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The Role for Runt Related Transcription Factor 2 (RUNX2) as a Transcriptional Repressor in Luteinizing Granulosa Cells

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Abstract

Transcription factors induced by the LH surge play a vital role in reprogramming the gene expression in periovulatory follicles. The present study investigated the role of RUNX2 transcription factor in regulating the expression of *Runx1, Ptgs2,* and *Tnfaip6* using cultured granulosa cells isolated from PMSG-primed immature rats. hCG or forskolin+PMA induced the transient increase in *Runx1, Ptgs2,* and *Tnfaip6* expression, while the expression of *Runx2* continued to increase until 48 h. The knockdown of the agonist-stimulated *Runx2* expression increased *Runx1, Ptgs2,* and *Tnfaip6* expression and PGE_2 levels in luteinizing granulosa cells. Conversely, the over-expression of RUNX2 inhibited the expression of these genes and PGE_2 levels. The mutation of RUNX binding motifs in the *Runx1* promoter enhanced transcriptional activity of the *Runx1* promoter. The knockdown and overexpression of *Runx2* increased and decreased *Runx1* promoter activity, respectively. ChlP assays revealed the binding of RUNX2 in the *Runx1* and *Ptgs2* promoters. Together, these novel findings provide support for the role of RUNX2 in down-regulation of *Runx1, Ptgs2,* and *Tnfaip6* during the late ovulatory period to support proper ovulation and/or luteinization.

Keywords

Runx2; Runx1; Ptgs2; Tnfaip6; Granulosa cells; LH; Ovary

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The authors have nothing to disclose.

1. INTRODUCTION

The preovulatory gonadotropin (FSH/LH) surge initiates multipath, yet intertwined processes that lead to the expansion of cumulus oocyte complex (COC), ovulation, and corpus luteum (CL) formation. These changes are accomplished by spatio-temporally regulated expression of genes in periovulatory follicles. Specific transcription factors induced by the LH surge directly control the dynamic changes in periovulatory gene expression, thus playing a central role in successful ovulation of COCs and luteal transformation [reviewed in (Richards, 2007; Russell and Robker, 2007; Sirotkin, 2010; Stouffer et al., 2007)].

Previously, we and others have demonstrated that two members of the Runt related transcription factor family, *Runx1* and *Runx2*, were highly induced by the LH surge in periovulatory follicles of rat, mouse, and human ovaries (Hernandez-Gonzalez et al., 2006; Jo and Curry, Jr., 2006; Park et al., 2010). However, their temporal expression pattern was distinct; *Runx1* expression was highly induced shortly after the LH surge, and declined rapidly around the time of ovulation (Jo and Curry, Jr., 2006), whereas *Runx2* expression progressively increased and reached the highest level in the forming CL (Park et al., 2010). *In situ* hybridization and immunohistochemical analyses further verified the tissue localization of *Runx1* and *Runx2* to granulosa and cumulus cells of periovulatory follicles and forming CL (Jo and Curry, Jr., 2006; Park et al., 2010).

RUNX1 and RUNX2 are members of the Core Binding Factor (CBF/PEBP2/RUNX) family. CBF is a heterodimeric transcription factor composed of α and β subunits; the α subunit is encoded by one of three *Runx* genes (*Runx1*, *Runx2*, and *Runx3*) and the β subunit is encoded by a single gene, *CBF*β. CBFβ does not bind to DNA directly, but enhances DNA binding of RUNX proteins (Bae et al., 1993; Ogawa et al., 1993; Speck et al., 1999). Both RUNX1 and RUNX2 have been shown to be essential for the development of various tissues by controlling cell proliferation and differentiation [reviewed in (Coffman, 2003; Otto et al., 2003)]. However, RUNX1 and RUNX2 contribute distinct biological processes by regulating tissue specific gene expression (Geoffroy et al., 2002; Lutterbach and Hiebert, 2000). For instance, RUNX1 plays a vital role in hematopoiesis by regulating specific hematopoietic genes (Lutterbach and Hiebert, 2000). RUNX2 is known to regulate the expression of specific osteogenic genes and is considered as a master regulator for osteogenesis (Komori, 2002; Komori, 2008; Komori, 2010).

Structurally, RUNX proteins contain multiple trans-activation and repression domains in addition to a conserved DNA-binding Runt domain (Durst and Hiebert, 2004; Westendorf, 2006). Therefore, RUNX proteins can increase or inhibit the transcriptional activity of their respective target genes in a context dependent manner [reviewed in (Cameron and Neil, 2004)]. RUNX proteins are commonly thought of as a potent transcriptional activators because early studies identified them as positive regulators for numerous genes in diverse cell types (Komori, 2010; Lutterbach and Hiebert, 2000; Otto et al., 2003; Pratap et al., 2006). More recently, their role as a transcriptional repressor has also been appreciated in a number of cell types (Otto et al., 2003; Shimizu et al., 2010; Sun et al., 2009; Vitolo et al., 2007). Intriguingly, among the list of the genes that are negatively regulated by RUNX proteins include *Runx* genes themselves (Brady et al., 2009; Brady and Farrell, 2009; Drissi et al., 2000). All three *Runx* genes contain multiple RUNX binding sites in their promoter regions (Rini and Calabi, 2001; Wong et al., 2011; Xiao et al., 2001). Indeed, experimental evidence has been accumulating in support of auto- or cross-regulatory mechanisms for their own expression (Brady et al., 2009; Brady and Farrell, 2009; Drissi et al., 2000; Ghozi et al., 1996; Spender et al., 2005; Wong et al., 2011). For instance, RUNX2 was shown to suppress its own expression by direct binding to its own promoter and 5’ UTR region in osteoblastic

cell lines (Drissi et al., 2000). In T-cell receptor activated cells, RUNX1 self-represses its own expression by acting on its distal promoter region (Wong et al., 2011). Moreover, a recent study by Spencer et al. (Spender et al., 2005) has reported cross-regulation between different RUNX family members; RUNX3 represses *Runx1* expression in human B cells.

Interestingly, our previous microarray data showed that the knockdown of *Runx2* by siRNA resulted in increased levels of mRNA for *Runx1* in cultured granulosa cells, suggesting intra-familiar regulation between these two transcription factors. The microarray data also revealed that *Ptgs2* and *Tnfaip6* expression were higher in *Runx2* siRNA-treated cells. Importantly, *Ptgs2* and *Tnfaip6* expression are transiently induced in periovulatory follicles and were found to be crucial for proper ovulation and/or COC expansion (Davis et al., 1999; Fulop et al., 1997; Fulop et al., 2003; Joyce et al., 2001; Ochsner et al., 2003; Sirois et al., 2004; Yoshioka et al., 2000).

In the present study, we hypothesize that RUNX2 plays a role in down-regulation of *Runx1*, *Ptgs2*, and *Tnfaip6* expression in luteinizing granulosa cells. To test this hypothesis, the temporal expression of these genes was first assessed in luteinizing granulosa cells in culture. Then, using the experimental approaches of silencing and over-expression of *Runx2*, we determined whether modulation of *Runx2* expression affects *Runx1*, *Ptgs2*, and *Tnfaip6* expression. In addition, we investigated the molecular mechanisms of RUNX2 regulation on *Runx1* expression at the promoter level in luteinizing granulosa cells.

2. MATERIALS AND METHODS

2.1. Materials

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

2.2. Animals

Sprague Dawley rats were obtained from Harlan, Inc. (Indianapolis, IN) and maintained at the University of Kentucky Laboratory Animal Resources. Animal protocols for the rat study were approved by University of Kentucky Animal Care and Use Committees. Immature rats (24–25 days old) were injected with pregnant mare serum gonadotropin (PMSG, 10 IU) to stimulate follicular development. Forty eight hours later, the animals were injected with human chorionic gonadotropin (hCG, 10 IU) to induce ovulation and formation of CL. Animals were killed at the time of hCG administration and defined times after hCG.

2.3. Isolation and culture of rat granulosa cells

Ovaries were collected from immature rats at 48 h post-PMSG and punctured to isolate granulosa cells as described previously (Park et al., 2008). The granulosa cells were pooled, filtered, and pelleted by centrifugation. The cells were cultured in Opti-MEM (Invitrogen) media supplemented with 0.05 mg/ml of gentamycin, and 1x ITS (insulin, transferin, and selenium). The cells were cultured in the absence or presence of various reagents at 37 °C in a humidified atmosphere of 5% CO_2.

2.4. Quantification of Runx1, Runx2, Ptgs2, and Tnfaip6 mRNA

Total RNA was isolated from rat ovaries and cultured granulosa cells using a Trizol™ reagent (Invitrogen) or RNeasy mini kit (QIAGEN, Inc., Valencia, CA), respectively. To measure levels of rat *Runx1*, *Runx2*, *Ptgs2*, and *Tnfaip6* mRNA, we used Real-time PCR as

described previously (Park et al., 2008). Oligonucleotide primers corresponding to each gene were designed using PRIMER3 software and listed in Table 1. The specificity for each primer set was confirmed by both running the PCR products on a 2 % agarose gel and analyzing the melting (dissociation) curve using the MxPro Real-time PCR analysis program (Stratagene, La Jolla, CA) after each Real-time PCR reaction. All samples were measured in duplicate or triplicate and the amplification efficiency of each transcript primer set was determined by running a standard curve. The relative abundance of the target transcripts was normalized to the endogenous reference gene *L32* for rats and calculated according to the Pfaffl method (Pfaffl, 2001).

2.5. Western blot analysis

Nuclear extracts and cytoplasmic fractions were isolated from ovaries or cultured granulosa cells using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA) as described previously (Jo et al., 2004). All lysates were denatured by boiling for 5 min and separated by SDS-PAGE on a 9 % polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membrane was incubated overnight at 4°C in 1% casein solution containing primary antibody against RUNX2 (PC287, Calbiochem, La Jolla, CA), RUNX1 (Ab-1,Calbiochem) or PTGS2 (Cayman Chemical, Ann Arbor, MI). Primary antibody against TATA binding protein (TBP, Abcam) and β-ACTIN (Cell Signaling Technology, Danvers, MA) was used as a loading control for nuclear and cytoplasmic fractions, respectively. The blots were incubated with the respective secondary HRP-conjugated antibody (Santa Cruz Biotechnology) for 1 h. Peroxidase activity was visualized using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL).

2.6. Cloning of the rat Runx1 promoter and generation of Runx1 promoter-reporter plasmid constructs

Genomic DNA was isolated from ear samples from rats using an easy-DNA kit (Invitrogen). A 369 bp (−283/+86), 861 bp (−775/+86), and 1349 bp (−1263/+86) fragments of the *Runx1* gene were amplified using the primers attached with restriction enzyme sites (*Kpn* I and *Hind* III) and cloned into the pGL3 basic vector (Promega, Madison, WI). Site-directed point mutations of the *Runx1* promoter were generated using a QuickChange II site-directed mutagenesis kit according to the manufacturer's protocol (Stratagene). The sequences of the oligonucleotide primers used to generate respective *Runx1* promoters containing mutations (shown in lowercase) are following: mutant A (5'- AGG CCC GCT CCA CCT GCg cta gcT ACA CGG TGC TCG CTC G −3'), mutant B (5'- AAC TGC GGC TCA ACT CCC ACC AAg cag gcG GAT CAG CCA CAA ACT TAA CC −3'). All constructs cloned into the expression vector were sequenced commercially to verify their authenticity (MWG Biotech, Inc., High Point, NC).

2.7. Knockdown of Runx2 mRNA by siRNA in granulosa cell cultures

Granulosa cells were isolated from ovaries collected at 48 h post-PMSG and transfected with siRNA as described previously (Park et al., 2010). Briefly, granulosa cells were cultured overnight to acclimatize the cells and the next day, cells were transfected with siRNA specific for *Runx2* (sense, GCA CGC UAU UAA AUC CAA Att; antisense, UUU GGA UUU AAU AGC GUG Ctg; Ambion, Inc.) or negative control siRNA (Stealth™ RNAi Negative Control Med GC; Invitrogen) using Lipofectamine ™ RNAiMAX (Invitrogen) according to the manufacturer's instruction. Transfected cells were incubated for 3 h before stimulating with forskolin (FSK; 10 μM) + phorbol 12-myristate 13-acetate (PMA; 20 nM) and further cultured for 24 or 48 h. At the end of culture, the cells were used to isolate total RNA or extract proteins.

2.8. Transient transfection and luciferase reporter assay

Granulosa cells isolated from immature rats (48 h post-PMSG) were transfected with respective firefly luciferase reporter plasmids (pGL3-basic vector or *pGL3-Runx1* promoter constructs) and Renilla luciferase vector (pRL-TK vector) using a Lipofectamine 2000 reagent (Invitrogen) as described previously (Liu et al., 2010). The next day, the cells were treated with FSK+PMA. Six hours later, the cells were harvested to measure Firefly and Renilla luciferase activities using a Dual-luciferase reporter assay system (Promega) and each reaction was monitored by Luminescence System in the Tecan Infinite 200 microplate reader (Tecan US, Durham, NC). Firefly luciferase activities were normalized to Renilla luciferase activities and each experiment was performed in quadruplicate at least 3 times.

2.9. Adenoviral mediated over-expression of RUNX2 in vitro

Granulosa cells were isolated from immature rats (48 h post-PMSG) as described above. The cells were incubated with adenoviruses expressing RUNX2 (Ad-RUNX2) (Ge et al., 2009) or containing null control vector (Ad-ψ) at 5 infectious units (IFU). The next day, cells were treated with FSK+PMA for 4 h and then collected for isolation of total RNA for Real-time PCR or proteins for Western blot analysis.

2.10. Measurements of PGE_2 level

Granulosa cells isolated from immature rats (48 h post-PMSG) were plated in a 96 well plate and transfected with *Runx2* siRNA or NC siRNA or infected with Ad-RUNX2 or Ad-ψ as described above. Upon completion of culture, PGE_2 levels were determined using the Biotrak Prostaglandin E_2 Enzyme Immunoassay system (Amersham Pharmacia Biotech, USA) according to the manufacturer's protocol at 620 nm wavelength.

2.11. Chromatin immunoprecipitation (ChIP) analysis

ChIP assay was performed on RUNX binding sites in rat *Runx1* and Ptgs2 promoter regions using a ChIP kit (Upstate Biotechnology) as described previously (Park et al., 2008). Briefly, chromatins isolated from cultured granulosa cells were immunoprecipitated overnight at 4 °C with anti-RUNX2 antibody (5 μg/reaction; Santa Cruz Biotechnology) or rabbit IgG (5 μg/reaction). The immunoprecipitated chromatin and 1:10 dilution of input chromatin were analyzed by PCR using the primers designed to amplify fragments spanning the RUNX motif in the *Runx1* gene [see Fig.4E, $RUNX^{-728}$ (forward 5'-GTG GGA GTG AGC GTG TGT AA-3', reverse 5'-TCG GAA GTC AGC CAC TGT C-3') and $RUNX^{-526}$ (forward 5'-GAG TGC ATG TCT GCC TGT GT-3', reverse 5'-ACT CTG GTT TGG GAA CGA TG-3')] and in the *Ptgs2* gene [see Fig.4F, $RUNX^{-260}$ (forward 5′-GGG GAA GCT GTG ACA TTC TC T-3′, proximal reverse, 5′-CCA TAG GGG CAG GCT TTA CT-3′ ')]. After 25–30 amplification cycles, PCR products were run on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

2.12. Statistical analyses

Results were expressed as mean ± SEM. Data were tested for homogeneity of variance by Levene test, and square root or log transformations were performed on data set that showed heterogeneous variance. All data were analyzed by Student's t-test, or ANOVA (one-way or two-way ANOVA) to determine the significant difference across time of culture or among treatments *in vitro*. Student t-test was used to assess differences of treatment between groups. If ANOVA revealed significant effects, the means were compared by Tukey's test, with $p < 0.05$ considered significant.

3. RESULTS

3.1. hCG induced the transient expression of Runx1, Ptgs2, and Tnfaip6 and the sustained expression of Runx2 in rat preovulatory granulosa cell cultures

In the previous microarray data, we found that the levels of mRNA for *Runx1, Ptgs2*, and *Tnfaip6* were increased in *Runx2* siRNA-treated cells, suggesting negative regulation of these gene expressions by RUNX2. To further study the relationship among these periovulatory genes, we first determined the expression profile of these genes in our granulosa cell culture model. Although the expression pattern of these genes has already been characterized in periovulatory ovaries (Hernandez-Gonzalez et al., 2006; Jo and Curry, Jr., 2006; Joyce et al., 2001; Park et al., 2010; Yoshioka et al., 2000), it is important to verify that LH/hCG induces the unique expression pattern of these genes in our rat granulosa cell cultures. As shown in Fig. 1, hCG increased the expression of *Runx1, Runx2, Ptgs2*, and *Tnfaip6*. The levels of *Runx2* mRNA increased at 6 h and continued to rise until 48 h (Fig. 1A), while *Runx1* expression was transiently increased with the highest level of mRNA at 6 h and declined to basal level at 24 h (Fig. 1B). The up-regulation of *Ptgs2* and *Tnfaip6* expression was also rapid and transient, which reached highest levels at 6 h (Fig. 1C&D). These results demonstrated that in our granulosa cell cultures, hCG treatment induced transient up-regulation *of Runx1, Ptgs2*, and *Tnfaip6* expression and gradual increases in *Runx2* expression similar to those documented in the *in vivo* system (Jo and Curry, Jr., 2006; Joyce et al., 2001; Park et al., 2010; Yoshioka et al., 2000).

3.2. Alteration of Runx2 expression affected Runx1, Ptgs2, and Tnfaip6 expression in granulosa cell cultures

Based on the unique, yet differential expression pattern among *Runx1, Runx2, Ptgs2*, and *Tnfaip6* in periovulatory granulosa cells, we hypothesized that the elevated expression of *Runx2* in the late ovulatory period facilitates down-regulation of *Runx1, Ptgs2*, and *Tnfaip6* expression. Therefore, we determined whether the knockdown of *Runx2* expression could affect *Runx1, Ptgs2*, and *Tnfaip6* expression in luteinizing granulosa cells. In our granulosa cell culture system, optimal gene silencing was achieved when the cells were acclimatized overnight and then transfected with *Runx2* siRNA. However, this overnight acclimation caused LH receptor down-regulation in cultured granulosa cells (Robert et al., 2003; Schwall and Erickson, 1983). Therefore, in many studies from our laboratory (Liu et al., 2009; Liu et al., 2010; Park et al., 2008; Park et al., 2010) and others (Li et al., 2009; Li et al., 2011; Shimada et al., 2007; Sriraman et al., 2003), the combination treatment of forskolin (FSK, activator of adenylate cyclase) and PMA (activator of PKC) was used to mimic the action of LH/hCG in inducing periovulatory genes in granulosa cell cultures. These agonist treatments stimulated the expression of *Runx1, Runx2, Ptgs2*, and *Tnfaip6* with a similar expression pattern compared to those observed in hCG-stimulated granulosa cell cultures (Supplemental Fig. 1). In the present study, we examined the effect of *Runx2* silencing at 24 h as well as 48 h after stimulation with these activators. As shown in Fig. 2A & E, *Runx2* siRNA effectively suppressed the stimulated expression of *Runx2* compared to that in negative control (NC) siRNA-treated cells in both mRNA and protein levels in luteinizing granulosa cells. Moreover, the knockdown of *Runx2* expression resulted in increases in levels of mRNA for *Runx1, Ptgs2* and *Tnfaip6* compared to that in NC siRNA-treated cells (Fig. 2B, C & D). Consistent with the mRNA data, RUNX1 protein level and PGE_2 concentrations in *Runx2* siRNA-treated cells were higher compared to that in NC siRNA-treated cells (Fig. 2E & F). We also observed similar increases in *Runx1, Ptgs2*, and *Tnfaip6* expression by *Runx2* knockdown at 48 h after the agonist treatment (Supplemental fig. 1).

Next, to determine whether the over-expression of *Runx2* affects *Runx1*, *Ptgs2*, and *Tnfaip6* expression, granulosa cells were infected with adenoviruses expressing *Runx2* (Ad-RUNX2) or null control vector (Ad-ψ) at 5 infectious units (IFU). The next day, the cells were stimulated with the activators for 4 h, the time period showing the high expression of *Runx1*, *Ptgs2*, and *Tnfaip6* and relatively low expression of *Runx2*. The over-expression of *Runx2* was confirmed at both mRNA and protein levels. As expected, the agonist treatment increased the expression of *Runx1*, *Ptgs2* and *Tnfaip6* in granulosa cells infected with Ad-ψ. But the adenovirus mediated over-expression of *Runx2* resulted in dramatic reduction of the agonist-stimulated expression of these genes (Fig. 3B–E). The levels of RUNX1 and PTGS2 protein as well as PGE_2 concentrations were also drastically decreased by RUNX2 over-expression (Fig. 3E & F).

3.3. Transcriptional activity of Runx1 promoter reporter constructs was regulated by Runx2 expression in granulosa cell cultures

Data from both silencing and over-expression experiments indicated that RUNX2 negatively affects *Runx1* expression. To further investigate the transcriptional regulation of *Runx1* expression, we cloned the rat *Runx1* promoter fragment (−1263/+86 bp) into the upstream of a firefly luciferase reporter construct. This construct contains 2 consensus RUNX binding motifs as well as binding sites for various transcription activators including Sp1, AP2, and c-Ets. This promoter construct was transfected into preovulatory granulosa cells isolated from PMSG-primed immature rats. The cells were then stimulated with FSK+PMA for 3, 6, 12, or 24 h. We found that the transcriptional activity of the *Runx1* promoter construct was highest at 6 h after FSK+PMA treatment and decreased by 24 h, which mimicked the profile of *Runx1* mRNA (Data not shown). To further analyze transcriptional contribution of different regions of the *Runx1* promoter, three different promoter fragments (−283/+86, −775/+86, and −1263/+86 bp) of the *Runx1* gene were cloned and transfected into preovulatory granulosa cells. The cells were stimulated with FSK+PMA for 6 h, a time point showing the highest transcriptional activity. The agonist treatment increased the luciferase activity of −283/+86 and −1263/+86 bp reporter constructs compared to that of the vehicle (Fig. 4A). The *Runx1* promoter construct (−1263/+86 bp) containing 2 RUNX motifs as well as binding sites for other transcription activators showed the highest transcriptional activity (Fig. 4A). Meanwhile, the *Runx1* promoter construct (−775/+86 bp) containing 2 RUNX binding motifs showed the lowest transcriptional activity compared to two other constructs. This data suggested suppressive attribute of RUNX binding motifs on *Runx1* transcriptional activity.

To assess whether the presence of RUNX binding motifs affects the transcriptional activity of the *Runx1* promoter, site-directed mutations of the distal binding site (−728/−723 bp, mutant A) and proximal binding site (−260/−255 bp, mutant B) as well as double mutation of these two sites (−728/−723 bp plus −260/−255 bp, mutant AB) were generated. These constructs were transfected into preovulatory granulosa cells and then stimulated with FSK +PMA for 6 h (Fig. 4B). The results showed that mutation of either the distal or proximal RUNX binding site increased *Runx1* promoter activities compared to the activities of the wild type construct (Fig. 4B). Moreover, the double mutations of both regions of consensus RUNX binding sites (mutant AB) showed the highest transcriptional activity (Fig. 4B). These findings indicated that RUNX binding sites are involved in the repression of *Runx1* promoter activity.

To further determine whether RUNX2 is involved in controlling the *Runx1* promoter activity, the expression of *Runx2* was altered by *Runx2* siRNA and adenovirus expressing *Runx2*. The cultured granulosa cells were transfected with the −1263/+86 bp *Runx1* promoter construct and then stimulated with FSK+PMA. The transcriptional activity of the

Runx1 promoter reporter construct was higher in *Runx2* siRNA-transfected granulosa cells compared to the cells transfected with NC siRNA, indicating that *Runx2* knockdown enhanced the promoter activity of the *Runx1* gene (Fig. 4C). In contrast, the over-expression of *Runx2* reduced the reporter activity of the *Runx1* promoter (Fig. 4D). Together, these data suggest that *Runx2* expression negatively affects the promoter activity of rat *Runx1* gene in luteinizing granulosa cells.

Next, we determined whether RUNX2 could directly interact with RUNX binding motifs in the *Runx1* promoters. ChIP assays were performed in preovulatory granulosa cells infected with Ad-RUNX2. As shown in Fig. 4E, PCR analyses revealed the enrichment of chromatin fragments spanning the distal RUNX binding site (−728), but not proximal RUNX binding site (−526) by immunoprecipitation with RUNX2 antibody. In addition, ChIP assays showed the binding of RUNX2 on the *Ptgs2* promoter region in same cultured granulosa cell samples (Fig. 4F)

3.4. Expression of HDACs in the ovary in vivo and granulosa cells in vitro

HDACs were found to act as co-repressors of RUNX2 in various cell types (Hug, 2004; Jensen et al., 2007; Jensen et al., 2008; Lamour et al., 2007; Shimizu et al., 2010; Sun et al., 2009; Westendorf, 2006). Therefore, the periovulatory expression of *Hdac1, Hdac3* and *rSinc3a* were assessed in whole ovaries collected at different times after hCG administration. *Hdac1, Hdac3* and *rSinc3a* mRNA were constitutively expressed throughout the periovulatory period (Fig. 5). Similar to the mRNA expression profile, HDAC3 protein expression was also constant in whole ovarian extracts collected at different time intervals after hCG administration (Fig. 5A). Similar to the *in vivo* data, the expression of these genes was readily detected, but not affected by hCG treatment in cultured granulosa cells (Fig. 5B).

4. DISCUSSION

The LH surge causes dynamic changes in gene expression in periovulatory follicle (Espey and Richards, 2002; Richards et al., 1998; Richards, 2007). Interestingly, for a certain cohort of genes, their expression rapidly increases, yet is transient during the preovulatory period [e.g., *Ptgs2, Tnfaip6, Pgr, Runx1, C/EBP*β (Fan et al., 2011; Jo and Curry, Jr., 2006; Joyce et al., 2001; Natraj and Richards, 1993; Yoshioka et al., 2000)], while for another group of genes, their expression increases progressively and continues in the forming CL (e.g., *Runx2, Cyp11a1, StAR, C/EBPa*)(Chen et al., 1999; Hernandez-Gonzalez et al., 2006; Park et al., 2010; Rodgers et al., 1987; Sirois and Richards, 1993). These two distinct patterns of gene expression implicates functional involvement of these genes in the ovulatory process (ovulatory genes) and luteal development (luteal genes), respectively.

As central mediators of LH-initiated cellular events, transcriptional factors induced in periovulatory follicles directly control temporal changes in periovulatory gene expression. So far, studies have primarily focused on identifying periovulatory genes that are up-regulated by LH-induced specific transcription factors (Fan et al., 2011; Jo and Curry, Jr., 2006; Robker et al., 2000). Moreover, little attention was given to the involvement of specific transcription factors in rapid down-regulation of ovulatory gene expression. In the present study, we demonstrated that RUNX2 contributes to rapid down-regulation of a distinctive set of transiently induced preovulatory genes such as *Ptgs2, Tnfaip6*, and *Runx1*. Moreover, the negative effect of RUNX2 on *Runx1* transcription involves the direct binding of RUNX2, at least on one of two RUNX binding motifs in the promoter of the rat *Runx1* gene.

RUNX proteins (RUNX1, 2, 3) are structurally similar and have been shown to exhibit overlapping transcriptional activity on several target genes in different experimental models (Cameron and Neil, 2004; Liu et al., 2010; Park et al., 2008; Zhang et al., 2009). However, in most of the cases the expression of *Runx* genes is temporally or spatially separated. Recent studies have provided experimental evidence of self- and cross-regulatory mechanisms responsible for the tightly controlled expression of *Runx* genes (Brady et al., 2009; Brady and Farrell, 2009; Drissi et al., 2000; Spender et al., 2005). As relevant examples to the present study, Wong et al. (Wong et al., 2011) reported that RUNX1 down-regulates its own expression by directly binding to a RUNX consensus site in the promoter upon T-cell receptor activation in CD4+ T cells. *Runx1* expression was also suppressed by RUNX3 in human B lymphoid cell lines and this suppression was thought to be important for B cell proliferation (Brady et al., 2009; Brady and Farrell, 2009; Spender et al., 2005). However, there has been, to our knowledge, no report of either positive or negative intra-familial regulation between *Runx1* and *Runx2*. Previously, we have reported that both *Runx1* and *Runx2* expression are up-regulated by hCG-activated signaling, yet with distinctive temporal expression pattern in periovulatory granulosa cells. Based on the previous microarray data showing the increased expression of *Runx1* by siRNA-mediated *Runx2* knockdown, we proposed negative regulation of *Runx1* by RUNX2 in periovulatory granulosa cells. Indeed, the present data from siRNA and over-expression experiments, along with promoter reporter and ChIP assays collectively pointed out that RUNX2 functions as a transcriptional repressor for the *Runx1* gene in luteinizing rat granulosa cells. In addition to *Runx1*, the present study also identified *Ptgs2* and *Tnfaip6* as two other potential target genes for RUNX2 in luteinizing granulosa cells. The levels of mRNA for both *Ptgs2* and *Tnfaip6* are rapidly, yet transiently increased by the LH surge in granulosa and cumulus cells of periovulatory follicles (Fulop et al., 1997; Joyce et al., 2001; Sirois et al., 1992; Yoshioka et al., 2000). This unique temporal expression pattern could be mimicked *in vitro* by hCG or FSK+PMA treatment, as confirmed in our granulosa cell culture model. Furthermore, the data from silencing and over-expression experiments could be interpreted that RUNX2 is a part of the transcriptional complexes controlling rapid down-regulation of *Ptgs2* and *Tnfaip6* expression. Both rat *Ptgs2* (Liu et al., 2009) and *Tnfaip6* harbor multiple consensus RUNX binding motifs in their promoter region, suggesting that RUNX2 could act directly at the promoter level to repress the expression of genes. However, there is another important fact to consider. In the early ovulatory phase, RUNX1, not RUNX2, was found to directly bind to RUNX binding motifs on the *Ptgs2* promoter and facilitate the LH-dependent increase in *Ptgs2* expression in preovulatory granulosa cell (Liu et al., 2009), suggesting that RUNX1 is involved in the up-regulation of *Ptgs2* expression during the early ovulatory period. Unlike *Ptgs2* expression, we found that reduction (~45%) of *Runx1* expression by *Runx1* siRNA had no effect on *Tnfaip6* expression in preovulatory granulosa cell cultures (Supplemental Fig. 3). Nonetheless, it is possible that RUNX2 could inhibit the expression of *Ptgs2* and *Tnfaip6* indirectly via down-regulating the expression of *Runx1* during the late ovulatory period. It is well established that the expression of *Ptgs2* and *Tnfaip6* in periovulatory follicles is vital for successful ovulation and/or cumulus expansion (Davis et al., 1999; Fulop et al., 2003; Lim et al., 1997). Given the relationship between *Runx2* and these important ovulatory genes, *Ptgs2* and *Tnfaip6*, the present data suggests a potential role for RUNX2 in fine-tuning the ovulatory process.

We previously demonstrated that RUNX2 is actively involved in the up-regulation of specific luteal genes such as *Rgc32, Mmp13, Ptgds, Fabp6* and *Abcb1a* in luteinized granulosa cells (Park et al., 2010). These studies, together with the present data, revealed the opposing action of RUNX2 in regulating periovulatory gene expression; RUNX2 increases the expression of luteal genes, while simultaneously suppresses the transcription of specific ovulatory genes in luteinizing granulosa cells. But, the present observation may not be surprising, given the fact that RUNX proteins have been often described as a context-

dependent transcription factor [reviewed in (Cameron and Neil, 2004)]. For example, RUNX2 was found to act either as an activator or repressor by recruiting and/or interacting with other co-regulators in the promoter region of target genes in non-ovarian cells (Cameron and Neil, 2004; Durst and Hiebert, 2004; Komori, 2010; Otto et al., 2003; Westendorf, 2006). Recent studies using molecular approaches have identified several co-regulators of RUNX2 such as p300 (Westendorf and Hiebert, 1999), SMADs (Zhang et al., 2000), pRB (Thomas et al., 2001) and C/EBPβ (Gutierrez et al., 2002) as a co-activator and histone deacetylase (HDACs) (Hug, 2004; Jensen et al., 2007; Jensen et al., 2008; Shimizu et al., 2010; Sun et al., 2009; Westendorf, 2006), transducin-like enhancer of split (TLE) proteins (Ali et al., 2010), mSin3a (Lutterbach and Hiebert, 2000), and yes associated protein (YAP)(Vitolo et al., 2007) as a co-repressor. Importantly, several of these proteins are also expressed in the periovulatory ovary, including p300/CBP (Ongeri et al., 2005), SMADs (Kaivo-oja et al., 2006), and C/EBPβ (Sterneck et al., 1997). The present data also added HDACs as potential co-repressors of RUNX2 in the periovulatory ovary. Therefore, it is conceivable that the dual actions of RUNX2 observed in our studies are likely mediated by the interaction with these co-activators or co-repressors expressed in granulosa cells of periovulatory follicles.

In conclusion, the expression of *Ptgs2*, *Tnfaip6*, and *Runx1* dramatically increases in preovulatory granulosa cells in response to the LH surge, yet rapidly declines before ovulation. This rapid down-regulation of *Ptgs2*, *Tnfaip6*, and *Runx1* during the late ovulatory period is likely orchestrated by multiple factors, including reduction of positive regulators and accumulation of negative regulators. Our findings provide new insight into the complex regulatory mechanisms of *Ptgs2*, *Tnfaip6*, and *Runx1* expression. RUNX2 acts as a direct transcriptional repressor for the *Runx1* gene. The inhibitory effect of RUNX2 on *Ptgs2* and *Tnfaip6* could be direct and/or indirect through down-regulating *Runx1* expression. The identify of and precise mechanisms of interaction between transcriptional regulators expressed in periovulatory follicles needs to be determined to fully appreciate dynamic transcriptional control of genes required for proper ovulation, cumulus expansion and luteal formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- RUNX2 knockdown increases the expression of *Runx1*, *Ptgs2*, and *Tnfaip6* in the ovary.
- RUNX2 over-expression represses *Runx1*, *Ptgs2*, and *Tnfaip6* expression in the ovary.
- RUNX binding motifs are relevant to *Runx1* promoter activity
- Runx2 knockdown increases transcriptional activity of the *Runx1* promoter
- RUNX2 represses *Runx1* expression by direct binding in the *Runx1* promoter.

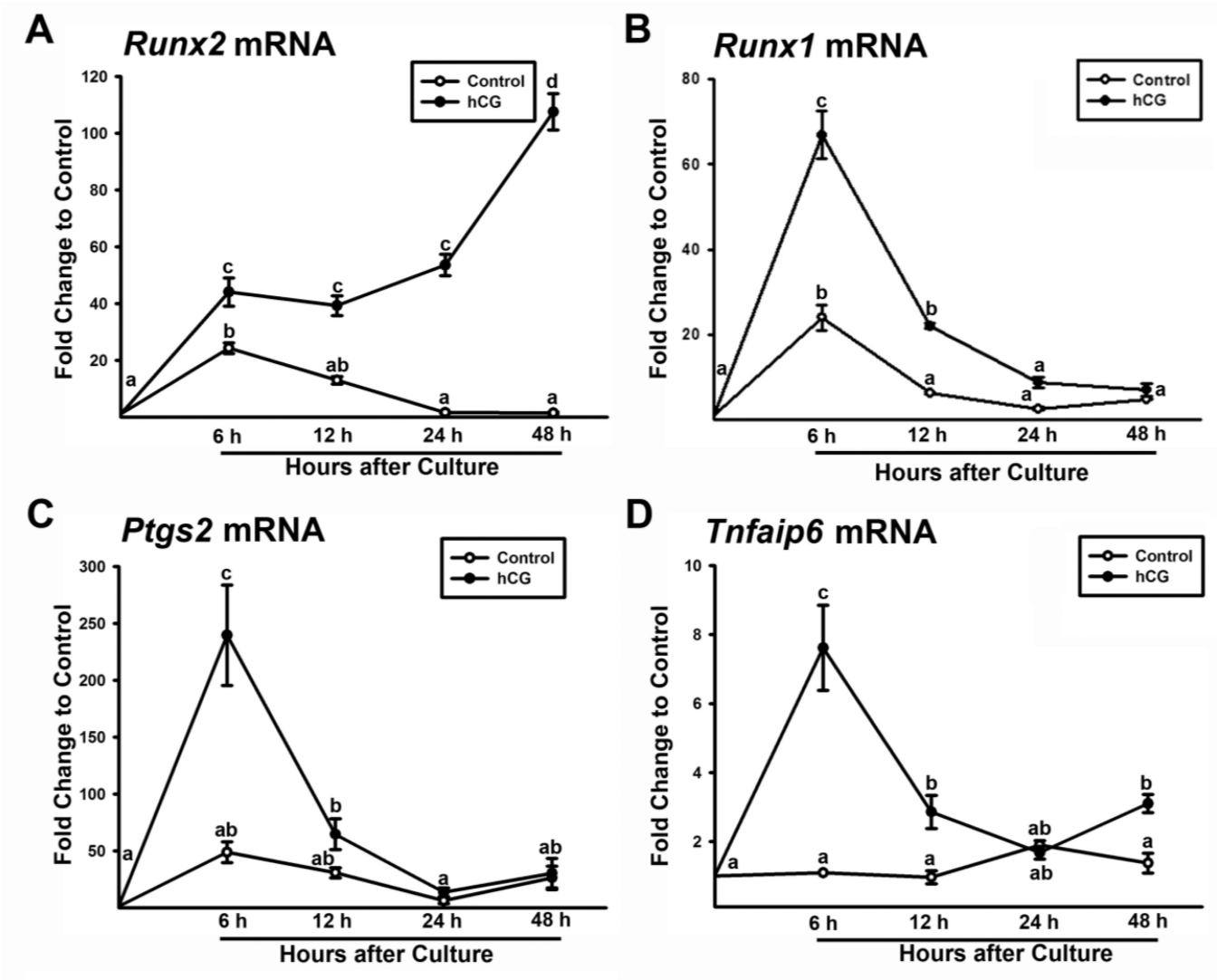

Figure 1. Stimulation of *Runx2, Runx1, Ptgs2* and *Tnfaip6* expression by hCG in granulosa cells in vitro

Granulosa cells isolated from rat preovulatory ovaries (48 h post-PMSG) were cultured for 0, 6, 12, 24, or 48 h in medium alone (Control) or with hCG (1 IU/ml). The levels of mRNA for *Runx2* (**A**), *Runx1* (**B**), *Ptgs2* (**C**) and *Tnfaip6* (**D**) were measured by Real-time PCR and normalized to the *L32* value in each sample (mean ± SEM; n = 3 independent culture experiments). No common superscripts are significantly different ($P < 0.05$).

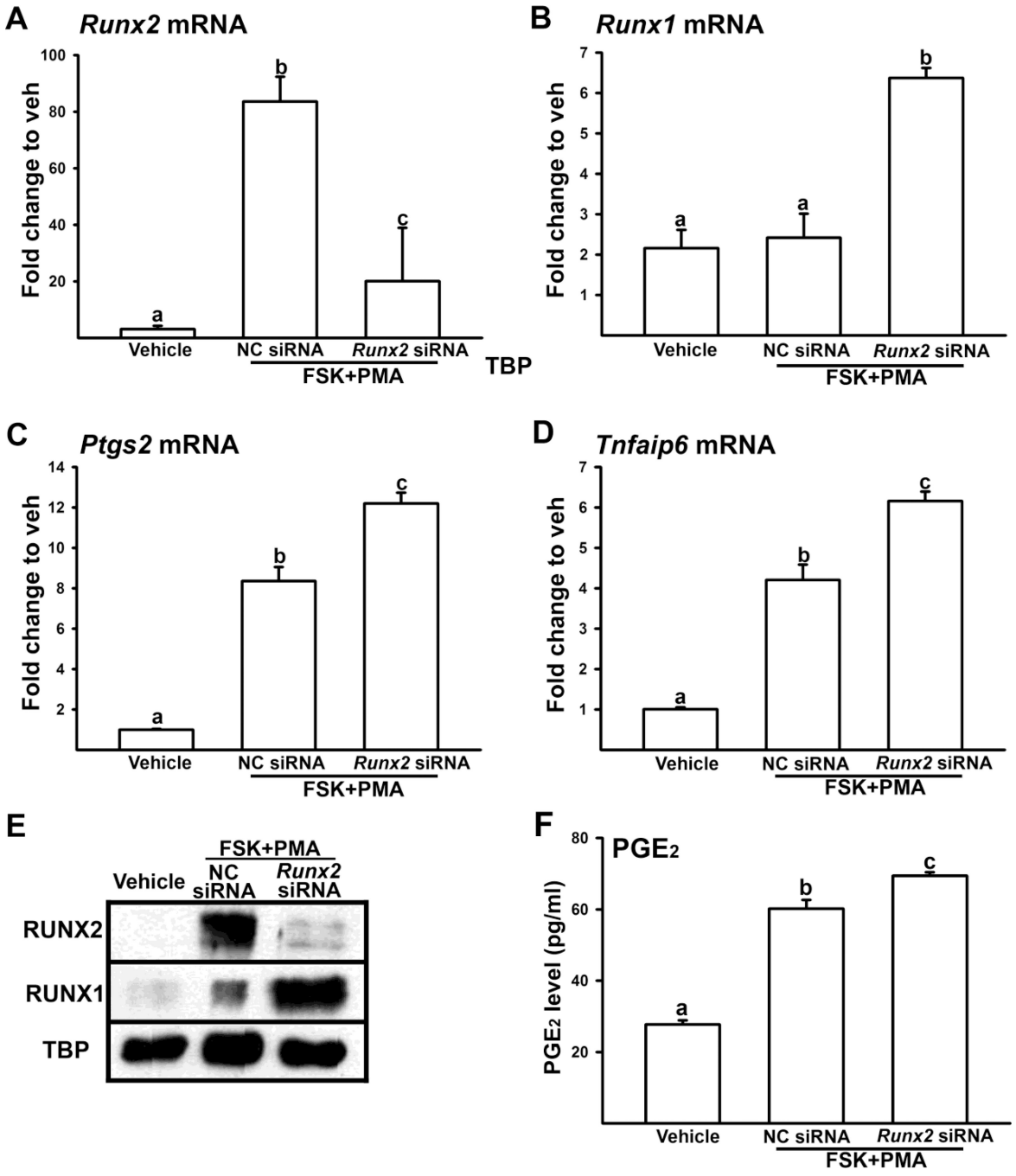

Figure 2. Regulation of *Runx1, Ptgs2,* and *Tnfaip6* expression by *Runx2* knockdown in cultured granulosa cells

Granulosa cells isolated from rat preovulatory ovaries (48 h post-PMSG) were transfected without siRNA (Vehicle) or with negative control siRNA (NC *siRN*A) or *Runx2* siRNA and stimulated with FSK+PMA for 24 h. The levels of mRNA for *Runx2* (**A**), *Runx1* (**B**), *Ptgs2* (**C**), and *Tnfaip6* (**D**) were measured by Real-time PCR and normalized to the *L32* value in each sample. **E**. RUNX1 and RUNX2 proteins in nuclear extracts were assessed by Western blot analyses. Each lane was loaded with 30 μg of nuclear fraction extracted from granulosa cell cultures transfected without or with siRNAs. The membrane was re-probed with a monoclonal antibody against TATA binding protein (TBP) to show the relative loading of

nuclear extracts. The blots are representatives of three separate experiments. **F**. Concentration of PGE_2 was measured from granulosa cells transfected without or with siRNAs. Experiments were repeated at least four times, each with different cultured granulosa cell samples (mean ± SEM). Bars with no common superscripts are significantly different ($P < 0.05$).

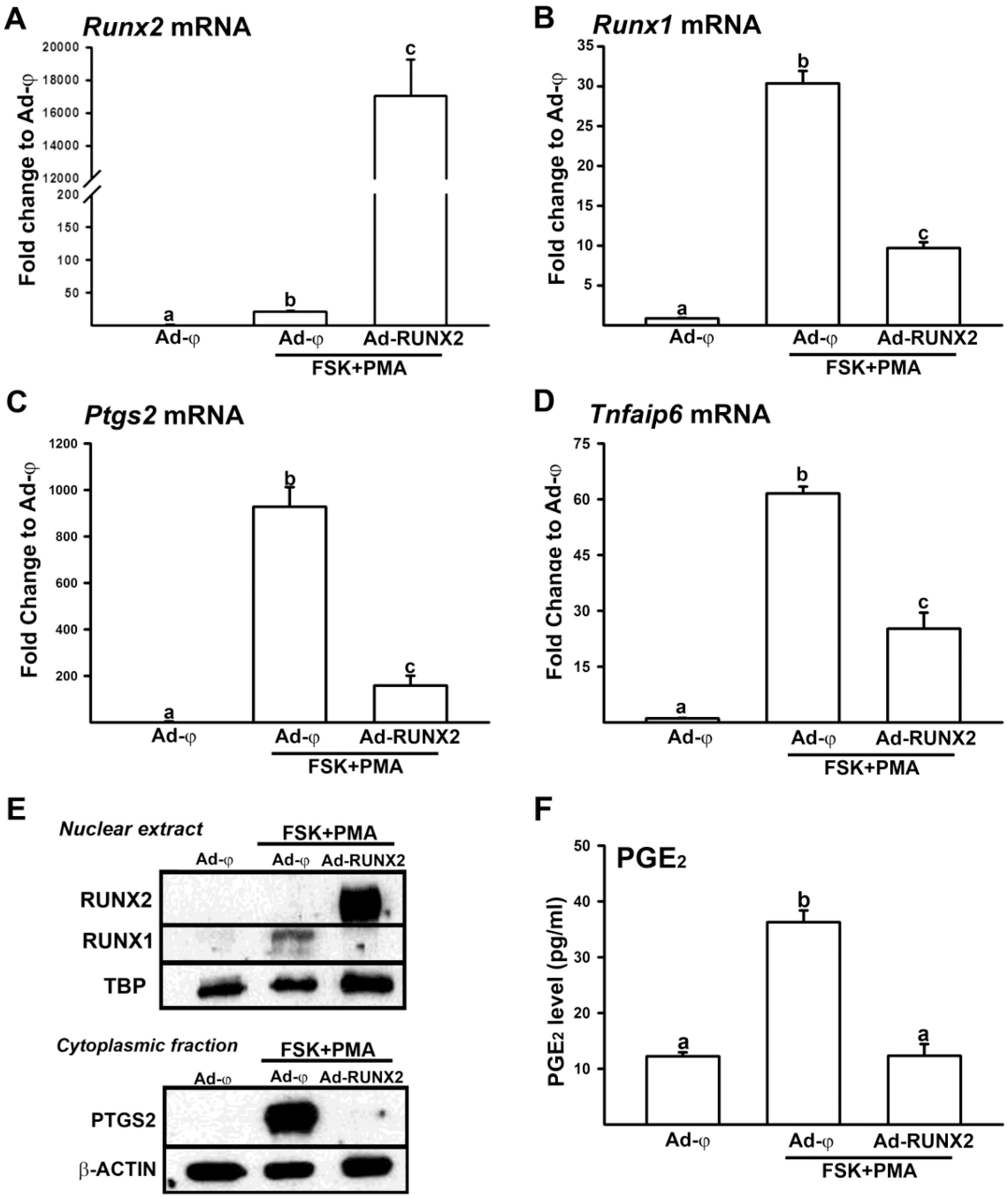

Figure 3. Regulation of *Runx1, Ptgs2,* and *Tnfaip6* expression by RUNX2 over-expression in cultured granulosa cells

Granulosa cells isolated from rat preovulatory ovaries (48 h post-PMSG) were infected with adenoviruses expressing *Runx2* (Ad-RUNX2) or containing null control vector (Ad-ψ) at 5 IFU, treated with FSK+PMA and further cultured for 4 h. The levels of mRNA for *Runx2* (**A**) *Runx1* (**B**), *Ptgs2* (**C**), and *Tnfaip6* (**D**) were measured by Real-time PCR and normalized to the *L32* value in each sample. **E**, Western blots show RUNX1 and RUNX2 protein in nuclear extracts and PTGS2 in cytoplasmic fractions isolated from granulosa cells infected with adenoviruses. Each lane was loaded with 30 μg of cellular fractions from granulosa cell infected with adenoviruses. The membrane was re-probed with a monoclonal

antibody against TATA binding protein (TBP) or β-ACTIN to show the relative loading. The blots are representatives of three separate experiments. **F**. Concentration of PGE_2 was measured from granulosa cells infected with adenoviruses. Experiments were repeated at least three times, each with different cultured granulosa cell samples (mean ± SEM). Bars with no common superscripts are significantly different ($P < 0.05$).

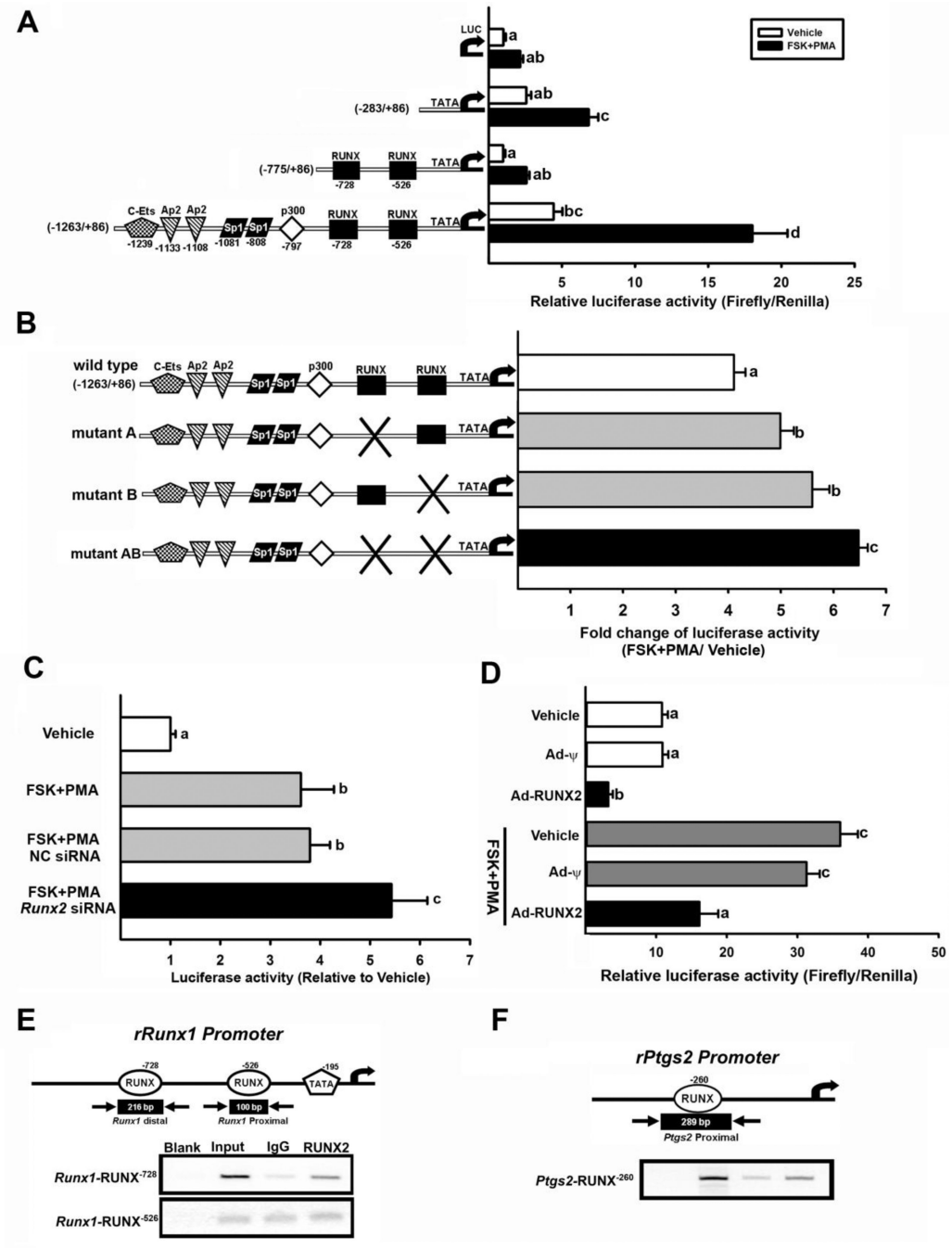

Figure 4. Regulation of transcriptional activity of *Runx1* promoter reporter constructs in cultured granulosa cells

Granulosa cells were isolated from gonadotropin-primed immature rats (48 h post-PMSG). **A**) The cells were transfected with empty luciferase reporter vector (LUC), −283/+86 bp, −775/+86 bp, or −1263/+86 *Runx1-luc*iferase reporter constructs, treated with FSK+PMA and further cultured for 6 h. Firefly luciferase activities were normalized to Renilla luciferase activities. **B**, The cells were transiently transfected with wild type, mutant A, mutant B, or mutant AB *Runx1*-luciferase reporter constructs and stimulated with FSK plus PMA for 6 h. Luciferase activity of each constructs was expressed as a fold change of FSK plus PMA treatment to vehicle treatment. **C**, The cells were transfected with −1263/+86

*Runx1-lucif*erase reporter constructs overnight, and then treated with NC siRNA or *Runx2* siRNA and stimulated with FSK plus PMA. Luciferase activity of FSK plus PMA-treated constructs was normalized to Vehicle treated construct. **D**, The cells were infected with adenoviruses expressing Runx2 (Ad-Runx2) or control null vector (Ad-ψ), and then transfected with −1263/+86 *Runx1*-luciferase reporter constructs and stimulated with FSK +PMA for 6 h. Firefly luciferase activities were normalized to Renilla luciferase activities. The experiments were repeated at least 3 times (mean ± SEM). Bars with no common superscripts are significantly different ($P < 0.05$). **E & F**, ChIP assays were performed using granulosa cells infected with adenoviruses expressing *Runx2* (Ad-RUNX2) at 5 IFU and then stimulated with FSK+PMA for 6 h. DNAs were analyzed by PCR using primers indicated in the text and represented as arrows in **E** & **F**. Amplified DNA fragments containing RUNX binding motifs are represented as black boxes with the indicated PCR product size. Experiments were repeated at least three times, each with different cultured granulosa cell samples.

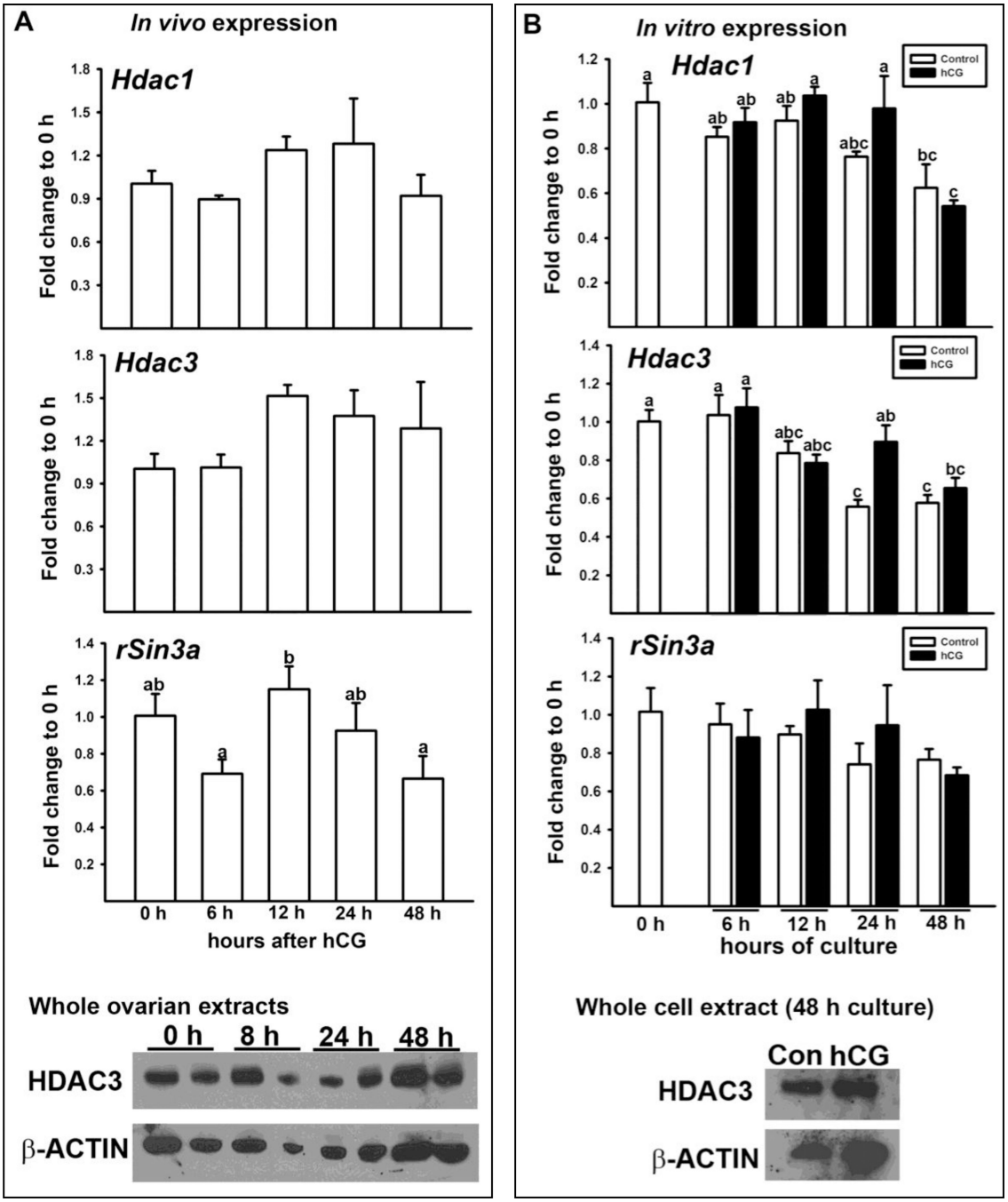

Figure 5. The ovarian expression of *Hdacs in vivo* and *in vitro*

A) Ovaries were collected before or at indicated hours (h) after hCG injection from PMSG-primed immature rats. **B**) Granulosa cells isolated from rat preovulatory ovaries (48 h post-PMSG) were cultured for 0, 6, 12, 24, or 48 h in medium alone (Control) or with hCG (1 IU/ml). The levels of mRNA for *Hdac1*, *Hdac3*, and *rSin3a* were measured by Real-time PCR and normalized to the *L32* value in each sample (mean ± SEM; n = 3 independent culture experiments). HDAC3 protein in whole cell extracts was assessed by Western blot analyses. The membrane was re-probed with β-ACTIN to show the relative loading of whole cell extracts.

Table 1

List of primers used for Real-time PCR

Gene Name	Reference (Accession no./Primer sets, 5'-3')
L32	BC061562 GAAGCCCAAGATCGTCAAAA AGGATCTGGCCCTTGAATCT
Runx2	NM_009820 GTTATGAAAAACCAAGTAGCCAGGT GTAATCTGACTCTGTCCTTGTGGAT
Runx1	NM_017325.1 AACCCTCAGCCTCAAAGTCA GGGTGCACAGAAGAGGTGAT
Ptgs2	U03389.1 TACCCGGACTGGATTCTACG AAGTTGGTGGGCTGTCAATC
Tnfaip6	BC158666.1 CGTCTTGCAACCTACAAGCA TTCGGGTTGTAGCAATAGGC