Egr1 Protein Acts Downstream of Estrogen-Leukemia Inhibitory Factor (LIF)-STAT3 Pathway and Plays a Role during Implantation through Targeting Wnt4*§

Xiao-Huan Liang1, Wen-Bo Deng1, Ming Li5, Zhen-Ao Zhao5, Tong-Song Wang1, Xu-Hui Feng1, Yu-Jing Cao1, En-Kui Duan5, and Zeng-Ming Yang1†

From the 1College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China, 2College of Life Science, Xiamen University, Xiamen 361005, China, 3State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Science, Beijing 100101, China, and 4Department of Biology, Shantou University, Shantou 515063, China

Background: The downstream molecules of estrogen-LIF-STAT3 pathway during implantation are still unclear.

Results: Egr1 is regulated by estrogen through LIF-STAT3 pathway in mouse uterus and regulates decidualization by targeting Wnt4.

Conclusion: We showed Egr1 as a downstream target of LIF-STAT3 pathway and its involvement in decidualization.

Significance: Our data could be a valuable source for future study on embryo implantation.

Embryo implantation is a highly synchronized process between an activated blastocyst and a receptive uterus. Successful implantation relies on the dynamic interplay of estrogen and progesterone, but the key mediators underlying embryo implantation are not fully understood. Here we show that transcription factor early growth response 1 (Egr1) is regulated by estrogen as a downstream target through leukemia inhibitory factor (LIF) signal transducer and activator of transcription 3 (STAT3) pathway in mouse uterus. Egr1 is localized in the subluminal stromal cells surrounding the implanting embryo on day 5 of pregnancy. Estrogen rapidly, markedly, and transiently enhances Egr1 expression in uterine stromal cells, which fails in estrogen receptor α knock-out mouse uterus. STAT3 is phosphorylated by LIF and subsequently recruited on Egr1 promoter to induce its expression. Our results of Egr1 expression under induced decidualization in vivo and in vitro show that Egr1 is rapidly induced after decidualogenic stimulus. Egr1 knockdown can inhibit in vitro decidualization of cultured uterine stromal cells. Chromatin immunoprecipitation data show that Egr1 is recruited to the promoter of wingless-related murine mammary tumor virus integration site 4 (Wnt4). Collectively, our study presents for the first time that estrogen regulates Egr1 expression through LIF-STAT3 signaling pathway in mouse uterus, and Egr1 functions as a critical mediator of stromal cell decidualization by regulating Wnt4.

The effective molecular cross-talk between a functional blastocyst and the receptive endometrium is critical for successful implantation, which is tightly governed by ovarian hormones. In rodents the transient surge of estrogen in the pre-implantation period remains largely unknown.

Leukemia inhibitory factor (LIF), a member of the interleukin-6 family, has been shown to be an important downstream mediator of estrogen, which can replace nidatory estrogen to induce implantation (3, 4). LIF deficiency leads to implantation failure (5). LIF binds its transmembrane receptor, which in turn induces the phosphorylation of signal transducer and activator of transcription 3 (STAT3). Phosphorylated STAT3 can dimerize and translocate into nucleus to regulate target gene by directly binding to their promoters (6, 7). LIF antagonist is able to reduce the phosphorylation of STAT3 and abolish embryo implantation (8). LIF is not only strongly expressed in the glandular epithelium but also in the stroma surrounding the implanting blastocyst at implantation sites (9). The stromal localization of LIF is similar to phosphorylated STAT3 expression pattern (10). Conditional knock-out or transient suppression of STAT3 results in implantation failure, which suggests the critical role of STAT3 during implantation (11–14). As a pleiotropic cytokine, LIF is also essential for mouse and human decidualization (15, 16). However, the direct target gene of LIF-STAT3 pathway in mouse uterus during implantation period remains poorly understood.

Based on our RNA sequencing data, early growth response 1 (Egr1) expression is strongly expressed at the implantation site in mouse uterus (17), suggesting a potential involvement of Egr1 expression during embryo implantation. Egr1 belongs to primed endometrium, for which estrogen receptor α (ERα)2 is the primary driver of estrogen action (1). The interaction between ERα and target genes has been studied in ovariectomized mouse uterus (2). However, the molecular mechanism underlying estrogen regulation of maternal uterus during implantation period remains largely unknown.

The abbreviations used are: ERα, estrogen receptor α; Egr1, early growth response 1; LIF, leukemia inhibitory factor; STAT3, signal transducer and activator of transcription 3; Wnt4, wingless-related murine mammary tumor virus integration site 4; PPT, propyl pyrazole triol; ERαKO, ERα knock-out; Dtprp, decidual/trophoblast prolactin-related protein; HBSS, Hank’s balanced salt solution; ICI, ICI 182780; ESC, endometrial stromal cells.

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† To whom correspondence should be addressed. Tel.: 20-85282010; E-mail: zmyang@scau.edu.cn.

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the Egr transcription factor family in which Egr2, Egr3, and Egr4 are also included. Egr family has a defining feature of a highly conserved DNA binding domain composed of three zinc finger motifs that bind to the promoter of target genes (18). Egr1 is rapidly induced by growth, differentiation, and apoptotic stimuli (19–21). Gene targeting experiments in mice have revealed essential functions of Egr transcription factors in reproductive processes (20). Egr1 is the only one of the family members that causes female infertility by knocking out (22). However, the expression, regulation, and function of Egr1 in mouse uterus are largely unknown.

In this study we showed that Egr1 is strongly and specifically expressed in stromal cells under the regulation of estrogen-LIF-STAT3 signaling pathway and involved in implantation and decidualization. Egr1 knockdown inhibits in vitro decidualization progression in cultured mouse stromal cells. Egr1 action during decidualization should be mediated by wingless-related murine mammary tumor virus integration site 4 (Wnt4). Our study shows for the first time that Egr1 acts as a mediator during the progression of implantation during early pregnancy.

MATERIALS AND METHODS

Animals Treatments—All of the mice (CD-1 strain) were maintained in a controlled environment (14 h of light and 10 h of dark cycle). All animal procedures were approved by the Institutional Animal Care and Use Committee of South China Agricultural University. Female mice were mated with fertile or vasectomized males to induce pregnancy or pseudopregnancy (day 1 is the day of the vaginal plug). On days 1–4, pregnancy was confirmed by recovering embryos from the oviducts or uteri. On days 5 and 6 of pregnancy the implantation sites were identified by intravenous injection of 0.1 ml of Chicago sky blue oil/mouse; Sigma) was given to progesterone-primed mice to terminate delayed implantation on day 7. The delayed group was similar to that of normal implantation.

Delayed implantation was induced in ovariectomizing pregnant mice at 0830–0900 on day 4 of pregnancy. Mice were sacrificed at different time points after infusion to collect uteri for further analysis. All experiments were repeated at least three times.

Real-time RT-PCR—For real-time RT-PCR, mouse uterine endometrium or cultured stromal cells were harvested with TRIzol reagent (Invitrogen), and RNA was extracted according to the manufacturer’s instructions. After digesting with RNase-free DNase I (Promega), 500 ng of total RNAs were reverse-transcribed into cDNA with PrimeScript reverse transcriptase kit (TaKaRa) on the Rotor-Gene Q system. The conditions used were as follows: 95 °C for 10 s followed by 45 cycles of 95 °C for 5 s and 60 °C for 34 s. All reactions were run in triplicate. The ΔΔCt method was employed to determine relative changes of gene expression compared with Rpl7. Primer sequences used were listed in Table 1.

To induce artificial decidualization, 10 μl of sesame oil (Sigma) was infused into one uterine horn on day 4 of pseudo-pregnancy, and the non-infused contralateral horn served as the control. Mice were sacrificed at different time points after infusion to collect uteri for further analysis. All experiments were repeated at least three times.

Western Blot—A Western blot was performed as described (23). In brief, uterine endometrium or cultured stromal cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.25% sodium deoxycholate). Protein concentration was quantified using the BCA kit (Applygen). Lysates were then resolved on a 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore). After blocking with 5% skim milk (Sangon, Shanghai, China), membranes were

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**TABLE 1**

| Primer sequences used for real-time PCR | Gene/binding site | Forward and reverse primers | Product length (bp) |
|----------------------------------------|------------------|-----------------------------|---------------------|
| Egr1                                   | AGCCGAAACACCCCTGGAG | GTCCTTTGCGCTGGTAA | 102 |
| Rpl7                                   | GCAGATAGTCACCACCTTGATC | ACCTTTGCCCCTCTACCTCTGTTA | 129 |
| Dtnp1                                  | ACTGAGTAAAACTGCATTCT | TGATCCCTGACCCCTGTTA | 119 |
| LIF                                     | AAAACCATAGTCGGCCCTCAAC | GTACTAGCACCACCTGATACG | 98 |
| STAT3                                   | GGCCATTACGGGACCAAAAG | GCTTGGCTCCACTAGTGGTTT | 113 |
| Wnt4                                   | TGCTAGTGGCGGTGGTTT | CTCCCTACCTGCGTGCATTG | 128 |
| Egr2                                   | GCCGAAACACCCCTGGAG | GTCCTTTGCGCTGGTAA | 144 |
| Egr3                                   | GGCAGAAGACGCGTGGAC | TGAGAGGCTGCCAACTGAG | 168 |
| Egr4                                   | GGCAGAAGACGCGTGGAC | TGAGAGGCTGCCAACTGAG | 138 |
| Egr1 promoter, –214 site                | GGGGAGGAGCTGGGAACTCCA | TGAGAGGCTGCCAACTGAG | 145 |
| Egr1 promoter, –1561 site               | CCCATCTGCTGCTGCTCCTCG | CTTAGCCCTGCCACCTGAGA | 193 |
| Wnt4 promoter, –250 site                | CTGACCTGCTGCTGCTGCTG | CTGACCTGCTGCTGCTGCTG | 94 |
| Wnt4 promoter, –735 site                | CTGACCTGCTGCTGCTGCTG | CTGACCTGCTGCTGCTGCTG | 81 |
| Wnt4 promoter, –1107 site               | CTGACCTGCTGCTGCTGCTG | CTGACCTGCTGCTGCTGCTG | 192 |
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probed with the corresponding antibodies for Egr1 (Cell signaling), phospho-STAT3 (Cell Signaling), total STAT3 (Cell Signaling), tubulin (Cell Signaling), Gapdh (Santa Cruz), and β-actin (Santa Cruz). Membranes were then incubated with the matched secondary antibodies conjugated with HRP (1:5000). Signals were detected with the ECL kit (Pierce).

*In Situ Hybridization*—Total RNAs from the mouse embryos on day 8 of pregnancy were reverse-transcribed and amplified with primers for mouse Egr1 (549–650 bp, GenBank™ accession number NM_007913). Primers were designed according to Web-based primer design program Primer 3. The amplified PCR fragment was recovered from the agarose gel, cloned into pGEM-T plasmid, and verified by sequencing. The plasmids were amplified with the primers for T7 and SP6 to prepare the templates for labeling antisense and sense probes. Digoxigenin-labeled cRNA probes were transcribed *in vitro* using a digoxigenin RNA labeling kit (Roche Applied Science).

*In situ* hybridization was performed as described previously (24). Frozen sections (10 μm) of uterus were mounted on 3-aminopropyltriethoxysilane (Sigma)-coated slides and fixed in 4% paraformaldehyde solution in PBS. Digoxigenin-labeled Egr1 sense probe was used to replace Egr1 antisense probe for negative control. Endogenous alkaline phosphatase activity was inhibited with 2 mM levamisole (Sigma). Membranes were then incubated with the secondary antibody for 1 h followed by a counterstain with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI). Fluorescence was observed under a fluorescence microscope (Leica). Negative control slides were immunolabeled with an equal concentration of anti-GFP antibody (Santa Cruz).

*Isolation of Mouse Uterine Stromal Cells and in Vitro Decidualization*—Stromal cells were isolated as previously described (26). Briefly, uterine horns were split longitudinally, washed with Hanks’ balanced salt solution (HBSS; Sigma), and digested with 1% (w/v) trypsin (Amresco) and 6 mg/ml dispase (Roche Applied Science) in HBSS for 1 h at 4 °C followed by 1 h at room temperature and 10 min at 37 °C. After rinsing 3 times with HBSS, the remaining tissues were incubated in 2 ml of HBSS containing 0.15 mg/ml collagenase I (Invitrogen) at 37 °C for 30 min. The digested uteri were shaken and filtered through a 70-μm wire gauze filter and centrifuged. The isolated cells were grown in DMEM/F-12 medium containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen). After an initial culture for 30 min, the medium was changed to remove unattached cells. The purity of stromal cells was verified with anti-vimentin (a marker for stromal cells) and anti-cytokeratin (a marker for epithelial cells), respectively. Immunofluorescence showed that the stromal cells we isolated were positive for vimentin and negative for cytokeratin (*supplemental Fig.* 1).

To perform LIF treatment, cells were plated in DMEM/F-12 with 10% FBS and allowed to attach overnight. Then the cells were cultured in serum-free DMEM/F12 overnight to reduce Egr1 expression. Cells were treated with 10 ng/ml LIF (Sigma) for different periods and collected with sterile PBS for further analysis. To block estrogen biosynthesis, the cells were starved and treated with letrozole for 24 h. Letrozole-treated cells were then treated with LIF for 3 h.

*In vitro* decidualization of endometrial stromal cells from day 4 pregnant uteri was performed as previously described (27). Briefly, uterine stromal cells isolated on day 4 of pregnancy were induced for *in vitro* decidualization with the medium supplemented with progesterone (1 μM) and estrogen (10 nM) in DMEM/F-12 containing 2% charcoal-treated FBS (Biological Industries). The control group received no hormonal supplementation.

*Transfections*—Transfection for STAT3 or Egr1 small interfering RNA (siRNA) was performed according to Lipo-
fectamine 2000 protocol (Invitrogen). siRNAs specific for STAT3 or Egr1 were purchased from Ribobio Co., Ltd. (Guangzhou, China). Briefly, for the 35-mm culture dish, 100 pmol of siRNA and 5 μl of Lipofectamine 2000 were diluted in 250 μl of Opti-MEM (Invitrogen). After 5 min of incubation, the diluted siRNA was mixed with diluted Lipofectamine 2000 and incubated for 20 min. The siRNA-Lipofectamine 2000 mixture was added onto cultured stromal cells and cultured for 6 h. STAT3 plasmid with continuous activation, which could activate transcription without induced tyrosine phosphorylation (28), was purchased from Addgene. According to manufacturer’s instructions, 4 μg of DNA and 10 μl of Lipofectamine 2000 were used for each 35-mm dish. Transfected cells were harvested for real-time RT-PCR or Western blot.

**Chromatin Immunoprecipitation (ChIP)**—Samples used for ChIP were either uterine endometrium from day 5 implantation sites or cultured mouse uterine stromal cells treated with LIF for 30 min. ChIP was conducted as previously described with modification (29). The endometrium of day 5 implantation sites was squeezed out using a bent syringe needle. LIF treatment of stromal cells was performed as described above. Uterine endometrium or stromal cells were washed once with PBS and placed in 10 ml of PBS containing 1% formaldehyde at room temperature for 10 min to covalently cross-link any DNA-protein complexes. The cross-link was terminated by adding 570 μl of 2.5 M glycine. The cells were washed 3 times with PBS and harvested using a cell scraper. The endometrium was homogenized with a glass homogenizer. After incubated in

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**FIGURE 2.** Expression levels of Egr family members in mouse uterine endometrium during peri-implantation. A, real-time RT-PCR analysis of Egr1 mRNA expression levels in mouse uterine endometrium on days 4 – 6 of early pregnancy. The data were normalized to Rpl7. Statistical analysis was performed with GraphPad Prism software, and a p value lower than 0.05 was considered statistically significant. The mRNA expression of Egr2 and Egr3 are shown in B and C. D, mRNA quantification of Egr1, Egr2, and Egr3 in the endometrium of implantation sites on day 4.5 of pregnancy. E, Egr1 protein levels in endometrium of days 4 – 6 were determined by Western blot. Gapdh served as a loading control. F, effects of progesterone on Egr1 mRNA expression in the uteri of ovariectomized mice. The data shown in this figure are representative of three or more independent experiments. Imp, implantation site; NI, inter-implantation site. *, p < 0.05.
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Mg-NI buffer (15 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 60 mM KCl, 0.5 mM DTT, 15 mM NaCl, and 300 mM sucrose) and Mg-NI-Nonidet P-40 buffer (Mg-NI buffer with 1% Nonidet P-40), cell pellets were resuspended in lysis buffer (1% SDS, 50 mM Tris-HCl, pH 8.0, and 0.5 mM EDTA) and sonicated by repeating a program (15 s on and 30 s off at 45% amplitude) 8 times using the Sonic Vibra Cell (Sonics & Materials). After the samples were centrifuged at 13,000 rpm for 10 min to pellet cell debris, the soluble chromatin was harvested. Immunoprecipitation was prepared with IgG (Cell signaling), anti-STAT3 (Santa Cruz), anti-Egr1 (Santa Cruz), or anti-AcH3 (Millipore) at 4 °C overnight. Protein A (GE) slurry was added to pull down the DNA-protein complexes. Relative levels of recruitment at different binding sites of gene promoter were determined by real-time RT-PCR and normalized to input DNA. Primer sets used for real-time RT-PCR are listed in Table 1.

RESULTS

Localization of Egr1 Expression in Mouse Uterus during Early Pregnancy—Our previous study showed that Egr1 was up-regulated at implantation sites in mouse uterus (31). We performed in situ hybridization to localize Egr1 mRNA expression in mouse uteri from days 1 to 8 of pregnancy. Egr1 mRNA signals were undetectable in the uteri from days 1–4 (data not shown). On days 4.5 and 5 of pregnancy, there was a high level of Egr1 mRNA expression in the stroma surrounding the implanting blastocyst. No Egr1 mRNA signal was observed at interimplantation sites on both days 4.5 and 5. From day 6, the Egr1 mRNA expression declined to a basal level (Fig. 1A). When Egr1 sense probe was used for hybridization, there was no detectable signal (Fig. 1A).

Immunofluorescence showed that Egr1 protein was detected in the subluminal stroma at implantation sites on day 5 of pregnancy, which confirmed the localization of Egr1 (Fig. 1B). There was no detectable signal when sections were incubated with anti-GFP antibody.

Using the delayed and activated implantation model, we found that there was no detectable Egr1 mRNA expression in mouse uterus under delayed implantation. When delayed implantation was terminated by estrogen, a high level of Egr1 mRNA was detected in the subluminal stromal cells surrounding the implanting embryo (Fig. 1C).

Expression of Egr Family Members in Mouse Uteri during the Peri-implantation Period—Real-time RT-PCR was performed to quantify mRNA expression level of Egr family members. Egr1, Egr2, and Egr3 mRNA levels were markedly higher in the endometrium of implantation sites than that of interimplantation sites on both days 4.5 and 5 of pregnancy (Fig. 2, A–C). The mRNA level of Egr4 was too low to be detected. Quantifications were then performed to determine the absolute copy numbers of Egr1, Egr2, and Egr3 in mouse uterine endometrium of implantation sites on day 4.5 of pregnancy. Egr1 mRNA showed the most abundant expression (Fig. 2D). Using Western blots, we confirmed the high level of Egr1 protein expression in the endometrium at implantation sites on days 4.5 and 5 of pregnancy (Fig. 2E). These results confirmed the up-regulation of Egr1 expression at implantation sites.

The Regulation of Estrogen on LIF-STAT3 Pathway Is ERα-dependent—Estrogen and progesterone are the two main hormones orchestrating the process of implantation. Progesterone treatment performed in ovariecromized mice suggested that progesterone was unable to induce uterine Egr1 expression (Fig. 2F), so we focused on estrogen in the following study. LIF is an essential mediator of estrogen and can replace nidatory estrogen in the activation of delayed implantation (4). LIF activates the phosphorylation of transcription factor STAT3,
which is also indispensable for implantation (12). As a previous study has shown two potential STAT3 binding sites in Egr1 promoter (31), we hypothesized that estrogen might control the expression of Egr1 through LIF-STAT3 pathway.

To test this hypothesis, we first investigated the effects of estrogen on LIF and STAT3 expression. Our results showed that the mRNA levels of both LIF and STAT3 were up-regulated by estrogen in ovariectomized mouse uteri at 3 h after estrogen administration (Fig. 3, A and B). We then used ICI 182780 (ICI), an ER antagonist, to determine if the effects of estrogen were ER-dependent. ICI pretreatment dramatically abrogated the estrogen-induced expression of LIF and STAT3 mRNA (Fig. 3, A and B).

Previous studies on ERα and ERβ knock-out mice demonstrated the essential role of ERα during embryo implantation but not ERβ (32). The primary role of ERα subtype in Egr1 up-regulation was further confirmed by the use of selective ERα agonist PPT. Our studies with PPT treatment further confirmed the ERα regulation on LIF and STAT3 mRNA expression (Fig. 3, C and D). LIF mRNA was strongly induced by PPT treatment for 3 h. In parallel, STAT3 mRNA expression was induced in mouse uterus after PPT injection and peaked at 3 h.

We then examined the effects of estrogen on LIF and STAT3 in WT and ERαKO mouse uteri. In the WT samples, LIF and STAT3 mRNA levels remained elevated 3 h after estrogen injection. However, the expression of LIF and STAT3 was not increased in uterine samples of ERαKO mice (Fig. 3, E and F). To further test whether STAT3 signaling pathway was activated, Western blot was performed to examine STAT3 phosphorylation in WT and ERαKO mouse uteri after estrogen treatment. The expression level of phospho-STAT3 was significantly elevated in samples from WT group, whereas the increase of phospho-STAT3 was not detected in the ERαKO group after estrogen treatment (Fig. 3, G).

Together, these studies reveal that estrogen could activate the LIF-STAT3 pathway in mouse uterus in an ERα-dependent manner.
Regulation of Estrogen on Egr1 Expression—In ovariectomized mouse uterus, estrogen stimulated a marked and rapid increase in Egr1 mRNA and protein expression (Fig. 4, A and B). The elevated Egr1 expression peaked at 3 h after estrogen treatment and then declined. In situ hybridization showed that the enhanced Egr1 mRNA expression was localized in uterine subluminal stromal compartments (Fig. 4 C). ICI pretreatment dramatically inhibited the estrogen-induced expression of Egr1 mRNA and protein (Fig. 4, D and E), indicating that the regulation was mediated through the ER.

PPT, an ERα-selective agonist ligand, did stimulate an up-regulation of Egr1 expression in ovariectomized mouse uteri (Fig. 4, F and G). The obvious up-regulations of both Egr1 mRNA and protein were observed at 3 h after PPT administration. To further confirm the role of ERα, we assessed estrogen regulation on Egr1 in ovariectomized ERα knock-out (ERαKO) mice and WT littermates. Egr1 expression displayed no obvious change in the uterine samples from ERαKO mice but significantly induced in littermates (Fig. 4, H and I). In situ hybridization showed that the enhanced Egr1 mRNA expression was localized in uterine subluminal stromal compartments (Fig. 4 C). ICI pretreatment dramatically inhibited the estrogen-induced expression of Egr1 mRNA and protein (Fig. 4, D and E), indicating that the regulation was mediated through the ER.

FIGURE 5. Expression of Egr1 is elevated in stromal cells by LIF. Cultured stromal cells of day 4 pregnancy were starved with serum-free culture medium overnight, then cultured in the absence (control) or presence of recombinant LIF (10 ng/ml). A, Egr1 mRNA was significantly induced at 30 min and peaked at 1 h after LIF treatment. *, p < 0.05. B, total protein lysates of ESC treated as above were used for Western blot analysis and immunoprobe for Egr1 and phospho-STAT3. C, Egr1 protein localization in endometrial stromal cells cultured in the presence or absence of LIF for 30 min. The intensity of Egr1 (green channel) and DAPI nuclear staining (blue channel) were captured by fluorescence microscopy. Egr1 protein is transported into the nucleus after treatment with LIF.

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FIGURE 6. Egr1 is directly regulated by STAT3 in stromal cells during implantation. A, STAT3 mRNA levels were measured by real-time RT-PCR in ESC transfected with either negative control or STAT3 siRNA for 24 h. B, Egr1 mRNA expression in STAT3 siRNA-transfected ESC. C, protein lysates extracted from cells transfected as above were used for Western blot analysis and immunoprobe with STAT3 and Egr1 antibodies. D, primary mouse ESC were transfected with a constitutively dimerizable STAT3 (STAT3.C) plasmid for 24 h and used for Western blots. Chromatin extracted from ESC treated with LIF for 30 min was immunoprecipitated with antibodies against STAT3, AcH3, and IgG. E, relative levels of the proximal STAT3 recruitment site were determined by real-time RT-PCR and normalized to input DNA. F, relative levels of the STAT3 proximal recruitment site in endometrium of day 5. NC, negative control. *, p < 0.05.
tion also showed that there was no difference for Egr1 mRNA expression between oil and estrogen group in ER\textsubscript{α} KO uteri (Fig. 4J). The level of Egr1 mRNA and protein levels was also increased after delayed implantation was activated by estrogen (Fig. 4, K and L). Our data indicated that ER\textsubscript{α} mediated the estrogen action on Egr1 induction.

**LIF Regulation on Egr1 Expression in Cultured Uterine Stromal Cells**—Mouse endometrial stromal cells (ESC) were treated with LIF to determine whether LIF could regulate Egr1 expression. Cultured ESC isolated on day 4 of pregnancy were starved in serum-free culture medium overnight and then treated with LIF for various time courses. Our real-time RT-PCR confirmed that Egr1 mRNA expression was highly induced by LIF, maximizing at 1 h after LIF treatment. Then Egr1 mRNA expression returned to near background level 3 h post-LIF treatment (Fig. 5A). Western blots confirmed that Egr1 protein was markedly induced at 30 min and 1 h after LIF treatment (Fig. 5B). We noticed that the induction of phospho-STAT3 began as early as 10 min and declined to a basal level at 1 h after LIF administration, indicating a potential role of STAT3 on Egr1 expression. As nucleus localization is prerequisite for the function of transcription factor, we examined whether LIF treatment could cause Egr1 translocation from cytoplasm to nucleus. Egr1 were obviously enriched in nuclei after LIF treatment (Fig. 5C), which provides further evidence that LIF plays an integral role in induction of Egr1 signaling.

**STAT3 Regulation on Egr1 in Stromal Cells**—Because phospho-STAT3 and Egr1 share a similar expression pattern in the subluminal stroma at day 5 implantation sites (10), we examined whether STAT3 could regulate Egr1 expression in uterine stromal cells. After STAT3 expression was down-regulated by STAT3 siRNA in the stromal cells, the expression of both Egr1 mRNA and protein was remarkably reduced compared with negative control (Fig. 6, A–C). When stromal cells were transfected with a consistently activated STAT3 plasmid, which is constitutively dimerized independently of tyrosine kinase (28), both STAT3 and Egr1 protein levels were significantly increased after STAT3 treatment (Fig. 6, D and E).

**FIGURE 7.** Egr1 is up-regulated upon decidualization. A, expression of Egr1 in oil-induced decidualization in vivo. Uterine levels of Egr1 protein were measured by Western blot at various times after stimulus. B, expression of Egr1 in mouse ESC decidualized in vitro. Cultured mouse ESC were either untreated or decidualized with Estradiol-17β and progesterone for the indicated time points. Egr1 expressions at protein levels were determined in parallel cultures by Western blot analysis. C, the real-time RT-PCR data show the -fold change in Dtprp transcript levels upon LIF treatment relatively to expression in control cells. D, the Dtprp mRNA level was down-regulated by STAT3 siRNA transfection. NC, negative control. E, Egr1 silence attenuated the hormones induction on Dtprp mRNA expression. F, effects of letrozole alone or in combination with LIF on the expression of Egr1 in stromal cells. AD, artificial decidualization. EP, estrogen plus progesterone. letro, letrozole. *, p < 0.05.
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(A) [Image showing Egr1 and Wnt4 expression]

(B) [Graph showing relative Wnt4 mRNA level for WT and ERαKO mice with Oil and E2 treatment]

(C) [Graph showing relative mRNA levels for Egr1 and Wnt4 with NC and siEgr1 treatment]

(D) [Diagram showing Wnt4 promoter sites 1, 2, and 3]

(E) [Bar graph showing % of input DNA of IP for IgG, Egr1, and AcH3 at site 1]

(F) [Bar graph showing % of input DNA of IP for IgG, Egr1, and AcH3 at site 2]

(G) [Bar graph showing % of input DNA of IP for IgG, Egr1, and AcH3 at site 3]

(H) [Bar graph showing relative Dppr mRNA level for NC, siWnt4, EP+NC, and EP+siWnt4]
enhanced (Fig. 6D). These gain- and loss-of-function data suggested that the expression of Egr1 is regulated by STAT3.

**STAT3 Recruitment in Egr1 Promoter—**Two STAT3 binding sites have been identified in the Egr1 promoter (31). In this study ChIP was performed to determine whether STAT3 was recruited to Egr1 promoter in response to LIF. In cultured stromal cells, there was more STAT3 binding recruited at the proximal −214-bp site of Egr1 promoter than that of the distal −1561-bp site after LIF treatment (Fig. 6, E and F). Then ChIP was performed with uterine endometrium from implantation sites on day 5 of pregnancy. STAT3 binding was significantly enriched at both the −214- and −1561-bp sites of the Egr1 promoter in day 5 endometrium (Fig. 6, G and H). These results confirmed the importance of STAT3 in Egr1 regulation during embryo implantation, indicating that LIF induces Egr1 expression through STAT3.

**LIF-STAT3-Egr1 Signaling Pathway Plays a Critical Role in Decidualization—**The decidual reaction can be induced experimentally by intrauterine injection of sesame oil on day 4 of pseudopregnancy in vivo (33, 34) or in cultured stromal cells with ovarian steroids (27, 35). After day 4 pseudopregnant mice were induced for artificial decidualization by injecting sesame oil into uterine lumen, Egr1 protein expression increased at 45 min and 2 h after oil injection and returned to a baseline level by 4 h (Fig. 7A). After stromal cells were induced for in vitro decidualization, the level of Egr1 protein had a rapid and obvious increase at 2 h and 4 h (Fig. 7B).

It has been reported that LIF can enhance decidualization of endometrial stromal cells in humans (16). In vivo experiments showed that polyethylene glycol (PEG)-conjugated LIF antagonist treatment reduced the decidual area of implantation site (15). To further examine the role of LIF during decidualization, stromal cells were treated with LIF. Dttp expression in stromal cells under in vitro decidualization was significantly increased by LIF, whereas LIF alone had no effects on Dttp expression in stromal cells (Fig. 7C).

To further explore the involvement of LIF-STAT3-Egr1 pathway in decidualization, we knocked down STAT3 and Egr1 expression with their siRNAs and examined the expression of Dttp, a decidualization marker during in vitro decidualization. Under in vitro decidualization, Dttp expression was significantly attenuated by STAT3 siRNA (Fig. 7D). Furthermore, Dttp expression was also inhibited by Egr1 siRNA (Fig. 7E). These observations indicated that the suppression of STAT3 and Egr1 perturbs the process of decidualization.

To study whether the LIF-STAT3 pathway acts downstream to mediate estrogen stimulation of Egr1 in a cell-autonomous manner, we treated stromal cells with luteozone (an aromatase inhibitor) to block estrogen effects. The results showed that Egr1 mRNA expression was significantly reduced in luteozone-treated cells (Fig. 7F). To further confirm the LIF-STAT3 involvement in estrogen effects, stromal cells were co-treated with both luteozone and LIF. LIF could rescue Egr1 expression compared with the luteozone-treated cells. These results further indicated the involvement of LIF-STAT3 pathway in estrogen regulation on Egr1 expression in stromal cells.

**Egr1 Regulates Decidualization via Wnt4—**In pursuing the molecular mechanisms through which Egr1 impacts decidualization, we noticed that the mRNA expression pattern of Egr1 and Wnt4 was similar at implantation site from previous studies (36, 37). Therefore, we performed in situ hybridization to examine whether the expression of these two molecules overlapped. As shown in Fig. 8A, the expression of both Egr1 and Wnt4 was identified in subluminal stromal cells surrounding the embryo in day 5 uteri. The expression level of Wnt4 was also elevated in samples from WT group, whereas no increase was detected in EραKO group after estrogen treatment (Fig. 8B). These results suggest the potential regulation of Egr1 on Wnt4 expression.

To test whether Egr1 regulates Wnt4 expression, we determined Wnt4 mRNA expression after Egr1 siRNA transfection and observed a significant decrease of Wnt4 mRNA level (Fig. 8C). To identify the mechanism of how Egr1 regulates Wnt4 expression, we performed an in silico analysis of Wnt4 promoters and found three potential Egr1 binding sites within the 1-kb 5’-flanking region to the transcription start site (Fig. 8D). To examine whether Egr1 directly binds to Wnt4 promoter, we performed ChIP with the endometrium of day 5 implantation sites. ChiP-quantitative PCR analysis revealed that the −250-, −735-, and −1107-bp binding sites were all enriched using the anti-Egr1 antibody (Fig. 8, E–G). These results support our hypothesis that Egr1 mediates Wnt4 expression directly. To further study the physiological significance of Wnt4, we transfected stromal cells with Wnt4 siRNA under in vitro decidualization and examined Dttp expression. The level of Dttp mRNA expression was significantly suppressed by silencing Wnt4 expression (Fig. 8H). Collectively, these results suggest that Egr1 may mediate decidualization by regulating Wnt4.

**DISCUSSION**

**Estrogen Is a Key Regulator of Egr1 Expression in Uterine Stromal Cells through the LIF-STAT3 Pathway—**Egr1 can be induced by estrogen in several cell lines, especially estrogen-related cancer cell lines, such as MCF-7 and Ishikawa cells (38). Our study showed the rapid and transient inductions of Egr1 by both estrogen and the Eρα agonist PPT. Using EραKO mice, we demonstrated the estrogen regulation on Egr1 in mouse uterus in an Eρα-dependent manner. As Eρα is the main functional subtype of ER in mouse uterus, we propose that the Eρα subtype is the primary effector on Egr1 induction.

LIF is a critical downstream target of estrogen with induction by estrogen treatment for 1–2 h in the WT mouse uterus (39). A previous study reported that LIF stimulates Egr1 expression in human endometrial stromal cells (40). In our study, we verified LIF stimulation on Egr1 expression in stromal cells but not in epithelial cells. In fact, LIF stimulation on Egr1 expression in epithelial cells was not observed in a recent study (41).

**FIGURE 8.** Egr1 regulation on Wnt4. A, expression of Egr1 and Wnt4 mRNA at implantation sites on day 5 of early pregnancy. B, estrogen regulation on Wnt4 mRNA in EραKO mouse uterus. C, mRNA expressions of Egr1 and Wnt4 were measured by real-time RT-PCR in ESC transfected with either NC or Egr1 siRNA for 24 h. NC, negative control. D, schematic diagram of Egr1 binding sites in Wnt4 promoter. ChiP assays were performed using anti-Egr1, anti-Ach3, and rabbit IgG. Real-time RT-PCR was used to amplify specific regions of the relative promoters. TSS, transcription start site. E, ChiP analysis for the recruitment of Egr1 onto the −250-bp site of Wnt4 promoter. F, ChiP analysis for the recruitment of Egr1 onto the −735-bp site of Wnt4 promoter. G, ChiP analysis for the recruitment of Egr1 onto the −1107-bp site of Wnt4 promoter. H, the Dttp mRNA level was down-regulated by Wnt4 siRNA transfection. *, p < 0.05.
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SYF cells (40). Our findings indicated estrogen regulation on Egr1 is mediated by LIF in uterine stromal cells.

LIF can activate the phosphorylation of STAT3 (6, 7). Our experiments in cultured stromal cells indicated that LIF can induce the phosphorylation of STAT3 in mouse uterine stromal cells followed by a subsequent induction of Egr1. It is important to note that, similar to LIF and Egr1, the phospho-STAT3 protein is also localized in the subluminal stromal surrounding the implanting blastocyst (10). It is reasonable to suppose that there is cross-talk between Egr1 and STAT3.

With loss- and gain-of-function experiments of STAT3 performed in cultured mouse uterine stromal cells, we verified the down-regulation and up-regulation on Egr1 expression, respectively. Two STAT3 binding sites conserved on both mice and humans have been identified in the Egr1 promoter (31). Our ChIP results also demonstrated that STAT3 is recruited to both sites at −214 and −1561 bp sites in Egr1 promoter in day 5 uterine endometrium or the LIF-treated stromal cells. Therefore, our studies establish a novel regulatory pathway in which estrogen regulates Egr1 expression through LIF and STAT3 in mouse uterus.

Estrogen-LIF-STAT3-Egr1 Pathway Plays Critical Roles during Decidualization—As the blastocyst implants, the stromal cells undergo extensive proliferation, differentiation, and remodeling to form the decidua, which is critical for embryo implantation and survival of the conceptus.

As a pleiotropic cytokine, LIF effects on decidualization have been studied in mouse and human endometrial stromal cells (15, 16). It was also observed that decidualization failed to be induced in LIF knock-out mice (41). Moreover, PEG-conjugated LIF antagonist (PEGLA) treatment reduces decidual area and desmin staining intensity in mouse uterus on day 6 of pregnancy (15). These observations suggest that LIF signaling plays an important role during decidualization. Under in vitro decidualization, we observed that LIF can augment the magnitude of decidualization. These results revealed that LIF is important for decidualization.

In mouse uterus on day 5 of pregnancy, besides the glandular epithelium, LIF is also expressed in subluminal stromal cells surrounding the blastocyst (9). Our data showed that Egr1 is expressed in a similar pattern to LIF in the subluminal stromal cells immediately surrounding the implanting blastocyst on day 5.

Because STAT3 and Egr1 are downstream of LIF signaling, they are likely to involve in the process of decidualization. Previous studies showed STAT3 is specifically activated during mouse and rat embryo implantation (10, 42) and in human endometrial stromal cells during decidualization (43). Recent studies characterized STAT3 as a regulator of differentiation in human stromal cell decidualization (44). Our studies in the cultured stromal cells showed that silencing either STAT3 or Egr1 attenuated in vitro decidualization, indicating their important roles in decidualization.

Our ChIP data showed that Egr1 is significantly enriched in Wnt4 promoter during decidualization. The level of Wnt4 is decreased significantly when Egr1 is knocked down. During early pregnancy, Wnt4 is expressed in the subluminal stromal cells at the time of implantation and becomes robust during decidualization (36, 37, 45). Wnt4 has been suggested to be important for stromal cell decidualization (27). The artificial decidual response is reduced upon uterine conditional ablation of Wnt4 (37). We also showed that Dtprp expression is inhibited after Wnt4 expression is silenced. Previous papers reported the estrogen regulation on Wnt4 in mouse uterus (30, 36, 45). This raises the possibility that LIF-STAT3-Egr1 mediates the expression of Wnt4 in response to estrogen.

In summary, Egr1 is identified as a downstream target of estrogen-induced LIF-STAT3 pathway in mouse uterus. Egr1 may regulate decidualization via Wnt4. Dtprp expression is significantly decreased when Egr1 is knocked down. Because Egr1 knock-out female mice are infertile (20), it is impossible to examine the action of Egr1 during early pregnancy. Uterus-specific Cre-LoxP mice should be required for further analysis on Egr1 role during decidualization. These new results have important implications for understanding the mechanism of embryo implantation.

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