ptgs2 mRNA expression increased in Nfil3 siRNA-transfected granulosa cells compared with its expression in granulosa cells treated with scrambled siRNA (Fig. 7C). Western blot analysis confirmed the siRNA data on the changes in mRNA expression (Fig. 7, B and C) or at the protein level (Fig. 7D).

Areg mRNA expression was inhibited by Nfil3 overexpression in granulosa cells

The EGF-like growth factors AREG, EREG, and betacellulin are potent stimulators of oocyte maturation and cumulus expansion (18, 19). The expression of Areg and Ereg is transient after the LH surge in the ovary (20). To investigate the role of Nfil3 in the transcriptional regulation of Areg, we generated three rat Areg promoter reporter constructs (−676/+16, −223/+16, and −65/+16 bp). These constructs were transfected into granulosa cells for 4 h, and the granulosa cells were then infected with either control Ad-GFP or Ad-Nfil3 for 24 h. The luciferase activity of the −65/+16 bp Areg promoter was not induced by FSK + PMA whereas FSK + PMA stimulated luciferase in the −223/+16 bp Areg construct (Fig. 8A). This observation suggested that the −223 bp/−65 bp region was important for FSK + PMA induced regulation of Areg promoter. The luciferase activity of the Areg promoters (−676/+16 and −223/+16 bp) was inhibited by overexpression of Nfil3, whereas Nfil3 had no effect on the −65/+16 bp Areg promoter activity. These findings are interpreted that the promoter region −223/−65 bp was important for Nfil3 regulation of Areg promoter activity. The promoter analysis showed a CREB-binding site located at −91 bp. To determine the importance of this consensus CREB-binding site, site-directed mutants were generated and transfected into cultured preovulatory granulosa cells. The mutation of the CREB-binding sequence decreased the induction of Areg promoter luciferase activity by FSK + PMA (Fig. 8A), which suggested that CREB is critical for the up-regulation by LH.

To determine whether Nfil3 specifically binds to the CREB-binding site in the Areg promoter in vivo, we per-
formed ChIP assays using chromatin samples extracted from periovulatory granulosa cells (0 and 12 h post-hCG) and amplified by PCR. ChIP assays revealed that immunoprecipitation of endogenous NFIL3-enriched chromatin fragments contained the CREB-binding sequence in the promoter region compared with that of normal rabbit IgG (Fig. 8B). This result indicated that endogenous NFIL3 protein is associated with the region containing the CREB-binding site in the Areg promoter in periovulatory granulosa cells.

hCG reduces hydroxyprostaglandin dehydrogenase mRNA expression in periovulatory rat ovaries and granulosa cells

HPGD is a key catabolic enzyme that controls the biological activities of PGs by catalyzing the first step of PG inactivation (21, 22). Gonadotropin-regulated HPGD has been proposed to play an important role in the control and timing of ovulation (23). Because NFIL3 regulates PTGS2 expression and PG synthesis, we sought to determine whether NFIL3 could impact Hpgd expression and potentially PG catabolism. Initially the in vivo pattern of Hpgd mRNA expression in the ovary and granulosa cells

<table>
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Granulosa cells from preovulatory ovaries were infected with Ad-NFIL3 or Ad-GFP for 24 h and then treated with FSK (10 μM) + PMA (20 nM) for 6 h. RNA was analyzed using Affymetrix Rat 230–2.0 oligonucleotide array sets. A subset of genes that were decreased more than 60% compared with cells treated with Ad-GFP are listed. PDZ, An acronym combining the first letters of three proteins - Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and Zonula occludens-1 protein (Zo-1); RING finger, a protein structural domain of zinc finger type which contains a Cys3HisCys4 amino acid motif which binds two zinc cations.

was investigated. Real-time PCR data showed that Hpgd expression decreased gradually after hCG treatment in the whole ovary (Fig. 9A). In granulosa cells collected in vivo after hCG administration, Hpgd mRNA expression did not change after hCG treatment (data not shown). The expression of Hpgd mRNA expression in theca-interstitial cells collected in vivo after hCG administration also decreased gradually (Fig. 9B). The results of in situ hybridization confirmed the real-time PCR data. Hpgd mRNA was mainly located in the theca cells, and its expression gradually decreased through 24 h after hCG treatment (data not shown). Collectively these data suggested that the expression of Hpgd mRNA is predominately located in the theca-interstitial cells.

NFIL3 inhibits Hpgd mRNA expression in theca-interstitial cells

To investigate the role of NFIL3 protein in the transcriptional regulation of Hpgd, we generated three rat Hpgd promoter reporter constructs (−779/+29, −182/+29, and −103/+29 bp). These constructs were transfected into theca-interstitial cells for 4 h and then cells were infected with either a control Ad-GFP or Ad-NFIL3 for 24 h. The basal activity of the −103/+29 bp Hpgd promoter was lower than the activity of the −182/+29 bp Hpgd promoter, which suggested that the −182 bp−103 bp region was important for stimulating Hpgd promoter activity (Fig. 10A). The luciferase activity of the −779/+29 and −182/+29 bp Hpgd promoters was inhibited by overexpression of NFIL3, whereas NFIL3 had no effect on the −103/+29 bp Hpgd promoter activity. These findings suggest that the promoter region from −182/−103 bp was important for the regulation of Hpgd and that NFIL3 inhibits Hpgd promoter activity.

Analysis of the Hpgd promoter showed a CREB-binding site located at −118 bp. To determine whether NFIL3 specifically binds to this CREB-binding site in the Hpgd
promoter in vivo, we performed ChIP assays using chromatin samples extracted from periovulatory theca-interstitial cells (0 and 8 h post-hCG). PCR analysis revealed that immunoprecipitation of endogenous NFIL3-enriched chromatin fragments contained the CREB-binding sequence in the promoter region compared with that of normal rabbit IgG (Fig. 10B). This result indicated that endogenous NFIL3 protein associated with the region containing the CREB-binding sequence of the Hpgd promoter in periovulatory theca-interstitial cells.

**Discussion**

The pituitary surge of LH/FSH initiates a cascade of events culminating in the precisely controlled breakdown of the follicle wall and release of the oocyte. This process of ovulation is associated with the temporal regulation of transcription of specific genes and is presumed to involve the synthesis and/or activation of specific proteases that degrade the follicle wall. Genes reported to be associated with ovulation include C/EBPβ (12), PGR (24, 25), PTGS2 (26–28), and the epidermal growth factors AREG and EREG (18). In the present study, we demonstrate that NFIL3 is induced by hCG and acts as a newly identified transcriptional regulator of periovulatory gene expression.

NFIL3 is a bZIP transcription factor that acts either as a transcriptional repressor or a transcriptional activator. The potential function of NFIL3 has emerged from reports of its ability to regulate cell survival (29), motoneuron growth (30), macrophage differentiation and activation (31), antiinflammatory responses (3), circadian rhythm regulation (3), and adrenal steroidogenesis (32). Many of these processes, cell survival and differentiation, inflammation, and steroidogenesis, are landmarks of the ovulatory process that prompted us to examine the expression of NFIL3 and its potential involvement across the periovulatory period.

In the present study, NFIL3 expression was induced in the ovary by hCG treatment. The induction of Nfil3 mRNA increased in the theca-interstitial cells before expression in the granulosa cells. This difference in the temporal induction of NFIL3 mRNA expression between these two cell compartments may reflect diverse roles for NFIL3 in granulosa and theca-intersti-
induction of PTGS2 expression in granulosa cells (13), which has been shown to be regulated by C/EBPβ (7). The level of mRNA for Ptgs2 is highest at 4 h after hCG treatment in granulosa cells in vivo (26, 36). After ovulation, the levels of PGs decrease quickly and are at basal levels in luteal cells (37). Down-regulation of Ptgs2 mRNA and PG levels has been observed by NFIL3 in other systems such as osteoblasts (38–40), which prompted us to explore the role of NFIL3 in regulating PGs in the ovary. Our findings demonstrate, for the first time, that overexpression of NFIL3 decreased Ptgs2 mRNA, PTGS2 protein, as well as PGE levels in granulosa cells whereas the converse approach of decreasing NFIL3 by siRNA increased Ptgs2 mRNA. Furthermore, the ability of NFIL3 to decrease the promoter activity of Ptgs2 further supports our hypothesis that NFIL3 is decreasing LH-induced Ptgs2 activity. The NFIL3 inhibition of PG synthesis, however, may not be complete due to levels of NFIL3 present, the inability of NFIL3 to completely displace C/EBPβ, or the ability of other transcriptional factors to regulate Ptgs2 induction (41).

In mouse osteoblasts, NFIL3 was reported to reduce PTH-induced activity of a Ptgs2 promoter-reporter construct by inhibiting C/EBPβ binding activity (14). Our attempts to determine whether NFIL3 was competing with C/EBPβ binding at the Ptgs2 promoter by mutating the C/EBPβ-binding site were unsuccessful because Ptgs2 promoter activity was dependent upon an intact C/EBPβ-biding site. However, both EMSA and ChIP analysis provided evidence of a potential NFIL3-C/EBPβ interaction because there was an enhanced binding of NFIL3 to DNA containing the C/EBPβ-binding site at 12 h after hCG when NFIL3 was maximally stimulated. Similarly, ChIP assays demonstrated that as NFIL3 binding increased, there was a corresponding decrease in C/EBPβ binding. Thus the possibility exists that NFIL3 regulates Ptgs2 promoter activity by inhibiting C/EBPβ-binding activity as previously described for osteoblasts (14).

NFIL3 may regulate the biological actions of PGs at multiple levels in different ovarian compartments. The synthetic regulation of PG production occurs by PTGS2; however, HPGD is considered to be a key catabolic enzyme that controls the biological activities of PGs (42,
HPGD is present in the primate periovulatory follicle in a pattern consistent with modulation of follicular PGE2 levels during the periovulatory interval, supporting the hypothesis that gonadotropin-regulated HPGD plays a role in the control and timing of ovulation in primates (42). In the present study, NFIL3 overexpression decreased Hpgd mRNA expression in granulosa cells and down-regulated Hpgd promoter activity in theca-interstitial cells. Thus, we would propose that although PTGS2 is induced primarily in the granulosa cells, HPGD may play a role in both granulosa and theca cells to regulate the biological actions of PGs. Furthermore, the regulation may occur through competition for, or interaction with, CREB-binding sites. Truncation of the Hpgd promoter revealed that the promoter region from −182 bp to approximately −103 bp was critical for Hpgd responsiveness to FSK + PMA treatment. A CREB-binding site was present within this region of the promoter, and NFIL3 could bind to the DNA containing the CREB-binding site located at −126 bp to approximately −116 bp as illustrated by ChIP analysis. We speculate that NFIL3 may inhibit HPGD expression by competing for CREB binding to the Hpgd promoter (3) or by interacting with CREB to form heterodimers that alter DNA binding (4). Although NFIL3 can heterodimerize with CREB (4), whether this heterodimer functionally inhibits DNA binding remains to be determined because heterodimerization of inducible cAMP early repressor with CREB was insufficient to inhibit the α inhibit subunit gene (15). Irrespective of the mechanism of NFIL3 action, the ability of NFIL3 to regulate HPGD indicates that NFIL3 may be an important regulator of both the synthesis and catabolism of PGs during the periovulatory period.

There is little to no data regarding the role of NFIL3 regulation of the EGF receptor ligands AREG or EREG in any model system. In the ovary, LH/hCG produces a transient up-regulation in mRNAs coding for Areg and Ereg after hCG administration (44). These EGF-like growth factors have been proposed to be involved in steroidogenesis, oocyte maturation, and cumulus expansion (45). In the present study, overexpression of NFIL3 decreased Areg and Ereg mRNA and also decreased the promoter activity of Areg. Similar to its action on the Hpgd promoter, NFIL3 may be competing for CREB-binding sites or heterodimerizing with CREB to regulate the Areg promoter.

In summary, we propose that the midcycle surge of LH induces NFIL3 expression, which results in the production of a transcriptional repressor. This is supported by the present findings that hCG induces the expression of Nfil3 mRNA, initially in the theca-interstitial compartment followed by expression in the granulosa cells. Evidence that NFIL3 functions as a transcriptional repressor is apparent from its ability to down-regulate gene expression when overexpressed. Furthermore, the interaction of NFIL3 with the DNA region containing the binding sites of C/EBPβ or CREB as seen by EMSA and ChIP assays supports the concept that NFIL3 is able to compete or interact with other members of the bZIP family to either fine tune or down-regulate key genes associated with the ovulatory process.

Materials and Methods

Materials and reagents

Unless otherwise noted, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Molecular biological enzymes, molecular size markers, oligonucleotide primers, pCRII-TOPO Vector, culture media, and Trizol were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA).

Tissue collection

All animal procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. Sprague Dawley rats (15 d old) were obtained from Harlan, Inc. (Indianapolis, IN). Animals were maintained on a 12-h light, 12-h dark cycle and provided water and rat chow ad libitum. On the morning of d 22−d 23, rats were injected with PMSG (910 IU sc). Animals were injected 48 h later with hCG (5 IU sc). Ovulation in this model occurs at 12−16 h after hCG (46). Ovaries were collected at 0 h (i.e., time of hCG administration) and 4, 8, 12, or 24 h after hCG administration (n = 3−4 animals per time point). Ovaries were stored at −70 C for later isolation of total RNA or protein, or placed in Tissue-Tek OCT compound (VWR Scientific, Atlanta, GA), snap frozen, and stored at −70 C until sectioned and processed for in situ hybridization analyses.

In situ hybridization of Nfil3 and Hpgd mRNA

In situ hybridization was performed as previously described (47). Oligonucleotide primers corresponding to cDNA for rat Nfil3 (forward, 5′-AAGCTTTGGACAGTGAGTTCG-3′; reverse, 5′-TTACCTGGAGTCCGAAGCCG-3′) and rat Hpgd (forward, 5′-ATGGCATAATCGGATTCACACG-3′; reverse, 5′-CTTCTAATGGCACCCATTGAGG-3′) were designed using PRIMER3 software (48). The PCR products were cloned into pCRII-TOPO Vector. Sense cRNA probes were used as control for nonspecific binding. One ovary from a minimum of three animals was used for in situ hybridization (n = 3−4 ovaries per time point). From each ovary a minimum of four sections per ovary were examined for a minimum of 12 sections per time point.

Rat granulosa cell culture

To isolate granulosa cells, ovaries were collected from rats 48 h after PMSG administration and processed as described previously (47). Briefly, granulosa cells were isolated by follicular puncture, pooled, filtered, pelleted by centrifugation at 200 × g for 5 min, and resuspended in Opti-MEM supple-
mented with 0.05 mg/ml of gentamycin and 1 × ITS (insulin, transferrin, and selenium). The cells were cultured in the absence or presence of various reagents discussed in detail below for different time points at 37 C in a humidified atmosphere of 5% CO2, FSK and PMA were added to mimic hCG action. This approach of using FSK + PMA was employed due to the downregulation of LH receptors in experiments in which cells were transfected for 24 h before treatment. At the end of each culture period, cells were collected and snap frozen for later isolation of total RNA and protein.

Isolation and culture of theca-interstitial cells

The theca-interstitial cells were isolated and cultured using previously described procedures (49, 50). In brief, the ovaries were dissected and punctured under a dissecting microscope to release granulosa cells and thoroughly flushed with medium to remove any remaining adhering granulosa cells. The remaining tissue was then minced and incubated for 90 min at 37 C in a medium containing 2.5 mg/ml collagenase plus 10 μg/ml deoxyribonuclease. After mechanical dispersion, the released theca-interstitial cells were centrifuged at 250 × g for 5 min and washed two times in Opti-MEM. Subsequently, the dispersed cells were subjected to a single 5-min unit gravity purification. The dispersed cells were then seeded in plates for further experimentation described below.

Real-time PCR quantification of mRNA

Total RNA was isolated from ovaries, granulosa cells, or theca-interstitial cells using the Trizol reagent according to the manufacturer’s protocol. Real-time PCR was used to measure levels of Areg, Nfil3, Ptgs2, and Hpgd mRNA in vitro and in vitro as described previously (47). Oligonucleotide primers corresponding to cDNA for rat L32 (forward, 5'-GAAGCCTTATGACGTCCAAA-3'; reverse, 5'-AGGATTCGCCCTTGAATCT-3'), rat Areg (forward, 5'-CGGAAAGGCAGAGGAACGG-3'; reverse, 5'-TGATGACATTTGGCCAGTACC-3'), rat Nfil3 (forward, 5'-AACCGCGGAAAAACACG-3'; reverse, 5'-TTCAAGGGAGCACTGACCA-3'), Hpgd (forward, 5'-CAAGCACTTTATTTTTTG-3'; reverse, 5'-AAATGACATTCAATCTCACC-3'), and rat Ptgs2 (forward, 5'-GATCATACATTGAGGACGTACC-3'; reverse, 5'-TTCTCATTTTTCCACC-3') were designed using PRIMER3 software. The specificity for each primer set was confirmed by both electrophoresis of the PCR products on a 2.0% agarose gel and analyzing the melting (dissociation) curve in the MxPro real-time PCR analysis program (Stratagene, La Jolla, CA) after each real-time PCR. The relative amount of each gene transcript was calculated using the 2–ΔΔCT method and normalized to the endogenous L32 reference gene.

Western blot analysis

Intact ovarian tissues were homogenized in radioimmuno precipitation assay buffer/protease inhibitor cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Granulosa cells were resuspended in radioimmuno precipitation assay buffer/protease inhibitor cocktail for 30 min on ice. Tissue or cell lysates were then centrifuged at 14,000 × g for 10 min. The supernatants were stored at −80 C until use. Protein (20 μg), measured by the Lowry method, was separated on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane (Whatman, Sanford, ME). Immunoblotting was performed by blocking nonspecific binding with 5% dry milk in Tris-buffered saline buffer containing 1% Tween 20 for 1 h as previously described (47). Blots were incubated with the primary antibody for Nfil3 (1:200, Santa Cruz Biotechnology), PTGS2 (1:1000, Cayman Chemical, Ann Arbor, MI) or β-actin (1:1000, Cell Signaling Technology, Danvers, MA), overnight at 4 C on a rocking platform. Blots were washed four times with PBS+0.1% Tween and incubated with respective secondary antibodies linked to horseradish peroxidase for 1 h. After extensive washing, blots were analyzed using an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ) and exposed to x-ray film.

Generation of Areg and Hpgd luciferase reporter vectors and granulosa or theca-interstitial cell infection

Genomic DNA was isolated from tail samples from rats using an easy-DNA kit (Invitrogen). Fragments of the Areg gene, a 692-bp (−676/+16), 239-bp (−223/+16), and 81-bp (−65/+16) fragment, were amplified using the primers attached with restriction enzyme sites (KpnI and HindIII). Fragments of the Hpgd gene, a 808-bp (−779/+29), 211-bp (−182/+29) and 132-bp (−103/+29) fragment were amplified using the primers attached with restriction enzyme sites (KpnI and Nhel). The fragments for the Areg and Hpgd gene were cloned into the pCRII-TOPO vector (Invitrogen) as described previously (51). Cloned fragments were digested with KpnI and HindIII enzymes (Hpgd fragments) and subcloned into a multiple cloning site of the pGL3 basic vector (Promega Corp., Madison, WI).

Site-directed point mutation of the Ptgs2 and Areg promoter was generated using a QuikChange II Site-Directed Mutagenesis kit according to the manufacturer’s protocol (Stratagene, La Jolla, CA). The sequence of the oligonucleotide primer used to generate the Ptgs2 promoter containing the C/EBPβ-binding site mutation (shown in lowercase) is as follows: 5'-GAGTAAAGCCTGCCCTATATGAGTaaGcaTgaATTGGAAGCGGAGATGGGGGAAAG-3'; the Areg promoter containing the CREB-binding site mutation (shown in lowercase) is as follows: 5'-CACCCAGGAGGGGCTcgcTCACCGGGCGGTGTT-3'. Granulosa cells or theca interstitial cells were isolated from immature rats (48 h after PMSG) as described above. The granulosa cells were transfected with respective firefly luciferase reporter plasmids (pGL3-Areg promoter or pGL3-Ptgs2 promoter constructs) and Renilla luciferase vector (pRL-TK vector) using a Lipofectamine 2000 reagent (Invitrogen). Theca interstitial cells were transfected with pGL3-HPGD promoter vector and Renilla luciferase vector. Ad-Nfil3 (described below) was added 4 h after transfection. Fresh culture media were added after 2 h and incubated for 24 h. The next day, granulosa cells were treated with FSK (10 μM), PMA (20 nM), or FSK + PMA for 6 h. Theca interstitial cells were not treated with FSK or PMA. The cells were harvested to measure firefly and Renilla luciferase activities using a dual-luciferase reporter assay system (Promega), and each reaction was monitored for 10 sec by the luminescence system in the Tecan Infinite 200 microplate reader (Tecan U.S., Durham, NC). Firefly luciferase activities were normalized by Renilla luciferase activities, and each experiment was performed in triplicate at least three times.
Generation of recombinant NFIL3 adenovirus vector and granulosa cell or theca-interstitial cell infection

The AdEasy XL Adenovirus vector system (Stratagene) was used for generation of the recombinant Ad-NFIL3 vector as previously described (36) to overexpress NFIL3 to explore its function. Briefly, the Nfll3 DNA was amplified by PCR using total RNA isolated from rat ovary. The specific primers for rat Nfll3 (forward, 5’-TTAGTCGACCCACCAGCTGAGAAATAATGC-3’; reverse, 5’-TTAGCGGCGCCTAACGTGAGTCCGAAGC-3’) were designed to contain SalI and NdeI sites. The PCR product was subcloned into the pShuttle-cytomegalovirus vector. The resultant plasmid encoded the Nfll3 gene under the control of a cytomegalovirus promoter. These plasmids were linearized using Pmel and then cotransformed into electro-competent BJ5183 bacteria with pAdEasy-1, which contains the viral backbone. The recombinant adenovector was linearized using PacI and transfected to Ad293 cells where viral particles were further amplified. The adenovirus was purified and then titered using an AdEasy Viral Titer Kit (Stratagene). The control Ad-GFP was a gift from Dr. Susan Kraner (University of Kentucky).

Rat granulosa cells or theca-interstitial cells were collected at 48 h after PMSG priming as detailed above and cultured in six well plates in Opti-MEM supplemented with 0.05 mg/ml of gentamycin and 1× ITS for 4 h before addition of the Ad-NFIL3 or Ad-GFP at a multiplicity of infection (MOI) of 50 pfu/cell. We routinely observe approximately a 70% infection efficiency of GFP-adenovirus in granulosa cells as well as theca-interstitial cells. The media were replaced 2 h later with fresh Opti-MEM. Next morning, theca-interstitial cells were collected for total RNA isolation or protein extraction, whereas granulosa cells were treated with FSK and PMA and further cultured for 6 h before collection.

Measurement of PGE2

Granulosa cells were exposed to Ad-GFP or Ad-NFIL3 for 24 h and subsequently treated with FSK and PMA for 6 h. Conditioned media containing total cellular lysates were collected for measuring PGE2 levels using a specific ELISA system (Amersham Biosciences, Piscataway, NJ). Briefly, granulosa cell samples or standard PGE2 was incubated for 1 h with specific anti-PGE2 reagent and peroxidase-labeled PGE2 in prepacked 96-well plates containing a goat antimusle solid phase. Unlabeled PGE2 (standard or unknown) and PGE2-peroxidase complex competed for a limited number of binding sites on PGE2-specific antibodies, and the amount of peroxidase-labeled ligand was inversely proportional to the concentration of added standard or unknown. After washing four times with washing buffer, tetramethylbenzidine/hydrogen peroxide substrate was added and incubated for 30 min. The reaction was stopped by acidifying the sample and the resultant color was read at 450 nm in a microtiter plate photometer. The concentration of unlabeled PGE2 in a sample was determined by extrapolation from a standard curve.

ChIP analysis

ChIP assay analysis was performed on NFIL3 in the Ptg2s, Areg, and Hpgd promoter region using a ChIP kit (Upstate Biotechnology, Inc., Lake Placid, NY) as described previously (52). The periovulatory granulosa cells were isolated from PMSG-primed immature rat ovaries at 0, 6, and 12 h after hCG injection. Theca-interstitial cells were isolated from PMSG-primed immature rat ovaries at 0 and 8 h after hCG treatment. The cells were treated with 1% formaldehyde and lysed in lysis buffer to release the nuclei. The nuclei were sonicated with a Fisher sonic dismembrator model 550 to obtain DNA fragments of an average length of approximately 100–500 bp. Chromatin was immunoprecipitated overnight at 4°C with C/EBPβ antibody (5 μg/reaction; Abcam) or NFIL3 antibody (5 μg/reaction; Santa Cruz Biotechnology) or normal rabbit IgG (5 μg/reaction; Santa Cruz Biotechnology) as a negative control. The immunoprecipitated chromatin and 1:10 dilution of input chromatin were analyzed by PCR using the primers designed to amplify the fragment spanning the C/EBPβ motif in the Ptg2s promoter (forward, 5’-GGTATTGCAATTGGAAGC-3’; reverse, 5’-CTTAGTAAAGTGACTCCAGG-3’), the fragment spanning the CREB motif in the Areg promoter (forward, 5’-TTAGAAACAACAGGAGTGG-3’; reverse, 5’-CGGTAGCAGTCTTATAGCC-3’), and the fragment spanning the CREB motif in the Hpgd promoter (forward, 5’-AAAGCTCTAGAAGCGAAAAGATCACCC-3’; reverse, 5’-GGGATGAAACTGTCAAACCAGC-3’). After 25–30 cycles of amplification, PCR products were run on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

EMSA5s

The wild-type probe of the C/EBPβ-binding motif (5’-TGC-CCCTATGGTGTTATGGCAATTGGAAGGAT-3’) on the Ptg2s promoter was labeled with biotin 3’-end DNA labeling kit (Fisher Scientific, Rockford, IL). The consensus C/EBPβ-binding sequence TTAGCAGA was replaced by GACTAGTC in mutant unlabeled probes. Nuclear protein from periovulatory granulosa cells was prepared using NE-PER Nuclear and Cytoplasmic Extraction Kit (Fisher Scientific). EMSA was performed using the LightShift chemiluminescent EMSA kit (Fisher Scientific). Briefly, 2 μl of nuclear extracts were incubated with different unlabeled competitor probes (4 pmol) and biotin end-labeled wild-type probe (20 fmol) in binding reaction buffer for 20 min. For supershift assays, 1 μl NFIL3 antibody (Santa Cruz) was added for another 30 min. Samples were loaded on a 4% native polyacrylamide gel in 0.5 x Tris-borate-EDTA buffer and electrophoresed at 100 V for 1–2 h. Binding reactions were transferred to a nylon membrane, UV cross-linked, and developed using a chemiluminescence nucleic acid detection system (Fisher Scientific).

DNA microarray analysis of granulosa cells after infection of Ad-NFIL3

The granulosa cells were exposed to Ad-NFIL3 or Ad-GFP at a MOI of 50 pfu/cell. Medium was replaced 2 h later with fresh Opti-MEM. At 24 h after adenovirus exposure, granulosa cells were treated with FSK + PMA for 6 h before collection for DNA isolation using a RNeasy kit according to the manufacturer’s instructions (QIAGEN Inc., Valencia, CA). Total RNA (5 μg) was used as a template for cDNA synthesis by the University of Kentucky Microarray Core facility as described previously (47). The Affymetrix Rat 230–2.0 oligonucleotide array set were hybridized, washed, and scanned using Affymetrix equipment and protocols (Affymetrix, Santa Clara, CA). The DNA microarray assays (accession number GSE26730) were performed on total RNA pooled from granulosa cells obtained from three separate experiments. The changes observed by DNA microarray analysis were confirmed by real-time PCR for a select subset of genes.
Knockdown of NFIL3 by siRNA in granulosa cells in vitro

Granulosa cells were collected 48 h after PMSG administration as described above. NFIL3 Stealth Select RNAi (Invitrogen) or Stealth RNAi Negative Control (Invitrogen) was transfected into granulosa cells using the Lipofectamine 2000 reagent (Invitrogen). At 4 h after the transfection, cells were treated with FSK + PMA and incubated for an additional 12 h at 37°C. The cells were collected and snap frozen for isolation of total RNA for real-time PCR.

Statistical analyses

All data are presented as means ± sem. ANOVA was used to test differences across time of culture, or among treatments in vitro. If ANOVA revealed significant effects, the means were compared by Duncan’s test, with \( P < 0.05 \) considered significant.

Acknowledgments

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