The Cebpd (C/EBPδ) Gene Is Induced by Luteinizing Hormones in Ovarian Theca and Interstitial Cells But Is Not Essential for Mouse Ovary Function

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The CCAAT/enhancer binding protein (CEBP) family of transcription factors includes five genes. In the ovary, both Cebpa and Cebpb are essential for granulosa cell function. In this study, we have explored the role of the Cebpd gene in ovarian physiology by expression and functional studies. Here we report that Cebpd (C/EBPδ) is expressed in the mouse ovary in a highly restricted temporal and spatial pattern. In response to luteinizing hormone (LH/hCG), CEBPD expression is transiently induced in interstitial cells and in theca cells of follicles from the primary to pre-ovulatory stage, and overlaps in part with expression of the alpha-smooth muscle actin protein. Efficient down-regulation of CEBPD was dependent on a functional Cebpd gene. Proliferating human theca cells in culture also express Cebpd. Cells from patients with polycystic ovarian syndrome (PCOS) exhibited higher Cebpd expression levels. However, deletion of Cebpd in mice had no overt effect on ovarian physiology and reproductive function. Very little is known at present about the molecular mechanisms underlying theca/interstitial cell functions. The expression pattern of CEBPD reported here identifies a novel functional unit of mouse theca cells of primary to tertiary follicles responding to LH/hCG together with a subset of interstitial cells. This acute stimulation of CEBPD expression may be exploited to further characterize the hormonal regulation and function of theca and interstitial cells.

INTRODUCTION

The ovary is a complex organ undergoing regular phases of reorganization in response to a variety of intrinsic and extrinsic factors. The main functional unit, the follicle is comprised of a central oocyte, mural and cumulus granulosa cell layers, and an outer layer of theca cells. Ovarian follicles reside within the interstitial stroma, which also gives rise to the specialized follicular cell types. The ovulatory surge of luteinizing hormone (LH), produced by the pituitary, triggers a series of dramatic morphological and physiological changes in the pre-ovulatory follicle, culminating in ovulation and subsequent differentiation of the follicular granulosa and theca cells into the luteal cells. While the LH receptor signals primarily through the cAMP/PKA pathway to influence gene expression [1] it also impacts signaling pathways involving Janus and phosphoinositol kinases, chloride currents, and calcium [2–5]. Transcription factors known to act downstream of LH receptor activation include STAT1 and STAT5 [2], GATA4 [6], EGR1 [7], and the CCAAT/enhancer binding protein proteins CEBPA and CEBPB [8].

The CEBP family of transcription factors is comprised of five proteins with a highly homologous carboxyterminal leucine-zipper/basic region domain required for dimerization and DNA binding. Each CEBP protein has unique properties regulating cell type-specific growth and differentiation. For example, within the hematopoietic system CEBPA is required for development of granulocytes, while lack of CEBPB affects differentiation of the B-cell lineage and monocytes. During adipocyte differentiation, CEBPB, -D, and A are expressed consecutively [9]. In the mammary gland, CEBPB promotes proliferation and differentiation [10], while CEBPD participates in the initiation of cell death [11]. In the ovary, both CEBPA and CEBPB are expressed in follicular granulosa cells, dynamically regulated by gonadotropins, and essential for follicular development, efficient ovulation and luteal differentiation. In particular, CEBPB is a downstream effector of the LH signaling pathway in granulosa cells [8]. The absence of significant levels of CEBPA and CEBPB in theca cells combined with the importance of these factors in mediating LH-triggered events in granulosa cells suggested the possibility that another member of the family may substitute for their function in theca cells. Therefore, we investigated the expression and function of CEBPD in the ovary.

Cebpd (C/EBPdelta, CELF, CRP3, NFIL-6beta) was first characterized as an acute phase inflammatory response gene. Expression of Cebpd is typically low to undetectable in most cell types and tissues, but is rapidly induced by a variety of extracellular stimuli, (e.g. growth hormone, insulin, IFNGamma, ...)
IL-1, IL-6, LPS, TNFα, noradrenaline and glutamate) [9,12]. In vitro and in vivo studies have implicated CEBPD in proliferation of osteoblasts [13,14], differentiation of lung epithelial cells [15–17], and growth arrest of mouse mammary epithelial cells [18]. While Cebpd-deficient mice display no overt phenotype, are fertile and achieve normal life spans, more detailed characterization revealed that the null mutation led to improved performance in the contextual fear conditioning test of long term memory [19], increased mammary ductal branching [20] and delayed mammary gland involution [11]. Furthermore, Cebpd-deficiency exacerbates the differentiation defect of Cebp-deficient adipocytes [21], and causes genomic instability in fibroblasts [22]. Thus, CEBPD appears to have highly diverse functions depending on cell type and specific physiological stimuli. Furthermore, because its expression is mostly activated by transient signals, the role of CEBPD in vivo may be modulatory and only uncovered when cells are investigated in response to specific stimuli. In this study we have addressed whether CEBPD plays a role in ovarian physiology through expression analyses and a thorough reproductive characterization of Cebpd-deficient mice.

RESULTS
Dynamic regulation of Cebpd mRNA expression in the mouse ovary

To address the role of CEBPD in the mouse ovary we first assessed its expression pattern in response to LH administration. We used an ovulation protocol in which mice were treated with pregnant mare serum gonadotropin (PMSG) to stimulate the coordinated development of multiple pre-ovulatory follicles, followed two days later by human chorionic gonadotropin (hCG) to mimic the ovulatory surge of LH. Northern blot analysis of whole ovary RNA revealed a highly restricted temporal expression pattern for Cebpd (Fig. 1). Expression of Cebpd mRNA was minimal prior to hCG treatment, but was highly induced 2–3 hours following hCG, before returning to pre-hCG levels by 6 hours where it remained through 8 hours. Similar data were obtained by Western analysis of whole ovary protein extracts (data not shown). Expression of prostaglandin-endoperoxide synthase 2 (Ptgs2, a.k.a. Cox-2, Pghs 2), a gene known to be induced by LH in granulosa cells [23], was evaluated as a positive control (Fig. 1). As expected, ovarian Ptgs2 expression increased following hCG treatment and then declined rapidly. In whole ovary extracts, Cebpd and Ptgs2 mRNA expression were regulated temporally in a similar pattern (Fig. 1). Similar expression data were obtained from 3 week old prepubertal and 5 week old postpubertal mice. Thus, we have used 5–6 week old mice for all other expression analyses in this study.

Analysis of Cebpd expression in Cebpb null mice

Since we previously identified an essential role of Cebpb in the LH responsiveness of granulosa cells [24], we assessed Cebpd expression also in Cebpb-deficient mice (Fig. 2). Cebpd mRNA expression was induced normally by hCG in Cebpb null mice. Expression levels at 0 h and 3 h after hCG treatment were statistically similar in WT and KO ovaries. However, Cebpd mRNA levels remained high in Cebpb knockout mice through five hours of hCG treatment, in contrast to the rapid loss seen in the controls (Fig. 2). By 8 hours following hCG, Cebpd-deficient and control ovaries expressed similar levels of Cebpd mRNA (data not shown). This pattern of normal induction by hCG but delayed down-regulation is similar to the defective down-regulation of Ptgs2 and aromatase mRNA expression in granulosa cells of Cebpb-deficient mice [24].

Localization of Cebpd expression in theca/interstitial cells

To determine which ovarian cell type(s) Cebpd was expressed in we employed in situ hybridization and immunohistochemistry. Figure 3 confirms the relative lack of Cebpd expression prior to the LH surge, and the dramatic induction of Cebpd mRNA levels following hCG administration. This expression was localized in the theca cell layer of follicles of varying sizes as well as in the stromal compartment. No evidence of granulosa cell expression was observed in any preparation. To better characterize Cebpd expression within the stroma, immunohistochemistry was performed. The anti-CEBPD rabbit polyclonal antibody detected nuclear CEBPD protein expression in theca and interstitial cells specifically in wild-type ovarian tissue but not in Cebpb knockout control tissue (Fig. 4A). As seen with Cebpd mRNA, maximal protein levels were observed 3 h after hCG treatment. However, 7 h following hCG-administration to mice CEBPD protein was still detected in interstitial cells, but less so in theca cells (Fig. 4A).

Figure 1: Cebpd mRNA expression is induced by hCG in mouse ovaries. Northern blot analysis of whole ovary total RNA isolated from 5 week-old (Exp.1) or 3 week-old (Exp.2) mice treated for 2 days with PMSG followed by the LH analog hCG for the indicated times (n.t. = no treatment). Ptgs2 was used as a positive control for the hCG response. Cyclophilin (Ppi) was used as loading control. doi:10.1371/journal.pone.0001334.g001

Figure 2: Down-regulation of Cebpd mRNA requires Cebpb. Phosphorimage quantitation of Northern blot analyses of whole ovary total RNA from 5 week-old Cebpb null mice (KO) or litter mate controls (WT) treated for 2 days with PMSG followed by hCG for the indicated times. Expression levels of Cebpd were normalized to cyclophilin (Ppi) expression and are shown in arbitrary units (a.u.; mean ± SEM; n = 3–4 mice per data point). **P = 0.01 by two-way ANOVA with follow-up comparisons among groups in the form of t-tests at each time point. doi:10.1371/journal.pone.0001334.g002
Thus, immunohistochemistry revealed expression kinetics that were not evident by analysis of whole ovary mRNA (Fig. 1) or protein extracts (data not shown). Most of the CEBPD-staining interstitial cells exhibit small, elongated nuclei. Theca externa cells express alpha smooth muscle actin (ACTA2) [25], and CEBPD is also expressed in vascular smooth muscle cells [26]. Thus, we sought to determine if CEBPD characterizes ACTA2 expressing interstitial cells. Analysis of parallel sections revealed that ACTA2 and CEBPD stain the same layers in primary to antral follicles (Fig. 4B). Similarly, some stromal areas that stain positive for ACTA2 also contain CEBPD-expressing cells (Fig. 4C).

Analysis of Cebpd null mutant mice

When Cebpd null mutant mice had first been generated [19], females and males were found to be fertile. Here we conducted a more detailed analysis to potentially uncover effects of Cebpd deficiency on ovarian physiology that may not be evident in assays of fertility. Comparison of Cebpd and control mice reproductive parameters are shown in Figure 5. No differences were observed between Cebpd knockout and control mice with respect to the size of their first litters (Fig. 5A). Regulation of the estrous cycle was assessed indirectly by mating the females to sterile males and recording mating activity. Both genotypes mated on average every
ten days, indicating normal mating behavior and a normal pseudopregnancy response to mating, which is reliant on luteal progesterone production (Fig. 5B). Levels of progesterone were assessed as a measure of follicular differentiation following ovulation. Cebpd null mice and controls produced similar levels of progesterone on day 5 of gestation and day 5 of pseudopregnancy (Fig. 5C). Lastly, examination of the ovarian histology of mice from age 70 to 400 days did not reveal abnormalities when compared to wild-type littermates (data not shown). Taken together, these data demonstrate that the Cebpd null mutation does not overtly affect reproductive function in female mice.

Since a physiological response may proceed normally despite the altered regulation of certain genes, we also analyzed the expression of several marker genes 4 hours after hCG treatment in knockout and wild-type ovaries (Fig. 6). No differences were observed for the mRNA levels of several steroidogenic marker genes, steroidogenic acute regulatory protein (Star), cytochrome P450sc (Cyp11a1), 17-α hydroxylase (Cyp17a1), and 3β-hydroxysteroid dehydrogenase (Hsd3b), as well as the theca-specific tissue-type plasminogen activator (Plat) gene [27]. Furthermore, Ptgs2, a gene primarily expressed in granulosa cells [27], was induced to similar levels in Cebpd knockout and control mice (Fig. 6). These data further confirm normal ovarian function in the absence of CEBPD.

Expression of CEBPD in cultured human theca cells

Analysis of cultured proliferating theca cells obtained from patients with polycystic ovarian syndrome (PCOS, n = 4) showed approximately 3-fold higher levels of Cebpd mRNA than theca cells isolated from women not afflicted with this syndrome (Fig. 7). These data confirm that Cebpd expression in theca cells is not a species-specific phenomenon as it occurs in mouse and human. Previous microarray experiments utilizing the same RNA preparations used in our experiments showed increased 17α-hydroxylase (Cyp17a1) expression in these PCOS-theca cells [28], confirming their pathological condition.
cellular distribution has not yet been characterized [8].

is detectable in whole ovary RNA, but its hormonal regulation or development. However, it is possible that loss of CEBPD was (which is critical for follicular steroidogenesis) or thecal cell might be expected, if it was essential for LH-induced steroidogenesis was not observed in interstitial cells [29]. Remarkably, loss of activation of Giot1 was detected only in theca interna cells and gonadotropin-inducible transcription factor 1 (transcription factor gene previously reported as activated by LH/hCG and may help to further characterize these ovarian cell types.

The expression data in Figures 1–3 were derived from mice of a group of interstitial cells within the ovarian stroma and in theca cells of preovulatory follicles, with the highest levels in the ovary by providing unique functions for its variety of cell types.

androgen levels and are in agreement with the chronically elevated expression in PCOS patient derived theca cells. Our results indicate that Cebpd expression was elevated in theca cells that are producing androgen and are in agreement with the chronically elevated androgen levels and Cyp17a1 gene expression seen in PCOS theca cells. However, our data are in contrast to an array-based expression analysis of biopsies, which reports 2-fold lower levels of Cebpd expression in PCOS tissue [31]. There are several potential reasons for this discrepancy. The cultured cells used here were derived from the theca layer of 5–6 mm follicles of both normal and PCOS women, while the biopsies were random and therefore of varying cell type composition that could be quite heterogeneous. In contrast, the cultured theca cells from normal and PCOS women would be more homogenous and comparable cell populations, and cultured cells are known to maintain some of their in vivo characteristics unique to PCOS such as elevated Cyp17a1. However, the potential effect of culture conditions affecting gene expression patterns can not be discounted either. Because of the relatively small number of samples in this analysis we primarily conclude that Cebpd expression in theca cells is not specific to mice but also true for human cells. Our present results lend support but far from confirm that CEBPD is possibly elevated in theca cells of PCOS women.

In summary, our data show that the Cebpd gene is rapidly induced by LH/hCG in theca cells of primary follicles through pre-ovulatory follicles as well as in interstitial cells. This observation demonstrates some common characteristics in the hormonal regulation between these cell populations. The data also suggest that in contrast to CEBPA and CEBPB, CEBPD function is not directly related to the ovulation process, but may rather have a role in the broad endocrine activity of the theca/interstitial compartment, or possibly the inflammatory response associated with ovulation. The physiologic/developmental role of LH/hCG responsive cells within the ovarian stroma compartment is presently not understood. CEBPD can be a useful marker for the early response of the theca/interstitial compartment to LH/hCG and may help to further characterize these ovarian cell types.

**MATERIALS AND METHODS**

Mice

The generation of mice [Mus musculus] with targeted deletion of Cebpd [19] and Cebp [24] has been described previously. Wild-type and mutant subjects were offspring from heterozygous breeding pairs. Cebp-deficient mice are not viable on a pure genetic background. Thus, the expression data in Figures 1–3 were derived from mice of a 129B6-F1 strain background, generated by mating heterozygous
mice that had been backcrossed into the 129S1 and C57BL/6 strain background, respectively. All other data were generated with mice on a pure 129S1 strain background, which exhibit similar Cebpd expression as the 129B6-F1 strain. Where indicated, mice were given intraperitoneal injections of pregnant mare serum gonadotropin (PMSG, 5 IU; SIGMA; equivalent to equine chorionic gonadotropins, eCG) or human chorionic gonadotropin (hCG, 5 IU, SIGMA). The mice were housed and bred in a specific pathogen-free facility with a 12 hour light cycle, and with chow and water ad libitum. All procedures were conducted in compliance with the guidelines of the Animal Care and Use Committee of the National Cancer Institute, MD, U.S.A.

**In situ analysis**

Ovaries were fixed in 4% paraformaldehyde and 5 μm sections were prepared from paraffin embedded tissues. *In situ* hybridization analysis was performed as described [32], using a cRNA probe representing the coding region for amino acids 1–101 of Cebpd.

**Immunohistochemistry**

Immunohistochemical staining was performed with the aid of an automated immunostainer (DakoCytomation, Carpinteria, CA). Formalin-fixed paraffin embedded tissue sections were mounted on glass slides and deparaffinized. Prior to staining, heat-induced antigen retrieval was performed by placing the slides into target retrieval solution, high pH (DakoCytomation), and steaming them in an commercial vegetable steamer at full temperature for 30 minutes (CEBPID) or by pressure cooking in TRIS/Citrate (pH 6) for 8 minutes (alpha-smooth muscle actin, ACTA2). Following the antigen retrieval procedure the slides were incubated with a CEBPD rabbit polyclonal antibody (ActiveMotif cat #39006; dilution 1:500) overnight at 4°C, or a monoclonal mouse antibody against ACTA2 (DAKO, Clone 1A4, dilution 1:500) for 1 hour. Detection was carried out on the automated system using an HRP/DA turnover polymer based rabbit detection system (Envision+, DakoCytomation) according to the manufacturer’s recommendations.

**Progesterone assay**

Serum was prepared from orbital eye bleeds and progesterone levels were assessed with the DSI-10-3900 ACTIVEL progesterone EIA kit (Diagnostic Systems Laboratories, Inc.), according to the manufacturer’s instructions. Each sample was assayed in duplicate. The coefficient of variation across experiments and genotypes was 0.35.

**RNA analysis**

Total ovarian RNA was prepared by homogenization of tissue in Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. The RNA was analyzed by Northern blotting as described [33] except that hybridizations were carried out in HyBlot plus solution (SigmaAldrich, Inc.). Radiolabeled DNA probes were prepared from isolated mouse cDNA clones for the indicated genes. The specific signals were recorded and quantified by phosphorimage analysis (Molecular Dynamics, ImageQuantTM).

**Human theca cell culture and real time PCR analysis**

Human theca interna tissue was obtained from follicles of women undergoing hysterectomy, following informed consent under a protocol approved by the Institutional Review Board of the Pennsylvania State University College of Medicine. In the experiments presented in this manuscript, 3rd and 4th passage (31–38 population doublings) theca cells isolated from size-matched follicles obtained from age-matched subjects were used as previously described [34,35]. All of the PCOS theca preparations studied came from ovaries of women with fewer than six menstrual periods per year and elevated serum total testosterone (T) or bioavailable T levels, as we previously described (26). The control (normal) theca cell preparations came from ovaries of fertile women with normal menstrual histories, menstrual cycles of 21–35 d, and no signs of hyperandrogenemia. The OB/Gyn department at Penn State Medical Center routinely schedules hysterecetomies of reproductive aged women during their follicular phase.

Total RNA (5 μg) isolated with Trizol reagent (Life Technologies, Inc.) was treated with DNase I (Promega, Madison, WI) followed by cDNA synthesis using the MMLV reverse transcriptase (Promega) and oligo dT primer (Promega) as previously described [35]. The resulting cDNAs were diluted 1:100 in sterile water and 1-μl aliquots were used in the quantitative real-time PCR reactions. The primers used to quantify Cebpd (Forward: 5'-GGTGCGGCTGGCCAGTTT-3'; Reverse: 5'-CTCGGAGTTTAAGTGAATGC-3') were designed with Primer Express software package that accompanies the Applied Biosystems Model 7900 sequence detector (PerkinElmer Life Science). The SyBr green reagent PCR Master Mix (Applied Biosystems) was used as described previously [36]. In order to account for differences in starting material, the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probe reagents from Applied Biosystems were used as described by the manufacturer. In order to quantify differences, the samples were compared to standard curves for each target amplicon and the average value for the triplicate was used in all subsequent calculations.

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**Author Contributions**

Conceived and designed the experiments: ES AH LC. Performed the experiments: ES AH MR SS LC. Analyzed the data: ES AH MR MR LC. Contributed reagents/materials/analysis tools: ES JM MR LC. Wrote the paper: ES LC.

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