Published in final edited form as:
Mol Endocrinol. 2006 September ; 20(9): 2156–2172.

Luteinizing Hormone-Induced RUNX1 Regulates the Expression of Genes in Granulosa Cells of Rat Periovulatory Follicles

Misung Jo and **Thomas E. Curry Jr.**
Department of Obstetrics and Gynecology, Chandler Medical Center, University of Kentucky, Lexington, Kentucky 40536-0298

Abstract

The LH surge induces specific transcription factors that regulate the expression of a myriad of genes in periovulatory follicles to bring about ovulation and luteinization. The present study determined 1) the localization of RUNX1, a nuclear transcription factor, 2) regulation of *Runx1* mRNA expression, and 3) its potential function in rat ovaries. Up-regulation of mRNA and protein for RUNX1 is detected in preovulatory follicles after human chorionic gonadotropin (hCG) injection in gonadotropin-treated immature rats as well as after the LH surge in cycling animals by *in situ* hybridization and immunohistochemical and Western blot analyses. The regulation of *Runx1* mRNA expression was investigated *in vitro* using granulosa cells from rat pre-ovulatory ovaries. Treatments with hCG, forskolin, or phorbol 12 myristate 13-acetate stimulated *Runx1* mRNA expression. The effects of hCG were reduced by inhibitors of protein kinase A, MAPK kinase, or p38 kinase, indicating that *Runx1* expression is regulated by the LH-initiated activation of these signaling mediators. In addition, hCG-induced *Runx1* mRNA expression was inhibited by a progesterone receptor antagonist and an epidermal growth factor receptor tyrosine kinase inhibitor, whereas amphiregulin stimulated Runx1 mRNA expression, demonstrating that the expression is mediated by the activation of the progesterone receptor and epidermal growth factor receptor. Finally, knockdown of *Runx1* mRNA by small interfering RNA decreased progesterone secretion and reduced levels of mRNA for *Cyp11a1*, *Hapln1*, *Mt1a*, and *Rgc32*. The hormonally regulated expression of *Runx1* in periovulatory follicles, its involvement in progesterone production, and regulation of preovulatory gene expression suggest important roles of RUNX1 in the periovulatory process.

Abbreviations

AML1, Acute myeloid leukemia 1; AREG, amphiregulin; *cdkn*, cyclin-dependent kinase inhibitor; C/EBPβ, CCAAT-enhancer binding protein β; CG, chorionic gonadotropin; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; *Hapln1*, hyaluronan and proteoglycan link protein 1; MEK, MAPK kinase; *Mt1a*, metallothionein 1a; PGR, progesterone receptor; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12 myristate 13-acetate; PMSG, pregnant mare serum gonadotropin; *Rgc32*, response gene to complement 32; siRNA, small interfering RNA; *Timp1*, tissue inhibitor of metalloproteinase-1

THE PREOVULATORY GONADOTROPIN surge induces profound changes in the preovulatory follicle, including breakdown of the extracellular matrix at the apex of the follicle to allow for release of the cumulus-oocyte complex, differentiation of follicular cells to luteal

Address all correspondence and requests for reprints to: Misung Jo, Department of Obstetrics and Gynecology, Chandler Medical Center, 800 Rose Street, Room MS 335, University of Kentucky, Lexington, Kentucky 40536-0298. E-mail: mjo2@uky.edu.

M.J. and T.E.C. have nothing to declare.

This work was supported by National Institutes of Health Grants NCRR P20 RR 15592 (to T.E.C. and M.J.) and RO3 HD051727-01 (to M.J.).

cells, and the follicular/luteal shift in steroidogenesis from estradiol to progesterone. These LH-induced preovulatory changes are mediated by the expression of specific genes, such as genes for inflammation-related proteins, matrix-remodeling enzymes, cell cycle inhibitors, and specific steroidogenic enzymes (reviewed in Refs. 1–3). Furthermore, transient induction of specific transcription factors by the LH surge [*e.g*. progesterone receptor (PGR) (4) and CCAAT-enhancer binding protein β (C/EBPβ) (5)] has been shown to be crucial for ovulation and/or luteinization. Recently, we have found that the expression of mRNA for the transcription factor, *Runx1*, is transiently increased in periovulatory ovaries of gonadotropin-stimulated immature rats after human chorionic gonadotropin (hCG) injection (6), suggesting that RUNX1 may be a key transcriptional modulator in periovulatory follicular changes. However, nothing is known about the regulatory mechanism of *Runx1* expression or its function in the ovary.

Runx1 mRNA is highly expressed in thymus, bone marrow, and various hematopoietic cell lines (7). RUNX1 has been shown to play an essential role in the differentiation of various cell types. For instance, RUNX1 is required for differentiation of myeloid progenitor cells into granulocytes (8,9), maturation of megakaryocytes (10), and differentiation of T and B cells (10,11). RUNX1 has been shown to be involved in nerve cell innervation (12), fibroblast transformation (13), and angiogenesis (14). In addition, the *Runx1* gene is the most frequent target of chromosomal translocation associated with leukemia, and the resultant mutant fusion proteins inhibit the normal transcriptional activity of RUNX1 in various hematopoietic cells (15). All of this evidence points to the involvement of RUNX1 in cell differentiation, maturation, and/or transformation.

RUNX1, also called AML1 (acute myeloid leukemia 1)/core binding factor A2/ polyomavirus enhancer binding protein 2αB, belongs to a family of runt-related transcription factors (RUNX/CBF/PEBP2). RUNX1 binds to a consensus recognition sequence, designated as a PEPB2 site [5′-PyGPyGGTPy-3′ (16)], in the promoter or enhancer binding regions of target genes, thus regulating their transcriptional activity. Several genes have been characterized as downstream targets of RUNX1 in various hematopoietic cells, although the transcriptional regulation of RUNX1 on these genes was found to be cell-type specific (17). Among these genes, a few have been found to be up-regulated in periovulatory ovaries, such as cyclin-dependent kinase inhibitor 1A (*cdkn1a*) (18,19), tissue inhibitor of metalloproteinase-1 (*Timp1*) (20,21), and matrix metalloproteinase 13 (*Mmp13*) (22,23). Studies with promoter regions of RUNX1 target genes have demonstrated the importance of the cooperation of RUNX1 with other transcription factors in regulating gene expression (19). Such transcription factors include activator protein-1 factors (23) and C/EBP (24), both of which are increased in preovulatory follicles after the LH surge (25,26). Based on these findings, we hypothesized that the LH-induced RUNX1 may play an important role in the differentiation of follicular cells to luteal cells via regulating the transcription of specific genes in the ovary. The present study tested this hypothesis by characterizing the expression of both mRNA and protein for RUNX1 during the peri-ovulatory period using ovaries from PMSG/hCG-primed immature mice and rats, as well as ovaries from cycling rats. In addition, *in vitro* experimental models were used to determine the regulatory mechanisms by which the LH surge induces *Runx1* expression and identify genes downstream of RUNX1 action in granulosa cells of periovulatory follicles.

RESULTS

Expression of *Runx1* mRNA and Protein during the Periovulatory Period in Rat Ovaries

We have previously demonstrated the transient induction of *Runx1* mRNA in granulosa cells of preovulatory follicles and theca cells of all antral follicles after hCG injection in PMSG-treated immature rat ovaries using *in situ* hybridization and Northern blot analyses (6). In the present study, to further determine whether the expression of *Runx1* mRNA is followed by its translation into protein, immunohistochemical analyses were performed using an antibody for

RUNX1 (AML1: N-20) in ovarian tissue sections from gonadotropin-treated immature rats. No staining for RUNX1 was detected in granulosa cells of preovulatory follicles in ovaries obtained before hCG injection (0 h post-hCG; Fig. 1A, panels a and b). After hCG injection, positive staining was evident in the nuclei of granulosa cells in preovulatory follicles, whereas granulosa cells of adjacent small follicles were not stained (Fig. 1A, panels c–f). After ovulation, positive staining was also observed in cells of corpora lutea (Fig. 1A, panels g and h). In all the ovarian sections examined, positive staining for RUNX1 was localized to the nuclei of theca cells of the majority of antral follicles (Fig. 1A, a–f). RUNX1 was up-regulated in periovulatory ovaries after hCG injection as demonstrated by Western blot analyses using a different anti-RUNX1 antibody (ab11903) and nuclear extracts from granulosa cells, residual ovarian tissues, or whole ovaries (Fig. 1B; two representative blots, a and b, are depicted). Two predominant sizes of RUNX1 (~ 53 kDa and ~ 57 kDa) were detected in granulosa cells only at 6 and 12 h after hCG injection, whereas in residual ovarian tissues the proteins were detected at all time points examined and in ovaries containing newly forming corpora lutea (24 h post-hCG). RUNX1 level was highest in granulosa cells obtained at 12 h post-hCG. We initially speculated that the approximately 57-kDa RUNX1 may be a phosphorylated form of RUNX1. However, when the nuclear extracts from 12 h post-hCG granulosa cells were incubated with alkaline phosphatase (1 unit; Promega Corp., Madison, WI), there was no shift in size in Western blot analyses (data not shown).

To confirm that the transient induction of mRNA and protein for RUNX1 in preovulatory follicles seen in the PMSG/hCG-induced immature animal model occurs in naturally cycling adult rats, ovaries were collected from cycling rats between the period of the endogenous gonadotropin surge and ovulation and examined by *in situ* hybridization and immunohistochemistry. A similar pattern of expression of *Runx1* mRNA in pre-ovulatory follicles was observed in cycling rats compared with that seen in the gonadotropin-primed model (Fig. 2A). For example, at the peak of the LH surge (1600 h proestrus), little expression of *Runx1* mRNA was detected in the rat ovary (Fig. 2A, panel b). In ovaries obtained at 4 and 8 h after the LH surge, however, high expression of *Runx1* mRNA was localized to granulosa cells of preovulatory follicles and thecal cells of the majority of antral follicles (denoted in Fig. 2A, panel d). After ovulation, newly forming corpora lutea also expressed *Runx1* mRNA, although the hybridization intensity for *Runx1* appeared to be lower compared with that observed in periovulatory follicles (Fig. 2A, panel f). Corpora lutea from previous estrous cycles lacked *Runx1* mRNA expression (Fig. 2A, panels b, d, and f).

We further confirmed that the up-regulation of *Runx1* mRNA in preovulatory follicles of ovaries from cycling rats resulted in the induction of RUNX1 protein in granulosa cells of preovulatory follicles after the LH surge (Fig. 2B). RUNX1 was barely detectable in granulosa cells of preovulatory follicles in cycling adult rat ovaries obtained at the peak of the LH surge (Fig. 2B, panels a and b) and in granulosa cells of small antral follicles (denoted in Fig. 2B, panels c, d, and e). However, by 4 and 8 h after the LH surge, intensive staining for RUNX1 was detected in nuclei of granulosa cells of periovulatory follicles (Fig. 2B, panels c and d). As expected, RUNX1 was also detected in cells of newly forming corpora lutea (Fig. 2B, e and f). Consistent with the results from immature rat ovaries, positive staining for RUNX1 was observed in the nuclei of theca cells of antral follicles (Fig. 2B, b and d).

We also examined the expression pattern of mRNA and protein for RUNX1 in mouse ovaries during the periovulatory period. Northern blot, *in situ* hybridization, and immunohistochemical and Western blot analyses of ovaries obtained from PMSG/hCG-stimulated immature mice revealed virtually identical expression patterns of mRNA and protein for RUNX1 in periovulatory ovaries compared with that in the rat ovary (data not shown).

Effects of LH on the Expression of Runx1 mRNA in Granulosa Cell Cultures

To determine whether the transient increase in levels of *Runx1* mRNA after hCG injection *in vivo* can be mimicked *in vitro* and whether the induction of *Runx1* mRNA is directly mediated by the action of LH, granulosa cells isolated from PMSG-primed immature rat ovaries (48 h post-PMSG) were cultured in the absence or presence of a luteinizing dose of hCG (1 IU/ml). Northern blot analysis revealed expression of multiple transcripts (6.3, 3.8, and 3.6 kb) of the *Runx1* gene in cultured rat granulosa cells (Fig. 3A). hCG treatment induced a dramatic, transient increase in levels of *Runx1* mRNA. The levels were highest at 3 and 6 h of culture and then declined to basal levels by 24 h (Fig. 3A). This transient expression pattern is similar to that of *Runx1* mRNA in rat granulosa cells of preovulatory follicles *in vivo* (6). To confirm the induction of RUNX1 protein by hCG, Western blot analysis was conducted with nuclear fractions extracted from the cultured granulosa cells. As expected, hCG induced a transient increase in RUNX1 protein (Fig. 3B). RUNX1 was detected in the granulosa cells collected at 6 h, further increased by 12 h, and then began to decline at 24 h after culture, indicating the time delay (~3 h) in RUNX1 protein accumulation compared with the profile of Runx1 mRNA expression. The highest level of RUNX1 was detected by 9–12 h of culture (data not shown). This profile of RUNX1 protein *in vitro* mimics that *in vivo* (Fig. 1B).

Intracellular Signaling Mechanism of Runx1 mRNA Induction *in Vitro*

The ovulatory LH stimulus has been shown to activate both protein kinase A (PKA) and protein kinase C (PKC) signaling pathways to induce the expression of periovulatory genes, such as prostaglandin-endoperoxide synthase 2 (*Ptgs-2*) (27) and progesterone receptor (*Pgr*) (28), in preovulatory granulosa cells *in vitro*. To investigate which intracellular signaling pathway(s) is involved in the up-regulation of *Runx1* mRNA in response to an ovulatory dose of hCG, we cultured granulosa cells from PMSG-primed immature rat ovaries in the absence or presence of forskolin, an activator of adenylate cyclase, and/or phorbol 12 myristate 13-acetate (PMA), an activator of protein kinase C to mimic the activation of PKA and PKC signaling by an ovulatory LH stimulus. The granulosa cells from rat preovulatory ovaries were cultured for 6 h, the time period of maximal *Runx1* mRNA expression in preovulatory follicular cells both *in vivo* and *in vitro*. As expected, hCG stimulated *Runx1* mRNA expression in cultured granulosa cells ($P < 0.05$; Fig. 4A). Treatments with forskolin, PMA (to a lesser degree), and forskolin + PMA (additively) stimulated *Runx1* mRNA expression ($P < 0.05$). The stimulatory effect of hCG on *Runx1* mRNA was reduced by treatment with specific inhibitors of PKA (H89, 10 μm), MAPK kinase (MEK, PD98059, 20 μm), and p38 kinase (SB2035850, 20 μm), but not by inhibitors of phosphatidylinositol 3-kinase (LY294002, 25 μm) or PKC (GF109203, 1 μm) ($P < 0.05$; Fig. 4B). Doses of these inhibitors have been shown to block the activation of their respective kinases in rat granulosa cell cultures (29,30). A recent study (30) has shown that hCG treatment induces the activation of MEK and p42/44 MAPK through the cAMP/PKA-dependent pathway, but also stimulates phosphorylation of p38 kinase in a PKA- and PKC-independent manner in rat granulosa cell cultures (incorporated in Fig. 4C). Taken together, our data indicate that the induction of *Runx1* mRNA expression in cultured granulosa cells is mediated by the LH-induced activation of various intracellular signaling molecules, including adenylate cyclase, PKA, MEK, and p38 kinase, and suggests the involvement of multiple signaling pathways for *Runx1* expression in peri-ovulatory granulosa cells (Fig. 4C).

Hormonal Regulation of Runx1 mRNA Expression in Cultured Granulosa Cells

To determine whether the LH-induced increase in *Runx1* mRNA levels in preovulatory granulosa cells requires *de novo* protein synthesis, granulosa cells were incubated for 6 h in the absence or presence of hCG, cyclohexamide [1 μg/ml; a concentration that blocked protein synthesis of rat granulosa cells in similar culture conditions (31) and in our preliminary study (data not shown)], or hCG + cyclohexamide. Cyclohexamide treatment completely blocked

the hCG-induced increase in levels of *Runx1* mRNA ($P < 0.05$; Fig. 5), whereas several hCG-induced genes were not affected (data not shown), demonstrating that the synthesis of a new protein(s) is prerequisite for the LH-induced *Runx1* mRNA expression. This result suggests that *Runx1* mRNA expression is dependent on the action of a newly synthesized protein(s) that is induced by the LH surge or an ovulatory dose of hCG.

The LH surge-induced production of progesterone and prostaglandins by preovulatory follicles has been proven to be essential for ovulation (32). A recent study demonstrated crucial roles for the LH surge-induced epidermal growth factor (EGF)-related peptides, such as amphiregulin, epiregulin, and betacellulin, in the ovulatory process (33,34). Furthermore, rapid and transient induction of *Ptgs2* mRNA (35) and *Pgr* mRNA (36), and EGF-related peptides (33,34) by an ovulatory dose of LH or hCG in rat preovulatory granulosa cells has been documented *in vivo* and *in vitro*. The expression pattern of these genes proceeds or is parallel to that of *Runx1* expression. We confirmed that hCG stimulates the transient up-regulation of levels of mRNA for *Ptgs2* (data not shown), *Pgr*, and amphiregulin (AREG) as well as the secretion of progesterone by preovulatory granulosa cells in our culture condition (Fig. 6, A and B). To further test whether the hCG-stimulated up-regulation of *Runx1* mRNA in cultured granulosa cells is mediated by the action of progesterone, prostaglandins, or EGF-related peptides, granulosa cells were cultured in the absence or presence of hCG, ZK98299 [10 μm; a specific PGR antagonist (31)], NS-398 [10 μm; a specific PTSG2 inhibitor (37)], AG1478 [1 μm; EGF receptor tyrosine kinase-selective inhibitor (33)], or hCG + each inhibitor/antagonist listed for 6 h and 12 h. The concentration of these inhibitors and the antagonists used in the present study has been shown to be effective in inhibiting the action of progesterone, prostaglandins, and EGF-related peptides in granulosa cells or follicle cultures (31,33,37). As expected, hCG stimulated *Runx1* mRNA expression in cultured granulosa cells (Fig. 6C). The hCG-induced *Runx1* mRNA accumulation was reduced approximately 55 and 50% by treatment with ZK98299 and AG1478, respectively ($P < 0.05$), whereas NS-398 treatment had no effect on *Runx1* mRNA expression in cultures at 6 h. Similar to the result from 6 h cultures, ZK98299 and AG1478 diminished the hCG-stimulated *Runx1* mRNA expression in granulosa cells cultured for 12 h ($P < 0.05$), but NS-398 had no effect on *Runx1* mRNA accumulation (data not shown), demonstrating the specific effect of ZK98299 and AG1478 on *Runx1* mRNA expression. Treatment with hCG + ZK98299 + AG1478 further decreased the levels of mRNA for Runx1 compared with that of hCG + ZK98299 or hCG + AG1478, but the level was higher than that of control (Fig. 6C).

To further determine whether the EGF-related peptides induced by the LH surge are involved in *Runx1* mRNA expression, we cultured granulosa cells in medium alone or with amphiregulin (100 nm, AREG), AREG + AG1478, or AREG + cyclohexamide. AREG stimulated the expression of *Runx1* mRNA compared with the control. The stimulatory effect of AREG on *Runx1* mRNA expression was blocked by cotreating with AG1478, confirming that the effect of AREG is mediated by the activation of EGF-receptor (Fig. 7A; $P < 0.05$). Interestingly, in granulosa cells that were pretreated with cyclohexamide for 1 h, AREG did not simulate *Runx1* mRNA expression. These data indicated the requirement of intermediate(s) induced by the action of AREG (or EGF-related peptides) for the up-regulation of *Runx1* mRNA. To further determine whether the stimulatory effect of AREG on *Runx1* mRNA expression is associated with the induction of PGR, three different experiments were performed. In the first experiment, granulosa cells were cultured in the absence or presence of AREG or AREG + ZK98299. The addition of ZK98299 reduced AREG-stimulated *Runx1* mRNA expression, indicating that AREG-induced Runx1 transcription is dependent, in part, on the activation of PGR (Fig. 7B; $P < 0.05$). Next, to determine whether AREG can induce *Pgr* mRNA expression, granulosa cells were cultured in medium alone, AREG, AREG + AG1478, or AG1478. The result demonstrated that AREG stimulated *Pgr* mRNA expression through the activation of EGF receptor (Fig. 7C; $P < 0.05$). Finally, to delineate the role of EGF signaling on the LH-

induced *Pgr* mRNA, the granulosa cells were cultured in the absence of presence of hCG, hCG + AG1478, or AG1478 alone. We found that the hCG-induced *Pgr* mRNA expression was inhibited by AG1478 (Fig. 7D; $P < 0.05$), suggesting that the LH-stimulated *Pgr* mRNA expression is, in part, mediated by EGF signaling in luteinizing granulosa cells *in vitro.* Based on these results, we proposed that the expression of *Runx1* mRNA is regulated by the LH-induced activation of EGF signaling directly as well as via induction of PGR. LH may act to stimulate an additional intermediate(s) to regulate *Runx1* mRNA expression (Fig. 7E).

Effects of Runx1 mRNA Reduction on Progesterone Production and Periovulatory Genes in Cultured Granulosa Cells

To begin to investigate the functional significance of the up-regulation of RUNX1 expression, granulosa cells isolated from preovulatory ovaries were transfected with small interfering RNA (siRNA) specific for *Runx1* to suppress the hCG-induced expression of *Runx1* mRNA. As determined by Northern blot and Western blot analyses, the levels of *Runx1* mRNA and protein were reduced in *Runx1*-specific siRNA-treated granulosa cells compared with that in control scrambled siRNA-treated cells (Fig. 8, A and B; $P < 0.001$). Transfection of siRNA to primary granulosa cells by electroporation was confirmed by BLOCK-iT Fluorescent Oligo (Invitrogen) (data not shown). No significant difference in levels of *Runx1* mRNA was detected between hCG alone and hCG + scrambled siRNA-treated cells (data not shown). We found that the reduction of *Runx1* mRNA levels by *Runx1* siRNA resulted in decreased progesterone accumulation in the conditioned culture media (Fig. 8C; $P < 0.01$).

To test whether the reduction of RUNX1 affects the expression of mRNA for *Cyp11a1*, an enzyme involved in progesterone production, as well as *Timp1* and *Cdkn1a*, both of which have previously been shown to be regulated by RUNX1 in fibroblast and hematopoietic cells, respectively, Northern membranes showing a decrease in *Runx1* mRNA levels were rehybridized with antisense riboprobes for these genes (Fig. 8D). We found that the levels of *Cyp11a1* mRNA were increased by hCG treatment (data not shown) and highest in granulosa cells cultured for 24 h. Furthermore, levels of *Cyp11a1* mRNA were reduced in *Runx1* siRNA-treated granulosa cells compared with that of control siRNA-treated cells by 24 h of culture (Fig. 8D; $P < 0.001$). *Timp1* mRNA expression was also stimulated by hCG treatment (data not shown), yet the stimulatory effect of hCG was transient; the levels of *Timp1* mRNA were higher in granulosa cells cultured for 4 h compared with the cells cultured for 24 h. Levels of *Timp1* mRNA did not differ between control siRNA and *Runx1* siRNA-treated cells at any time of culture (Fig. 8D; $P > 0.05$). We found that hCG stimulated the expression of *Cdkn1a* mRNA in cultured granulosa cells (data not shown), but the *Cdkn1a* mRNA level was not affected by *Runx1* siRNA at any time of culture (Fig. 9B).

To identify the possible target genes of RUNX1 action, preliminary DNA microarray analyses using Affymetrix Rat expression Arrays 230 2.0 (Affymetrix, Santa Clara, CA) were performed with total RNA isolated from siRNA-treated granulosa cells cultured for 10 h (highest protein level of RUNX1 was detected) or 24 h (n = 1). Consistent with previous Northern blot data, the microarray data showed no changes in *Timp1* mRNA levels at the time points studied and a 20% reduction of *Cyp11a1* mRNA levels by *Runx1* siRNA treatment only at the 24-h time point compared with that of scrambled siRNA-treated cells. From the microarray data, we further selected three genes that have been shown to be expressed in periovulatory follicles by other investigators (38,39) or the Rat Ovarian Gene Expression database (6) and were regulated by *Runx1* siRNA treatment in the microarray analysis for further investigation. These genes include metallothionein 1a (*Mt1a*) (38) and hyaluronan and proteoglycan link protein 1 (*Hapln1*) (39). In addition, we selected response gene to complement 32 (*Rgc32*) as a newly identified gene that was decreased by *Runx1* siRNA treatment. Northern blot analyses showed that the levels of mRNA for *Mt1a* and *Rgc32* increased over 24 h of culture, whereas

Hapln1 mRNA expression was transient in that the level was high at 10 h and declined by 24 h of culture (Fig. 9B). Levels of mRNA for these genes were stimulated by hCG treatment in our granulosa cell cultures (data not shown). *Mt1a* mRNA level was reduced in *Runx1* siRNA-treated cells compared with that of control siRNA-treated cells collected at 24 h of cultures (Fig. 9C), whereas the level of *Hapln1* mRNA was lower in *Runx1* siRNA-treated cells than in control siRNA at 10 h of cultures (Fig. 9D). *Runx1* siRNA treatment decreased hCG-stimulated *Rgc32* mRNA expression to 61% and 72% at 10 and 24 h of culture, respectively (Fig. 9E).

DISCUSSION

In response to the LH surge, preovulatory follicles undergo rapid and dramatic changes in the expression patterns of a myriad of genes (40). For instance, many genes that are highly expressed in growing preovulatory follicles are turned off by the LH surge, whereas the expression of numerous genes that are involved in ovulation and luteinization increases dramatically during the periovulatory period (reviewed in Refs. 1 and 32). Therefore, it is conceivable that transcriptional regulators induced in periovulatory follicles after the LH surge play a crucial role in controlling the transcriptional activation/repression of periovulatory genes. Such transcriptional modulators previously identified in periovulatory ovaries include PGR (31), C/EBPβ (5), and early growth response 1 (41). Herein, we report RUNX1 as a transcriptional regulator involved in peri-ovulatory gene expression; this is based on experimental evidence of LH surge-induced, hormone-regulated expression of *Runx1* in periovulatory follicles and alteration of periovulatory gene expression by suppression of hCG-induced RUNX1 expression.

In the present study, we demonstrated the rapid induction and transient expression of RUNX1 protein during the periovulatory period in rodent ovaries of gonadotropin-stimulated immature mice and rats. Using cycling adult rats, we confirmed the induction of RUNX1 in periovulatory follicles and newly forming corpora lutea. In addition, RUNX1 protein was detected in theca cells of antral follicles even before the LH surge in cycling rats and before hCG injection in the gonadotropin-induced model. Previously, we reported a minor and transient increase in *Runx1* mRNA levels at 12 h after PMSG injection (6). This increase is due to the expression of *Runx1* mRNA in the theca layer of antral follicles as determined by *in situ* hybridization studies (Jo, M., unpublished data). Thus, we speculated that the thecal expression of RUNX1 protein is initiated before hCG injection or the LH surge, presumably during the follicular growth stimulated by the LH-like activity of PMSG in gonadotropin-treated immature rats or LH in cycling animals.

Using primary granulosa cell cultures, we demonstrated that hCG induces a rapid and transient expression of *Runx1* mRNA, which was followed by that of RUNX1 protein, as determined by immunoblotting. These expression patterns of both mRNA and protein for RUNX1 *in vitro* are strikingly similar to those seen *in vivo*, demonstrating that the up-regulation of RUNX1 expression results from the direct action of LH on its cognate receptor in preovulatory granulosa cells. Importantly, the induction of *Runx1* mRNA in cultured granulosa cells afforded us an experimental model in which the LH-initiated signaling pathway involved in *Runx1* expression was further investigated. It is well recognized that the binding of LH to its receptor activates adenylate cyclase and leads to cAMP-mediated events, predominantly through the PKA-signaling pathway (32). In addition, the involvement of other kinase-signaling pathways has been implicated for mediating the complex series of LH-induced periovulatory processes, including PKC (27), phosphatidylino-sitol 3-kinase (42), and receptor tyrosine kinase (43). A previous study by Salvador *et al.* (30) has shown that the activation of LH receptor by administration of hCG to rat preovulatory granulosa cells *in vitro* leads to the activation of MEK and p42/44 MAPK through the PKA-dependent pathway and also stimulates p38 kinase

phosphorylation by a PKA- and PKC-independent pathway. In this study, we demonstrated that *Runx1* mRNA expression in cultured granulosa cells is dependent on the LH-induced activation of PKA, MEK, and p38 kinase, implicating the involvement of multiple signaling pathways, presumably the PKA-dependent pathway (cAMP/PKA/MEK) as well as the PKA- and PKC-independent pathway (p38 kinase) (depicted in Fig. 4C).

In addition to the involvement of multiple signaling pathways, the LH-induced Runx1 expression appears to require the action of autocrine and/or paracrine factors, which are synthesized in preovulatory granulosa cells in response to the LH surge. For instance, the present data show that the hCG-induced *Runx1* mRNA accumulation was completely abolished by cyclohexamide, demonstrating that the induction of *Runx1* mRNA requires the synthesis of a new protein(s). We hypothesized that the newly synthesized protein(s) could be PGR, EGF-related peptides, or PTGS2, all of which have been shown to be rapidly and transiently induced in granulosa cells of preovulatory follicles in response to the ovulatory level of LH *in vivo* and *in vitro* (28,34,36). This hypothesis was supported in part by the present finding that blocking PGR by a specific antagonist (ZK98299) and inhibiting the activation of the EGF receptor (AG1478) by an EGF receptor tyrosine kinase-selective inhibitor reduced the hCG-induced *Runx1* mRNA expression in cultured granulosa cells. Furthermore, the present data demonstrating the stimulatory effect of AREG on *Runx1* mRNA expression and its complete abolishment by AG1478 confirmed the involvement of EGF signaling on *Runx1* mRNA expression. Interestingly, this stimulatory effect of AREG was completely repressed when the *de novo* protein synthesis was blocked by cyclohexamide, indicating that a newly synthesized protein(s) is required for the up-regulation of *Runx1* mRNA. We explored the possibility that PGR may be one of the synthesized proteins induced by AREG and required for *Runx1* mRNA expression. This speculation was derived from our finding that the stimulatory effect of AREG on *Runx1* mRNA expression was reduced by a PGR antagonist. Our novel finding demonstrating the ability of AREG to stimulate *Pgr* mRNA expression and AG1478 to inhibit the hCG-induced *Pgr* mRNA expression provides support for this hypothesis. The fact that the levels of *Runx1* and *Pgr* mRNA were partially reduced by each inhibitor used in the present study implicates the presence of multiple pathways involved in the LH-induced gene expression. Taken together, these data suggest that the up-regulation of *Runx1* mRNA expression in periovulatory granulosa cells is mediated by the coordinate actions of multiple autocrine/paracrine factors that are induced by the LH surge, including actions of progesterone/PGR and EGF-related peptides/EGF receptor (depicted in Fig. 7E).

RUNX1 has been shown to play an essential role in differentiation of various cell types (8, 10,11,13). In the ovary, the LH surge promotes differentiation of follicular cells to luteal cells that predominantly produce and secrete progesterone [reviewed in (44)]. In addition, luteinizing granulosa cells have been shown to exit the cell cycle and subsequently stop dividing (18,45). Robker and Richards (18) have demonstrated that during the LH-induced terminal differentiation of granulosa cells (luteinization), the expression of a cell cycle promoter, cyclin D2, was rapidly down-regulated, whereas cell cycle inhibitors (*e.g. Cdkn1a* and *Cdknlb*) were dramatically increased in rat periovulatory granulosa cells. Previously, the expression of *Cdkn1a* has been shown to be regulated by RUNX1 in hematopoietic cells (19). Based on our finding of the rapid induction of RUNX1 expression by the LH surge and a previous report of the up-regulation of *Cdkn1a* mRNA expression in periovulatory granulosa cells (18), we speculated that RUNX1 may be involved in differentiation of luteinizing granulosa cells, perhaps by regulating the expression of *Cdkn1a*. However, we found that the levels of *Cdkn1a* mRNA were not affected by the reduction of RUNX1 expression in siRNA-treated granulosa cell cultures. Considering that RUNX1 expression was not completely blocked by siRNA treatment in the present study, it is possible that only complete abolishment of RUNX1 would exert an effect on *Cdkn1a* gene expression or it may be that RUNX1 regulates the transcription of *Cdkn1a* gene in a cell type-specific manner.

Another potential role of RUNX1 in the ovary may be the follicular/luteal shift in steroidogenesis toward progesterone production. For instance, we found that the suppression of RUNX1 by siRNA resulted in decreased progesterone accumulation as well as a reduction of the level of *Cyp11a1* mRNA, a key steroidogenic enzyme that is involved in converting cholesterol to progesterone. In the preovulatory ovary, LH/hCG has been shown to stimulate the expression of *Cyp11a1* mRNA and protein *in vivo* and *in vitro* (46,47) in luteinizing granulosa cells, which produce predominantly progesterone. Therefore, our novel findings suggest that RUNX1 plays a role in the follicular/ luteal shift in steroidogenesis in luteinizing granulosa cells, possibly through regulating the expression of genes for steroidogenic enzymes such as *Cyp11a1*.

One of the important findings in this study was the identification of candidate genes that are regulated by RUNX1 in periovulatory granulosa cells. Herein, we reported three genes, *Mt1a*, *Hapln1*, and *Rgc32*, as potential downstream targets of RUNX1. Metallothionein is a zinc-binding protein that acts to regulate the availability of zinc and other heavy metal ions in cells, thus known to play an important role in cellular metabolism, redox balance, and various cellular processes such as proliferation, inflammation, and apoptosis (reviewed in Ref. 48). A recent study by Espey *et al.* (38) has documented that *Mt1a* mRNA is induced in periovulatory follicles and highly expressed in corpora lutea of gonadotropin-stimulated immature rat ovaries. These authors suggested potential roles for this protein in regulating the activity of metalloproteinases, steroidogenesis, and inflammatory processes during the periovulatory period (38). HAPLN1 is a glycoprotein that interacts with both hyaluronic acid and proteoglycan (*e.g.* versicans) to form aggregates in a hyaluronan-rich extracellular matrix. In the ovary, HAPLN1 was localized exclusively to the extracellular matrix and cytoplasm of cumulus granulosa cells of periovulatory follicles in rodent ovaries (39,49), indicating the involvement of this protein in cumulus-oocyte complex expansion. *Rgc32* was first identified as a gene that is induced by sublytic complement activation in oligodendrocytes (50). A recent study by Badea *et al.* (50) showed that RGC32 increased the activity of $p34^{cdc2}$ kinase and the overexpression of this protein enhanced S-phase entry in aortic smooth muscle cells, suggesting the role of RGC32 as a cell cycle regulator. Rat Ovarian Gene Expression database (6) and our Northern blot data (Park, E., unpublished data) showed that *Rgc32* mRNA expression is increased in ovulating follicles of rodent ovaries. Therefore, it is of interest to further determine whether RGC32 is involved in changes in the cell cycle of luteinizing granulosa cells. Although the results from the present study suggest the involvement of RUNX1 in the gene expression of *Mt1a*, *Hap1n1*, and *Rgc32*, there has been no report for the binding site of RUNX1 in the known promoter/enhancer regions of these genes. A computational search (TFSEARCH, http://www.cbrc.jp/research/db/TFSEARCH.html) revealed the presence of potential binding sites for RUNX1 within 1000-bp genomic sequences 5′ of the first transcription start site of *Hapln1* and *Mt1a* gene of rats. As for the *Rgc32* gene, rat genomic sequence of this gene is not presently available, although in the mouse *Rgc32* gene, three possible binding sites for RUNX1 were found. However, further experimental analyses (*e.g.* promoter analysis and chromatin immunoprecipitation assay) are needed to determine whether these genes are direct targets of RUNX1 in luteinizing granulosa cells.

In summary, the present study demonstrated the rapid and transient induction of *Runx1* mRNA by the LH surge in periovulatory ovaries and the concomitant increase in RUNX1 protein in granulosa cells of periovulatory follicles and luteal cells in newly forming corpora lutea. We provide experimental evidence that the hCG-induced *Runx1* mRNA expression is dependent on the activation of the EGF receptor by EGF-related peptides and PGR activation, revealing coordinate regulation of *Runx1* mRNA expression by these two key mediators of the periovulatory process. Finally, the findings that reduction of hCG-induced RUNX1 expression by siRNA resulted in decreased progesterone accumulation as well as a reduction in levels of mRNA for *Cyp11a1*, *Mt1a*, *Hapln1*, and the novel gene, *Rgc32*, provide strong support for the

role of RUNX1 in the follicular/luteal shift in steroidogenesis and periovulatory gene expression in luteinizing granulosa cells.

MATERIALS AND METHODS

Materials

Unless otherwise noted, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO), or Calbiochem (La Jolla, CA). Molecular biological enzymes, BenchMark Prestained Protein ladder, oligonucleotides, and Trizol were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA).

Animals

All animal procedures were approved by the University of Kentucky Animal Care and Use Committee. In the present study, gonadotropin-treated immature female mice and rats, as well as sexually mature adult female rats exhibiting regular 4-d estrous cycles were used.

Immature female Sprague Dawley rats and C57BL/6 mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and provided with water and chow *ad libitum*. Rats were maintained on a 14-h light, 10-h dark cycle, and mice were maintained on a 12-h light, 12-h dark cycle. The animals (22 d old) were injected with PMSG (10 IU to rats, 5 IU to mice) s.c. to stimulate follicular development and 48 h later with hCG (5 IU to rat, 2.5 IU to mice) s.c. to induce ovulation and subsequent formation of corpora lutea. In these models (51), ovulation occurs approximately 12–16 h post-hCG administration. Animals were killed at 0 h (at the time of hCG administration) and defined times after hCG administration (n = 3–4 animals/time point). Ovaries were collected and stored at −70 C for later isolation of total RNA, 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 and immunohistochemical analyses. For western immnuoblot analyses, fresh ovaries were used to extract nuclear fractions from granulosa cells or residual ovarian tissues.

Adult female Sprague Dawley rats (150–180 g body weight, 2 months old) were purchased from Harlan Sprague Dawley, Inc., and housed as described above. Stages of the estrous cycle were determined by daily examination of vaginal cytology, and only animals showing at least two consecutive 4-d cycles were used for the experiment. Rats were killed at 1600 h, 2000 h, and 2400 h on proestrus, and 0400 h on estrus. In this colony of rats, the peak of the LH surge was at 1600 h on proestrus. Ovaries were collected, placed in Tissue-Tek OCT compound, and stored at −70 C until sectioned and processed for *in situ* hybridization and immunohistochemistry.

Isolation and Culture of Granulosa Cells

To isolate granulosa cells, ovaries were collected from immature rats 48 h after PMSG administration and processed as described previously (21). Briefly, granulosa cells were isolated by the method of follicular puncture. The cells were pooled, filtered, pelleted by centrifugation at 200 × *g* for 5 min, and resuspended in defined medium consisting of DMEM-Ham's F-12 medium supplemented with 1% BSA, 0.01% pyruvic acid, 0.22% bicarbonate, gentamycin (0.05 mg/ml), and ITS (insulin, transferrin, and selenium). The cells were cultured in the absence or presence of various reagents for 0, 3, 6, 12, or 24 h at 37 C in a humidified atmosphere of 5% CO_2. When reagents were dissolved in dimethylsulfoxide (DMSO) and added to the culture medium, the same concentration of DMSO was added to medium for the control cells. The final concentration of DMSO in cultures was less than 0.05%. At the end of each culture period, cells were collected and processed for extraction of nuclear fractions or snap-frozen for later isolation of total RNA.

siRNA Knockdown of Runx1 mRNA in Granulosa Cells *in Vitro*

Granulosa cells were collected from immature rats 48 h after PMSG administration described above and resuspended in siPORT siRNA electroporation buffer (Ambion, Inc., Austin, TX). The cells were mixed with siRNA specific for *Runx1* (sense, 5′-GGCUCCUACCAAUUCUCCAtt-3′; antisense, 5′-UGGAGAUUGGUAGGAGCCtg-3′; Ambion, Inc.) or Silencer negative control siRNA (scrambled siRNA; Ambion Inc.) and then electrophorated at 400 V, 100 μsec, and one pulse using a Multiporator (Eppendorf, Hamburg, Germany). These conditions were chosen based on the results of preliminary optimization experiments. The electroporated cells (~5×10^5 cells and 100 nm of siRNA per well) were cultured for 1 h in the defined medium described above before hCG (1 IU/ml) treatment, and further cultured for 4, 10, or 24 h at 37 C in a humidified atmosphere of 5% CO_2. At the end of each culture period, cells were collected and snap frozen for isolation of total RNA for Northern blot analyses or processed to isolate nuclear extracts for Western blot analyses. Conditioned culture media were collected and stored at −20 C and measured for progesterone by RIA.

DNA Microarray

Total RNA was extracted from the granulosa cells treated with *Runx1* siRNA or scrambled siRNA and cultured for 10 or 24 h using a RNeasy Mini kit (QIAGEN, Inc., Valencia, CA) according to the manufacturer's instructions and quantified by spectrophotometry. The integrity of total RNA was verified by visualizing the intact and distinct 28S rRNA and 18S rRNA stained with ethidium bromide in 1.5% agarose-formaldehyde gel. The total RNA was transferred to a nitrocellulose membrane. The reduction of *Runx1* mRNA in *Runx1* siRNA-treated granulosa cells was confirmed by Northern blot analyses. Total RNA (5μg) pooled from two independent experiments was used as a template for cDNA synthesis, and biotinylated antisense cRNA probe was prepared as described by the manufacturers of the SuperScript System Kit (Invitrogen) and the ENZO BioArray HighYield RNA labeling Kit (Enzo Diagnostics, Farmingdale, NY). Unincorporated nucleotides were removed from the riboprobe preparation using the RNeasy Mini Kit. The integrity of the riboprobe was checked by gel electrophoresis. The Affymetrix Rat 230 2.0 genechip array was hybridized, washed, and scanned using Affymetrix equipment and protocols (Affymetrix; DNA Microarray Core Facility, University of Kentucky, Lexington, KY).

Generation of the Plasmid-Containing cDNA for Rat Cdkn1a, Hapln1, Mt1a, Pgr, and Rgc32

Partial cDNAs corresponding to each of the reference sequences listed in Table 1 were generated by RT-PCR and cloned into the pCRII-TOPO Vector (Invitrogen) as described previously (6). Oligonucleotide primer pairs were designed based on published sequence data (the primer sequence for each gene is listed in Table 1). DNA sequences of cloned rat partial cDNAs for the selected genes were verified commercially (MWG Biotech, Inc., High Point, NC).

Quantification of *Runx1, Pgr, Areg, Timp1, Cyp11a1, Cdkn1a, Hapln1, Mt1a*, and *Rgc32* mRNA

Total RNA was isolated from whole ovaries or cultured granulosa cells using Trizol reagent according to the manufacturer's protocol and quantified by spectrophotometry. Northern blot analyses were carried out as described previously (52). Plasmids containing rat cDNAs for *Runx1* (6), *Areg* (6), *Timp1* (53), *Cyp11a1* (generously provided by Dr. J. S. Richards, Baylor College of Medicine, Houston, TX), mouse cDNA for ribosomal protein *L32* (kindly provided by Dr. O. K. Park-Sarge, University of Kentucky), *Cdkn1a*, *Hapln1*, *Pgr*, *Mt1a*, and *Rgc32* were linearized with appropriate enzymes. Anti-sense riboprobes were transcribed using [α-^{32}P]UTP (10 mCi/ml; NEN brand from PerkinElmer, Boston, MA) and SP6 or T7 RNA polymerase (Ambion, Inc.), as appropriate. Northern membranes were hybridized with ^{32}P-

labeled antisense riboprobes in Ultrahyb hybridization buffer (Ambion, Inc.) at 68 C for at least 16 h. Excess probe was removed by washing with a stringent buffer (0.1×SSC, 0.1% sodium dodecyl sulfate) twice at 68 C for 60 min. The membrane was exposed to a phosphorimaging plate and quantified with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The relative levels of *Runx1* mRNA or the gene of interest were normalized to *L32* mRNA levels. *Pgr* mRNA levels were also measured by semiquantitative RT-PCR, as described previously (31).

In Situ Hybridization of Runx1 mRNA

Ovaries collected from PMSG-primed immature mouse ovaries or naturally cycling adult rats were sectioned at 10 μm and mounted on Probe On Plus slides (Fisher Scientific, Pittsburgh, PA). *In situ* hybridization was carried out as described previously (54). Briefly, plasmids containing cDNA for rat *Runx1* were linearized with *SaI* I and *Bam*HI to generate sense and antisense riboprobes, respectively. Linearized plasmids were labeled with [α-^{35}S]UTP (10 mCi/ml; MP Biomedicals, Inc., Costa Mesa, CA) and appropriate RNA polymerases. The sections were hybridized overnight with 1×10^6 cpm ^{35}S-labeled riboprobe/slide in a humidified chamber at 55 C. The next day, slides were washed and treated with RNase A (0.1 mg/ml) for 30 min at 45 C. Tissue sections were washed again at high stringency, dried, dipped in Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY), and exposed at 4 C for 4 wk. To visualize the hybridized riboprobes, slides were developed with Kodak D19 and counterstained with hematoxylin solution. Tissues were examined with an Eclipse E800 Nikon microscope (Nikon Corp., Melville, NY) under bright- and dark-field optics. One ovary from each of three animals was used for *in situ* hybridization. At least 12 sections per ovary were analyzed for each antisense probe, making a total of at least 36 tissue sections analyzed for each time point. A sense riboprobe, used as a control for nonspecific binding, was included for each ovary and each time point.

Immunohistochemistry

Frozen ovaries collected from untreated or gonadotropin-treated immature mice and rats were sectioned at 8 μm, thaw-mounted on Probe On Plus slides, and air dried. The sections were fixed in acetone for 10 min. The endogenous peroxidase activity was quenched by immersing the sections in methanol containing 0.3% H_2O_2. Immunohistostaining was performed using the Vectorstain Elite ABC (avidin-biotin complex) kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. Briefly, after a PBS rinse, the sections were incubated with normal rabbit serum for 30 min and then treated with the primary RUNX1 antibody (AML1; N-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted to 2 μg/ml in PBS for 16 h at 4 C. For the negative control, the sections were incubated with the primary antibody preadsorbed with a blocking peptide for AML1 (Santa Cruz Biotechnology, Inc.) or without primary antibody. The sections were washed with PBS and then incubated with antigoat biotin-conjugated secondary antibody for 30 min. The biotinylated antibody complex was incubated with a peroxidase-labeled ABC for 1 h at room temperature by use of the ABC Elite reagent and then visualized with substrate-chromagen solution (3,3′-diaminobenzidine). One ovary from each of three animals was used for immunohistochemistry. At least eight sections per ovary were analyzed by immunohistochemistry, making a total of at least 24 tissue sections analyzed for each time point.

Western Blot Analysis

Nuclear extracts were isolated from granulosa cells, the residual ovarian tissue (ovary after expelling granulosa cells), or whole ovaries obtained from gonadotropin-stimulated immature rats or cultured rat granulosa cells using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instruction. Briefly, the cells were collected in ice-cold PBS/

phosphatase inhibitors (Active Motif), pelleted by centrifugation at 200 × *g* for 5 min, and resuspended in hypotonic buffer to swell the cell membrane for 15 min. Then, 25 μl of detergent was added to release cytoplasmic proteins into the supernatant. To collect nuclear pellet, the cell suspensions were pelleted by centrifugation at 14,000 × *g* for 30 sec. The supernatant was removed, and the nuclei were lysed with 50 μl Complete Lysis buffer/protease inhibitor cocktail for 30 min on ice. After centrifugation for 10 min at 14,000 × *g*, supernatants (nuclear fraction) were aliquoted and stored at −80 C until use. For the ovarian tissues, they were homogenized in hypotonic buffer/dithiothreitol + detergent with prechilled Dounce homogenizers. Homogenized tissues were pelleted by centrifugation at 850 × *g* for 10 min. The pellets were resuspended in hypotonic buffer and processed as described above. Nuclear extracts were denatured by boiling for 5 min and separated by SDS-PAGE on an 8% polyacryl-amide gel and then transferred onto nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). Immunoblotting of RUNX1 protein was performed using Vectorstain Elite ABC kit (Vector Laboratories, Inc.). Primary antibody [rabbit poly-clonal antibody to RUNX1 (ab11903, 1:1000 dilution; Abcam, Cambridge, MA) was added in 1% casein solution and incubated overnight at 4 C. Peroxidase activity was visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL). Jurkat nuclear extracts (Active Motif) were included as a positive control sample for RUNX1 protein and prestained protein size marker (Invitrogen) in most Western blots.

RIA

Concentrations of progesterone in culture media were measured using an Immulite kit (Diagnostic Products, Los Angeles, CA). Assay sensitivity was 0.02 ng/ml. The intraassay and interassay coefficients of variation were 9.6% and 10%, respectively.

Statistical Analyses

All data are presented as means ± sem. One-way ANOVA was used to test differences in levels of *Runx1* mRNA across time of tissue collection, time of culture, or among treatments *in vitro*. If ANOVA revealed significant effects of time of tissue collection, time of culture, or treatment, the means were compared by Duncan's test, with $P < 0.05$ considered significant. The experiments examining the effect of *Runx1* siRNA in granulosa cell cultures were analyzed by paired *t* tests.

Acknowledgements

We thank Dr. O.K. Park-Sarge for providing plasmids containing cDNAs for *L32* and ZK98299 and Dr. J. S. Richards for providing plasmids containing cDNAs for *Cyp11a1*.

References

1. Espey LL, Richards JS. Temporal and spatial patterns of ovarian gene transcription following an ovulatory dose of gonadotropin in the rat. Biol Reprod 2002;67:1662–1670. [PubMed: 12444039]
2. Richards JS, Russell DL, Ochsner S, Espey LL. Ovulation: new dimensions and new regulators of the inflammatory-like response. Annu Rev Physiol 2002;64:69–92. [PubMed: 11826264]
3. Richards JS. Ovulation: new factors that prepare the oocyte for fertilization. Mol Cell Endocrinol 2005;234:75–79. [PubMed: 15836955]
4. Robker RL, Russell DL, Espey LL, Lydon JP, O'Malley BW, Richards JS. Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. Proc Natl Acad Sci USA 2000;97:4689–4694. [PubMed: 10781075]
5. Sterneck E, Tessarollo L, Johnson PF. An essential role for C/EBPβ in female reproduction. Genes Dev 1997;11:2153–2162. [PubMed: 9303532]

6. Jo M, Gieske MC, Payne CE, Wheeler-Price SE, Gieske JB, Ignatius IV, Curry TE Jr, Ko C. Development and application of a rat ovarian gene expression database (rOGED). Endocrinology 2004;145:5384–5396. [PubMed: 15297439]
7. Satake M, Nomura S, Yamaguchi-Iwai Y, Takahama Y, Hashimoto Y, Niki M, Kitamura Y, Ito Y. Expression of the Runt domain-encoding PEBP2 α genes in T cells during thymic development. Mol Cell Biol 1995;15:1662–1670. [PubMed: 7862157]
8. Tanaka T, Tanaka K, Ogawa S, Kurokawa M, Mitani K, Nishida J, Shibata Y, Yazaki Y, Hirai H. An acute myeloid leukemia gene, AML1, regulates hemopoietic myeloid cell differentiation and transcriptional activation antagonistically by two alternative spliced forms. EMBO J 1995;14:341–350. [PubMed: 7530657]
9. Kitabayashi I, Yokoyama A, Shimizu K, Ohki M. Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation. EMBO J 1998;17:2994–3004. [PubMed: 9606182]
10. Ichikawa M, Asai T, Saito T, Yamamoto G, Seo S, Yamazaki I, Yamagata T, Mitani K, Chiba S, Hirai H, Ogawa S, Kurokawa M. AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. Nat Med 2004;10:299–304. [PubMed: 14966519]
11. Taniuchi I, Osato M, Egawa T, Sunshine MJ, Bae SC, Komori T, Ito Y, Littman DR. Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. Cell 2002;111:621–633. [PubMed: 12464175]
12. Zhu X, Yeadon JE, Burden SJ. AML1 is expressed in skeletal muscle and is regulated by innervation. Mol Cell Biol 1994;14:8051–8057. [PubMed: 7969143]
13. Kurokawa M, Tanaka T, Tanaka K, Hirano N, Ogawa S, Mitani K, Yazaki Y, Hirai H. A conserved cysteine residue in the runt homology domain of AML1 is required for the DNA binding ability and the transforming activity on fibroblasts. J Biol Chem 1996;271:16870–16876. [PubMed: 8663420]
14. Takakura N, Watanabe T, Suenobu S, Yamada Y, Noda T, Ito Y, Satake M, Suda T. A role for hematopoietic stem cells in promoting angiogenesis. Cell 2000;102:199–209. [PubMed: 10943840]
15. Downing JR, Higuchi M, Lenny N, Yeoh AE. Alterations of the AML1 transcription factor in human leukemia. Semin Cell Dev Biol 2000;11:347–360. [PubMed: 11105899]
16. Thornell A, Hallberg B, Grundstrom T. Binding of SL3–3 enhancer factor 1 transcriptional activators to viral and chromosomal enhancer sequences. J Virol 1991;65:42–50. [PubMed: 1985206]
17. Otto F, Lubbert M, Stock M. Upstream and downstream targets of RUNX proteins. J Cell Biochem 2003;89:9–18. [PubMed: 12682904]
18. Robker RL, Richards JS. Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. Mol Endocrinol 1998;12:924–940. [PubMed: 9658398]
19. Lutterbach B, Hiebert SW. Role of the transcription factor AML-1 in acute leukemia and hematopoietic differentiation. Gene 2000;245:223–235. [PubMed: 10717473]
20. Bertrand-Philippe M, Ruddell RG, Arthur MJ, Thomas J, Mungalsingh N, Mann DA. Regulation of tissue inhibitor of metalloproteinase 1 gene transcription by RUNX1 and RUNX2. J Biol Chem 2004;279:24530–24539. [PubMed: 15051730]
21. Mann JS, Kindy MS, Edwards DR, Curry TE Jr. Hormonal regulation of matrix metalloproteinase inhibitors in rat granulosa cells and ovaries. Endocrinology 1991;128:1825–1832. [PubMed: 1848503]
22. Komar CM, Matousek M, Mitsube K, Mikuni M, Brannstrom M, Curry TE Jr. Effects of genistein on the periovulatory expression of messenger ribonucleic acid for matrix metalloproteinases and tissue inhibitors of metalloproteinases in the rat ovary. Reproduction 2001;121:259–265. [PubMed: 11226050]
23. Hess J, Porte D, Munz C, Angel P. AP-1 and Cbfa/ runt physically interact and regulate parathyroid hormone-dependent MMP13 expression in osteoblasts through a new osteoblast-specific element 2/ AP-1 composite element. J Biol Chem 2001;276:20029–20038. [PubMed: 11274169]
24. Zhang DE, Hetherington CJ, Meyers S, Rhoades KL, Larson CJ, Chen HM, Hiebert SW, Tenen DG. CCAAT enhancer-binding protein (C/EBP) and AML1 (CBF α2) synergistically activate the

macrophage colony-stimulating factor receptor promoter. Mol Cell Biol 1996;16:1231–1240. [PubMed: 8622667]

25. Sharma SC, Richards JS. Regulation of AP1 (Jun/ Fos) factor expression and activation in ovarian granulosa cells. Relation of JunD and Fra2 to terminal differentiation. J Biol Chem 2000;275:33718–33728. [PubMed: 10934195]
26. Sirois J, Richards JS. Transcriptional regulation of the rat prostaglandin endoperoxide synthase 2 gene in granulosa cells. Evidence for the role of a cis-acting C/EBP β promoter element. J Biol Chem 1993;268:21931–21938. [PubMed: 8408049]
27. Morris JK, Richards JS. Luteinizing hormone induces prostaglandin endoperoxide synthase-2 and luteinization *in vitro* by A-kinase and C-kinase pathways. Endocrinology 1995;136:1549–1558. [PubMed: 7895665]
28. Natraj U, Richards JS. Hormonal regulation, localization, and functional activity of the progesterone receptor in granulosa cells of rat preovulatory follicles. Endocrinology 1993;133:761–769. [PubMed: 8344215]
29. Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS. Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-lnduced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. Mol Endocrinol 2000;14:1283–1300. [PubMed: 10935551]
30. Salvador LM, Maizels E, Hales DB, Miyamoto E, Yamamoto H, Hunzicker-Dunn M. Acute signaling by the LH receptor is independent of protein kinase C activation. Endocrinology 2002;143:2986–2994. [PubMed: 12130564]
31. Ko C, In YH, Park-Sarge OK. Role of progesterone receptor activation in pituitary adenylate cyclase activating polypeptide gene expression in rat ovary. Endocrinology 1999;140:5185–5194. [PubMed: 10537148]
32. Richards JS, Russell DL, Ochsner S, Hsieh M, Doyle KH, Falender AE, Lo YK, Sharma SC. Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. Recent Prog Horm Res 2002;57:195–220. [PubMed: 12017544]
33. Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 2004;303:682–684. [PubMed: 14726596]
34. Ashkenazi H, Cao X, Motola S, Popliker M, Conti M, Tsafriri A. Epidermal growth factor family members: endogenous mediators of the ovulatory response. Endocrinology 2005;146:77–84. [PubMed: 15459120]
35. Ko C, Park-Sarge OK. Progesterone receptor activation mediates LH-induced type-I pituitary adenylate cyclase activating polypeptide receptor (PAC(1)) gene expression in rat granulosa cells. Biochem Biophys Res Commun 2000;277:270–279. [PubMed: 11027674]
36. Sirois J, Simmons DL, Richards JS. Hormonal regulation of messenger ribonucleic acid encoding a novel isoform of prostaglandin endoperoxide H synthase in rat preovulatory follicles. Induction in vivo and in vitro. J Biol Chem 1992;267:11586–11592. [PubMed: 1597485]
37. Elvin JA, Yan C, Matzuk MM. Growth differentiation factor-9 stimulates progesterone synthesis in granulosa cells via a prostaglandin E2/EP2 receptor pathway. Proc Natl Acad Sci USA 2000;97:10288–10293. [PubMed: 10944203]
38. Espey LL, Ujioka T, Okamura H, Richards JS. Metallothionein-1 messenger RNA transcription in steroid-secreting cells of the rat ovary during the periovulatory period. Biol Reprod 2003;68:1895–1902. [PubMed: 12606366]
39. Kobayashi H, Sun GW, Hirashima Y, Terao T. Identification of link protein during follicle development and cumulus cell cultures in rats. Endocrinology 1999;140:3835–3842. [PubMed: 10433245]
40. Leo CP, Pisarska MD, Hsueh AJ. DNA array analysis of changes in preovulatory gene expression in the rat ovary. Biol Reprod 2001;65:269–276. [PubMed: 11420249]
41. Espey LL, Ujioka T, Russell DL, Skelsey M, Vladu B, Robker RL, Okamura H, Richards JS. Induction of early growth response protein-1 gene expression in the rat ovary in response to an ovulatory dose of human chorionic gonadotropin. Endocrinology 2000;141:2385–2391. [PubMed: 10875238]
42. Carvalho CR, Carvalheira JB, Lima MH, Zimmerman SF, Caperuto LC, Amanso A, Gasparetti AL, Meneghetti V, Zimmerman LF, Velloso LA, Saad MJ. Novel signal transduction pathway for

luteinizing hormone and its interaction with insulin: activation of Janus kinase/signal transducer and activator of transcription and phospho-inositol 3-kinase/Akt pathways. Endocrinology 2003;144:638–647. [PubMed: 12538627]

43. Conti M, Hsieh M, Park JY, Su YQ. Role of the EGF network in ovarian follicles. Mol Endocrinol 2005;20:715–723. [PubMed: 16051667]
44. Murphy BD. Models of luteinization. Biol Reprod 2000;63:2–11. [PubMed: 10859235]
45. Quirk SM, Cowan RG, Harman RM. Progesterone receptor and the cell cycle modulate apoptosis in granulosa cells. Endocrinology 2004;145:5033–5043. [PubMed: 15271877]
46. Goldring NB, Durica JM, Lifka J, Hedin L, Ratoosh SL, Miller WL, Orly J, Richards JS. Cholesterol side-chain cleavage P450 messenger ribonucleic acid: evidence for hormonal regulation in rat ovarian follicles and constitutive expression in corpora lutea. Endocrinology 1987;120:1942–1950. [PubMed: 3106012]
47. Oonk RB, Krasnow JS, Beattie WG, Richards JS. Cyclic AMP-dependent and -independent regulation of cholesterol side chain cleavage cytochrome P-450 (P-450scc) in rat ovarian granulosa cells and corpora lutea. cDNA and deduced amino acid sequence of rat P-450scc. J Biol Chem 1989;264:21934–21942. [PubMed: 2480959]
48. Miles AT, Hawksworth GM, Beattie JH, Rodilla V. Induction, regulation, degradation, and biological significance of mammalian metallothioneins. Crit Rev Biochem Mol Biol 2000;35:35–70. [PubMed: 10755665]
49. Sun GW, Kobayashi H, Terao T. Expression of link protein during mouse follicular development. J Histo-chem Cytochem 1999;47:1433–1442.
50. Badea T, Niculescu F, Soane L, Fosbrink M, Sorana H, Rus V, Shin ML, Rus H. RGC-32 increases p34CDC2 kinase activity and entry of aortic smooth muscle cells into S-phase. J Biol Chem 2002;277:502–508. [PubMed: 11687586]
51. Ying SY, Meyer RK. Ovulation induced by PMS and HCG in hypophysectomized immature rats. Proc Soc Exp Biol Med 1972;139:1231–1233. [PubMed: 5023317]
52. Jo M, Komar CM, Fortune JE. Gonadotropin surge induces two separate increases in messenger RNA for progesterone receptor in bovine preovulatory follicles. Biol Reprod 2002;67:1981–1988. [PubMed: 12444077]
53. Simpson KS, Byers MJ, Curry TE Jr . Spatio-temporal messenger ribonucleic acid expression of ovarian tissue inhibitors of metalloproteinases throughout the rat estrous cycle. Endocrinology 2001;142:2058–2069. [PubMed: 11316773]
54. Curry TE Jr, Song L, Wheeler SE. Cellular localization of gelatinases and tissue inhibitors of metalloproteinases during follicular growth, ovulation, and early luteal formation in the rat. Biol Reprod 2001;65:855–865. [PubMed: 11514351]

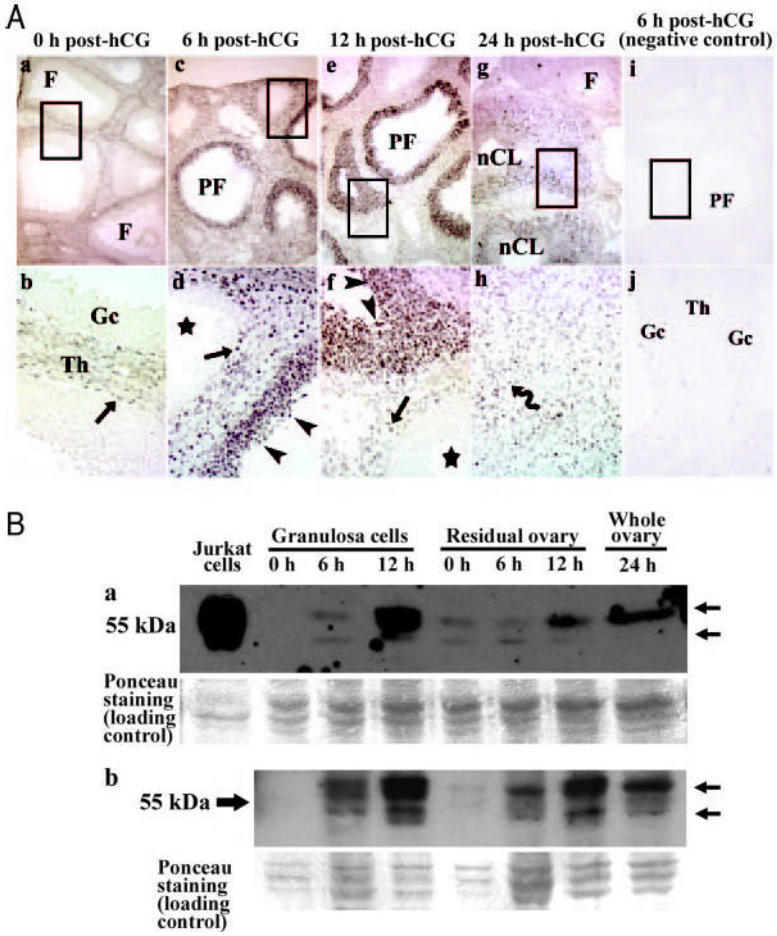

Fig. 1. Expression Profile of RUNX1 in Gonadotropin-Primed Immature Rat Ovaries

A, Immunohistochemical localization for RUNX1 protein during the periovulatory period in gonadotropin-primed immature rat ovaries. Sections of rat ovaries obtained at 0 h (48 h post-PMSG, a and b), 6 h (c, d, i, and j), 12 h (e and f), or 24 h (g and h) after hCG injection were immunostained with the antibody for RUNX1 (AML-1: N-20). The *boxed areas* in panels a, c, e, g, and i are magnified in panel b, d, f, h, and j, respectively. The tissue section depicted in i and j was incubated with the primary antibody preadsorbed with the blocking peptide for AML1 as a negative control. F, Follicle; Gc, granulosa cell layer; Th, theca cell layer; PF, periovulatory follicles; nCL, newly forming corpus luteum. *Arrowheads* indicate positive stained nuclei of granulosa cells. *Arrows* indicate positive stained nuclei of theca cells. *Wavy arrows* indicate positively stained nuclei of luteal cells. *Stars* indicate small antral follicles lacking RUNX1 immunoreactivity in the granulosa cells. Original magnification of a, c, e, g, and i is ×150. Original magnification of b, d, f, h, and j panels is ×400. B, Western blot analysis of RUNX1 protein in granulosa cells, residual ovarian tissue, or whole ovaries of immature rats obtained at indicated times after hCG injection. *Arrows to the right* of panel B) indicate different forms of RUNX1 detected. Each lane was loaded with 25 μl of nuclear fraction (~30 μg) extracted from ovaries of each animal. The membrane was stained with Ponceau-S to show the relative loading of sample protein. The blots (a and b in panel B) are representatives of four separate experiments (n = 4 animals/time point).

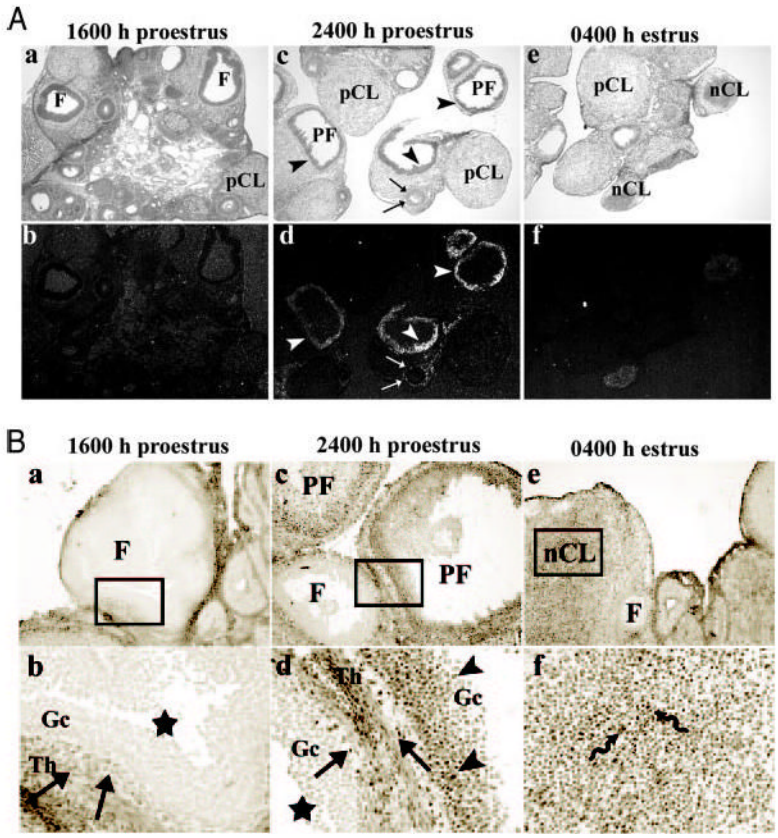

Fig. 2. Expression Profile of RUNX1 in Naturally Cycling Rat Ovaries

A, *In situ* hybridization analysis of *Runx1* mRNA during the periovulatory period in ovaries from naturally cycling rats. Representative bright-field (a, c, and e) and corresponding dark-field (b, d, and f) photomicrographs are depicted. Ovaries were collected at 1600 h (a and b; at the peak of the LH surge) and 2400 h (c and d) on proestrus, and 400 h on estrus (e and f). *Arrows* in d indicate a small antral follicle expressing Runx1 mRNA in the theca layer. *Arrowheads* in d indicate *Runx1* mRNA expression in periovulatory follicles. Original magnification of all slides is ×40. B, Immunohistochemical localization for RUNX1 during the periovulatory period in ovaries from naturally cycling rats. Sections of rat ovaries obtained at 1600 h (a and b; at the peak of the LH surge) and 2400 h (c and d) on proestrus, and 400 h on estrus (e and f) were immunostained with the RUNX1 antibody (AML-1: N-20). *Arrows*, *arrowheads*, and *wavy arrows* indicate theca cells, granulosa cells, and luteal cells, respectively, that show positive staining for Runx1 protein. *Stars* indicate small follicles lacking RUNX1 immunoreactivity in the granulosa cells. The *boxed areas* in panels a, c, and e were magnified in panels b, d, and f, respectively. Original magnification of a, c, and e panels is ×150. Original magnification of b, d, and f panels is ×400. Gc, Granulosa cell layer; Th, theca cell layer; PF, periovulatory follicles; nCL, newly forming corpus luteum; pCL, corpus luteum from previous estrous cycles; F, follicle.

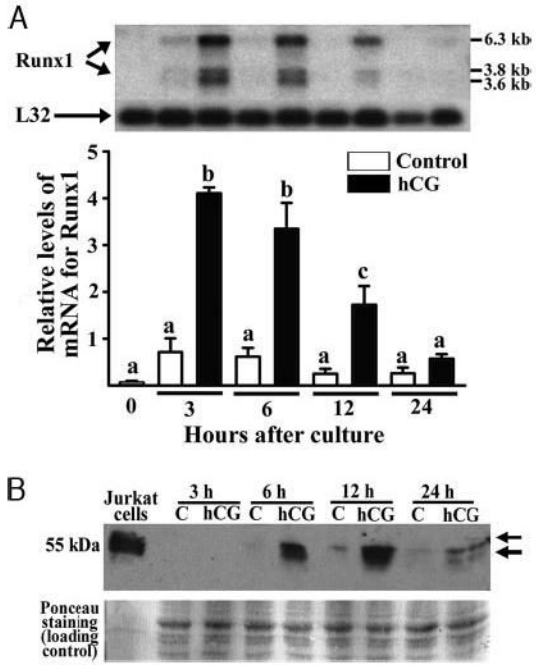

Fig. 3. Transient Up-Regulation of mRNA and Protein for RUNX1 in Granulosa Cells by hCG *in Vitro*

A, Autoradiograph of a representative Northern blot analysis shows multiple transcripts for the *Runx1* gene and ribosomal protein *L32* mRNA in granulosa cells from rat preovulatory ovaries (48 h post-PMSG) cultured in medium alone (Control) or with hCG (1 IU/ml) for 0, 3, 6, 12, or 24 h. The levels of *Runx1* mRNA were calculated by combining the intensity of all three different transcripts of the *Runx1* gene detected in the Northern blot. Relative levels of *Runx1* mRNA were normalized to the *L32* band in each sample (mean ± sem; n = 3 independent culture experiments). *Bars with no common superscripts* are significantly different ($P < 0.05$). B, Western blot analysis of RUNX1 protein in preovulatory granulosa cells cultured in medium alone (Control) or with hCG for 3, 6, 12, or 24 h. *Arrows to the right* of panel B indicate the different forms of RUNX1 detected. Protein concentrations were controlled by plating an equal number of cells per well for each treatment and then loading an equal volume (30 μl) of nuclear extracts to each lane. The membrane was stained with Ponceau-S to show the relative loading of sample protein. Panel B is a representative of four separate culture experiments. C, Control.

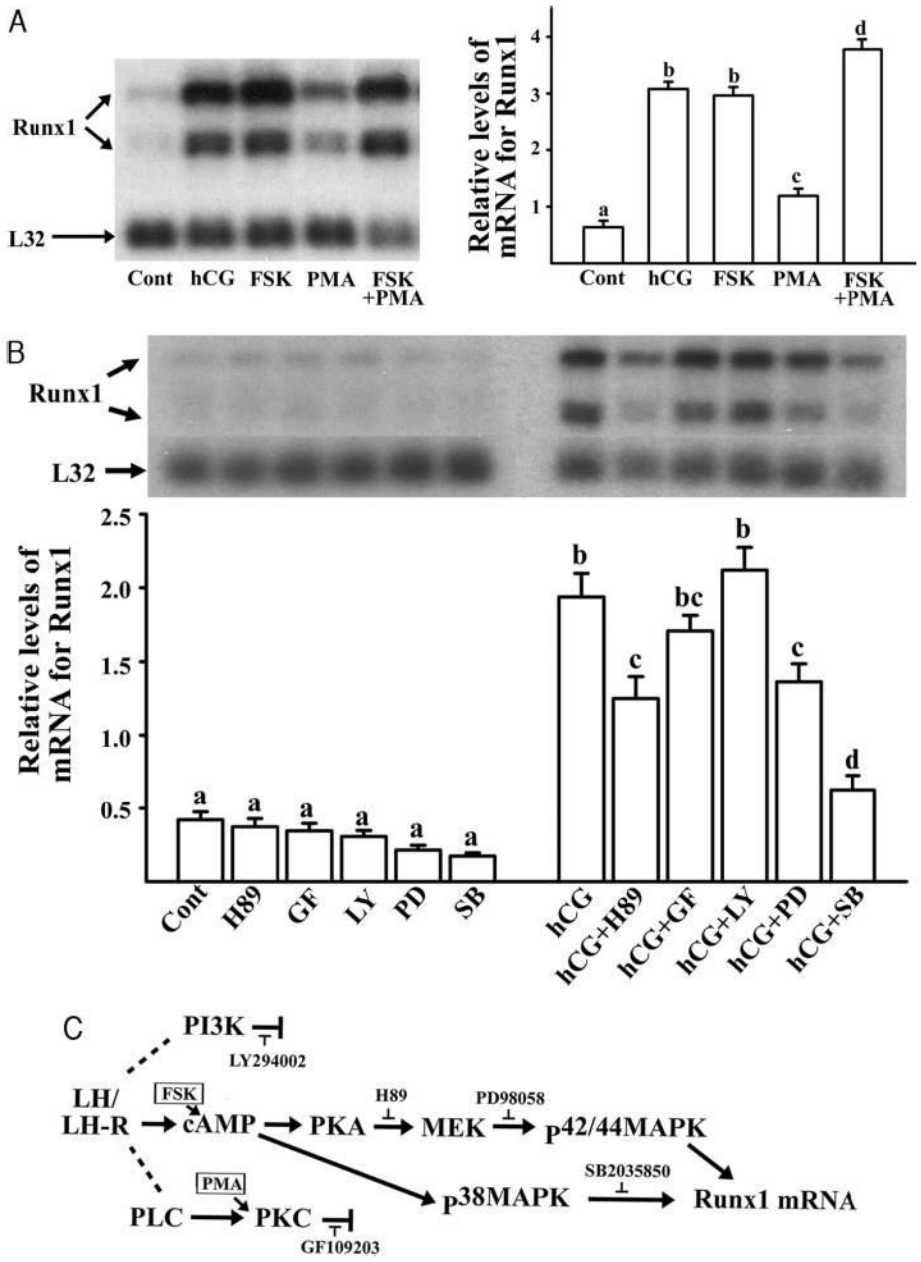

Fig. 4. Regulation of *Runx1* mRNA Expression by Agonists or Inhibitors of Various Intracellular Signaling Modulators in Granulosa Cells *in Vitro*

A, Autoradiograph of a representative Northern blot analysis shows mRNA for *Runx1* and ribosomal protein *L32* in granulosa cells from rat preovulatory ovaries (48 h post-PMSG) cultured in medium alone (Cont) or with hCG (1 IU/ml), forskolin (FSK, 10 μm), PMA (20 nm), or FSK + PMA for 6 h. The levels of *Runx1* mRNA were measured as described in Fig. 3. Relative levels of *Runx1* mRNA were normalized to the L32 band in each sample (mean ± SEM; n = 7 independent culture experiments). B, Granulosa cells from rat preovulatory ovaries (48 h post-PMSG) were cultured with medium alone (Cont), inhibitors of various signaling molecules [an inhibitor of PKA (H89, 10 μm), PKC (GF109203X[GF], 1 μm), phosphatidylinositol 3-kinase (LY294002[LY], 25 μm), MEK (PD98059[PD], 20 μm), and

p38 kinase (SB2035850[SB], 20 μm)], hCG, or hCG + inhibitors of various signaling molecules for 6 h. Levels of *Runx1* mRNA were measured by Northern blot analyses. Relative levels of *Runx1* mRNA were normalized to the *L32* band in each sample (mean ± sem; n = 4 independent culture experiments). *Bars with no common superscripts* are significantly different ($P < 0.05$). C, The experimental results are summarized in the *diagram*. The LH surge or an ovulatory dose of hCG stimulates *Runx1* mRNA expression via activating PKA, MEK, and p38 kinase. PI3K, Phosphatidylinositol 3-kinase; PLC, phospholipase C.

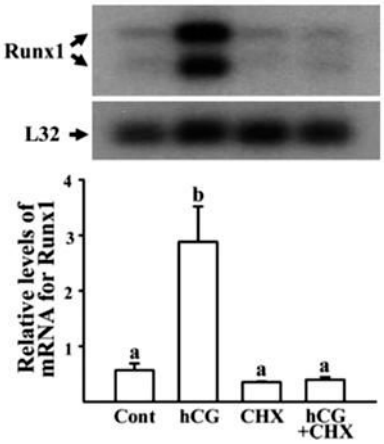

Fig. 5. Complete Inhibition of the hCG-Induced *Runx1* mRNA Accumulation by Cyclohexamide

Granulosa cells from rat preovulatory ovaries (48 h post-PMSG) were cultured in medium alone (Cont) or with cyclo-hexamide (1 μg/ml, CHX), hCG (1 IU/ml), or hCG + CHX for 6 h. Relative levels of *Runx1* mRNA were normalized to the L32 band in each sample in Northern blots (mean ± sem; n = 4). *Bars with no common superscripts* in each panel are significantly different ($P < 0.01$).

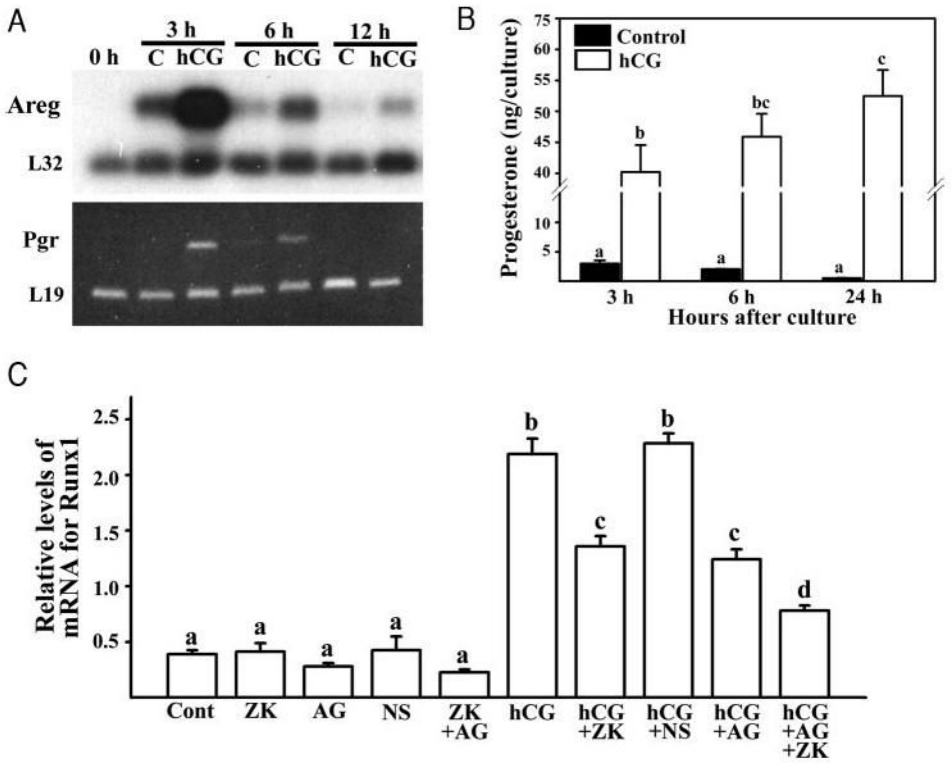

Fig. 6. Regulation of *Runx1* mRNA Expression by Hormones

A, A representative Northern blot and RT-PCR show a transient increase in levels of mRNA for *Areg* and *Pgr*, respectively. *L32* and *L19* were used as an internal control for each assay. Granulosa cells from rat preovulatory ovaries (48 h post-PMSG) were cultured in medium alone (C) or with hCG for 0, 3, 6, 12, or 24 h. B, hCG stimulates progesterone production in cultured granulosa cells. Concentrations of progesterone were measured in preovulatory granulosa cell conditioned culture media collected at 3, 6, or 24 h. *Bars with no common superscripts* are significantly different ($P < 0.05$). C, hCG-induced *Runx1* mRNA expression was reduced by a PGR antagonist (ZK98299) and an EGF receptor tyrosine kinase-selective inhibitor (AG1478), as determined by Northern blot analyses. Granulosa cells from rat preovulatory ovaries (48 h post-PMSG) were cultured in medium alone (Cont) or with NS-398 (1 μm; NS, a specific inhibitor of prostaglandin-endoperoxide synthase 2), ZK98299 (1 μm; ZK, a progesterone receptor antagonist), AG1478 (1 μm; AG, an EGF receptor tyrosine kinase-selective inhibitor), ZK + AG, hCG (1 IU/ml), hCG + NS, hCG + ZK, hCG + AG, hCG + ZK + AG for 6 h. *Bars with no common superscripts* in each panel are significantly different ($P < 0.05$).

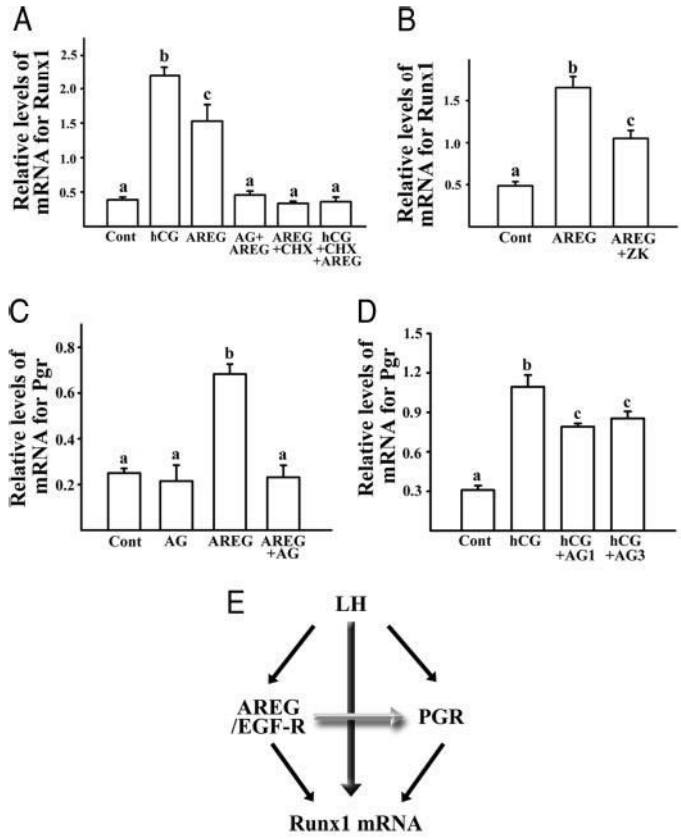

Fig. 7. Induction of *Runx1* mRNA by AREG Was Partially Mediated by the Activation and Induction of PGR

Granulosa cells from rat preovulatory ovaries (48 h post-PMSG) were cultured in medium alone (Cont) or with agonists and/or inhibitors for 6 h to detect *Runx1* mRNA and 4 h to measure the levels of *Pgr* mRNA. Concentrations of agonists and inhibitors are as follows: hCG (1 IU/ml), AREG (0.1 mg/ml), AG1478 (AG1, 1 μm; AG3, 3 μm), ZK98299 (1 μm, ZK), and cyclohexamide (1 μg/ml, CHX). The levels of *Runx1* and *Pgr* mRNA were determined by Northern blot analyses. *L32* was used as an internal control. Relative levels of mRNA for the gene were normalized to the *L32* band in each sample (mean ± sem; n = 3 or more independent culture experiments). A, The activation of EGF-receptor by AREG stimulated *Runx1* mRNA expression, and cyclohexamide blocked AREG-induced *Runx1* mRNA expression. B, The stimulatory effect of AREG on *Runx1* mRNA expression was inhibited by ZK98299. C, AREG stimulated *Pgr* mRNA expression. D, The stimulatory effect of hCG on *Pgr* mRNA expression was reduced by AG1478. *Bars with no common superscripts* in each panel are significantly different ($P < 0.05$). E, Hypothetical model of *Runx1* mRNA regulation by LH. Cont, Control; EGF-R, EGF receptor.

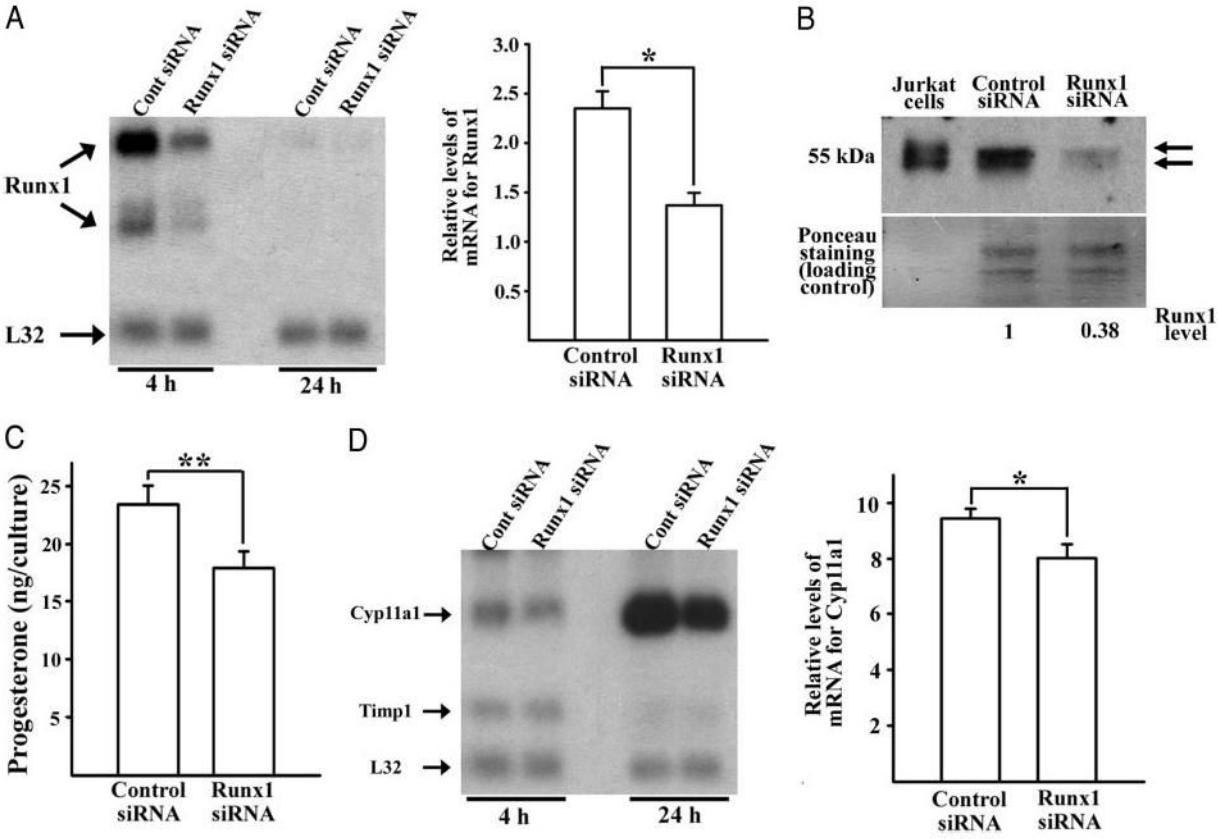

Fig. 8. Effects of Reduction in RUNX1 by *Runx1* siRNA in Cultured Granulosa Cells

Granulosa cells obtained from rat preovulatory ovaries (48 h post-PMSG) were cultured with hCG (1 IU/ml) + control siRNA (scrambled siRNA) or hCG + *Runx1* siRNA. A, Autoradiograph of a representative Northern blot shows mRNA for *Runx1* and *L32* in the granulosa cells cultured for 4 and 24 h. Relative levels of *Runx1* mRNA were normalized to the *L32* band in each sample (mean ± sem; n = 7 independent culture experiments). B, Western blot analysis of RUNX1 protein in preovulatory granulosa cells cultured with hCG (1 IU/ml) + control siRNA (scrambled siRNA) or hCG + *Runx1* siRNA for 10 h. *Arrows to the right* of panel B indicate the different forms of Runx1 detected. Protein concentrations were controlled by plating an equal number of cells per well in each treatment and then loading an equal volume (30 μl) of nuclear extract in each lane. The membrane was stained with Ponceau-S to show the relative loading of sample protein. Panel B is a representative of four separate culture experiments. C, Concentrations of progesterone in granulosa cell culture media collected at 24 h after hCG treatment (mean ± sem; n = 6 independent culture experiments). D, Autoradiograph of a representative Northern blot shows mRNA for *Cyp11a1*, *Timp1*, and *L32* in granulosa cells cultured for 4 and 24 h. Relative levels of *Cyp11a1* mRNA were normalized to the *L32* band in each sample (mean ± sem; n = 7 independent culture experiments). *, $P < 0.001$; **, $P < 0.01$. Cont, Control.

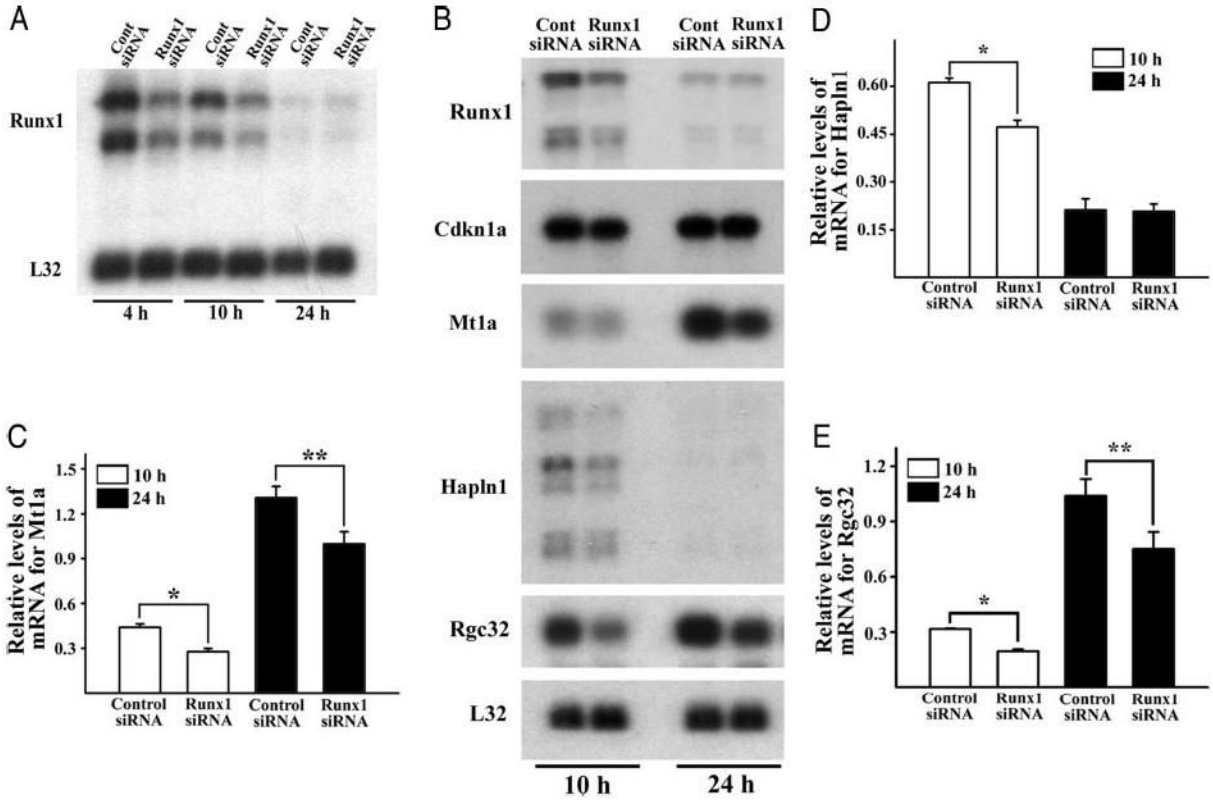

Fig. 9. Regulation of Periovulatory Gene Expression by Suppression of RUNX1 via siRNA in Cultured Granulosa Cells

Granulosa cells obtained from rat preovulatory ovaries (48 h post-PMSG) were cultured with hCG (1 IU/ml) + control siRNA (scrambled siRNA) or hCG + *Runx1* siRNA. A, Autoradiograph of a representative Northern blot shows mRNA for *Runx1* and *L32* in the granulosa cells cultured with hCG + siRNA for 4, 10, and 24 h. B, Autoradiograph of a representative Northern blot shows mRNA for *Runx1*, *Cdkn1a*, *Mt1a Hapln1*, *Rgc32*, and *L32* (internal control) in the granulosa cells cultured with hCG + siRNA for 10 and 24 h. Relative levels of *Mt1a* (C), *Hapln1* (D), and *Rgc32* (E) mRNA were normalized to the *L32* band in each sample (mean ± sem; n = 4 independent culture experiments). *, $P < 0.001$; **, $P < 0.01$. Cont, Control.

Table 1

List of Cloned cDNAs Used for Northern Blot Analyses

Gene Name	Reference (Accession no./Sequence Sites/Primer Sets, 5′–3′)	Length (bp)
Cdkn1a	U24174/124–550/AGCAAAGTATGCCGTCGTCT GGCGCTTGGAGTGATAGAAA	427
Hapln1	NM_019189.1/280–672/GTCTTCGTTTCCATGGGCTA CTCACGTGGTTTGGTGATTG	393
Mt1a	BC058442/153–370/ACCTCCTGCAAGAAGAGCTG ACATGCTCGGTAGAAAACGG	218
Rgc32	AF036548/69–486/CACGTGTCACTGAGCAACCT TGTCCAGATCAGCGATGAAG	418