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Early growth response 1 transcriptionally primes the human endometrial stromal cell for decidualization

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Abstract

Mouse studies support a role for endometrial early growth response 1 (EGR1) in uterine receptivity and decidualization, which are processes controlled by estrogen and progesterone. However, the importance of this transcription factor in similar cellular processes in human is unclear. Analysis of clinical samples indicate that endometrial EGR1 levels are decreased in the endometrium of women with recurrent implantation failure, suggesting tight control of EGR1 levels are necessary for normal endometrial function. Therefore, we used siRNA-mediated knockdown of *EGR1* expression in cultured human endometrial stromal cells (hESCs) to assess the functional role of EGR1 in hESC decidualization. Protein expression studies revealed that EGR1 is highly expressed in pre-decidual hESCs. However, EGR1 protein levels rapidly decrease following administration of an established deciduogenic hormone stimulus containing estradiol, medroxyprogesterone acetate, and cyclic adenosine monophosphate. Intriguingly, *EGR1* knockdown in pre-decidual hESCs blocks the ability of these cells to decidualize later, indicating that EGR1 is required to transcriptionally program pre-decidual hESCs for decidualization. Support for this proposal comes from the analysis of transcriptome and cistrome datasets, which shows that EGR1 target genes are primarily involved in transcriptional regulation, cell signaling, and proliferation. Collectively, our studies provide translational support for an evolutionary

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conserved role for human endometrial stromal EGR1 in the early events of pregnancy establishment.

Keywords

Early growth response 1; human endometrial stromal cells; decidualization; RNA-sequencing; ChIP-sequencing; pregnancy

1. Introduction

As an immediate early response transcription factor to a myriad of external stimuli, early growth response 1 (EGR1) modulates transcriptional programs obligate for proliferation, differentiation, and programmed cell death within a broad spectrum of physiological and pathophysiological contexts [1]. A member of the evolutionary conserved EGR family of Cys2-His2-type zinc finger transcription factors (containing also EGR2, EGR3, and EGR4), EGR1 directly controls gene transcription of target genes through binding GC-rich DNA motifs *via* its three zinc finger DNA binding domains. Because EGR1 is required for the expression of the luteinizing hormone beta subunit in the gonadotropes of the anterior pituitary gland, ablation of *Egr1* in the female mouse causes infertility primarily due to impairments in ovulation and luteinization [2, 3]. In the case of the uterus, recent studies revealed that Egr1 is induced by estrogen in the endometrial luminal epithelium and stroma during early pregnancy [4–7]. Mouse studies also showed that luminal epithelial Egr1 is essential for uterine receptivity [7] and that Egr1 in pre-decidual stromal cells in the subluminal region is necessary for complete decidualization in response to an artificial decidualogenic stimulus [4, 5, 7].

At the RNA level, we recently demonstrated that *EGR1* is expressed in pre-decidual human endometrial stromal cells (hESCs) in culture; however, *EGR1* transcript levels significantly decline in response to an established hormone decidualogenic stimulus [8]. While these findings suggest that downregulation of EGR1 expression is a prerequisite for decidualization, the role of EGR1 in pre-decidual hESCs remained an open question. Intriguingly, we also found in a previously published dataset that *EGR1* transcript levels are decreased in the endometrium of women with recurrent implantation failure [9]. Therefore, we used RNA interference of *EGR1* in cultured hESCs and found that abrogation of EGR1 in pre-decidual hESCs impairs their ability to decidualize when exposed to a hormone decidualogenic stimulus. Furthermore, application of RNA-sequencing (RNA-seq) and chromatin immunoprecipitation followed by sequencing (ChIP-seq) revealed that EGR1 in pre-decidual hESCs regulates the expression of a large number of genes involved in transcriptional regulation, cell signaling, and proliferation, cellular processes predicted to prime these cells for immediate proliferation and subsequent differentiation in response to the decidualogenic signal.

2. Materials and methods

2.1. Cell culture

Following a human protocol prospectively approved by the Institutional Review Board at Baylor College of Medicine and in accordance with the guidelines of the declaration of Helsinki [10], primary hESCs were isolated and maintained as described [11]. Unless otherwise stated, cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA). Primary hESCs were used for initial validation of gene function. The telomerase-immortalized T-HESC cell line was purchased from the American Type Culture Collection (Manassas, VA (CRL-4003)) and cultured in DMEM/F12 without phenol red with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 100 units/ml penicillin, 0.1mg/ml streptomycin, 0.5 µg/ml of puromycin (Sigma-Aldrich), and Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS-A). The identity of this cell line has been confirmed by short tandem repeat (STR) profiling by the Baylor College of Medicine Tissue Culture Core. Because of the number of cells required, the T-HESC cell line was used for ChIP-seq experiments and parallel RNA-seq. Importantly, the T-HESC cell line has been shown to decidualize similarly to primary hESCs *in vitro* [12].

2.2. *In vitro* decidualization

Primary hESCs and T-HESCs were plated into 6-well plates and transfected with small interfering (si)RNAs for 48 hours in OptiMEM with 2% charcoal-stripped FBS (sFBS) [11]. If cells were not transfected with siRNAs, they were incubated in OptiMEM with 2% sFBS for 48 hours prior to hormone treatment. For *in vitro* decidualization, cells were treated with a combination of 17β-estradiol (1×10^{-8} M), medroxyprogesterone acetate (1×10^{-6} M (primary hESCs) and 5×10^{-6} M (T-HESC cells)), and N6,2-O-dibutyryl adenosine 3,5-cyclic monophosphate sodium salt (5×10^{-5} M), (EPC) as described in [11]. Non-targeting (NT) and *EGR1* siRNAs were purchased from Dharmacon (Lafayette, CO (D-001810–10-05)) and Qiagen Inc. (Germantown, MD (GS1958)) respectively. For short-term (hours) EPC treatments, EPC was diluted in DMEM without serum and without phenol red. For long-term (days) EPC treatments, EPC was diluted in Opti-MEM without phenol red supplemented with 2% sFBS.

2.3. Isolation of RNA and quantitative reverse transcriptase (RT)-PCR

Total RNA was isolated with the RNeasy Plus kit with DNA removal columns (Qiagen Inc.) according to the manufacturer's instructions. Complementary (c)DNA was prepared with SuperScript VILO Master Mix (Thermo Fisher Scientific); cDNA of interest was quantified by Taqman real-time PCR as described in [11]. Taqman assays used in this study are listed in Supplemental Table ST1

2.4. RNA sequencing

Before RNA-seq, the purity and integrity of RNA was evaluated with a NanoDrop spectrophotometer (Thermo Fisher Scientific) and a 2100 Bioanalyzer with RNA chips (Agilent Technologies, Santa Clara, CA) respectively. For each experimental group, RNA samples from three independent experiments were used. Sequencing libraries were prepared

with the TruSeq Stranded mRNA kit (Illumina Inc., San Diego, CA) from 250 ng of RNA. Quality analysis of libraries was performed on 4200 TapeStation with D1000 ScreenTape assays (Illumina Inc.). Libraries were quantified with KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA). Following equimolar pooling, libraries were quantified on the 2100 Bioanalyzer (using the High Sensitivity DNA Kit and DNA chips) and KAPA Library Quantification Kit for Illumina platforms. Sequencing of libraries was performed on the NextSeq 500 platform (Illumina Inc.). Paired-end 75 base pair (bp) sequencing reads were generated at mid-output and mapped to the human genome (UCSC hg19) using STAR [13] with NCBI RefSeq genes as the reference. The Bioconductor package containing EdgeR was used to analyze the read counts to detect differentially expressed genes between *EGR1* and *NT* siRNA groups [14]. The false discovery rate (FDR) of differentially expressed genes was estimated using the Benjamini and Hochberg method [15]. Differentially expressed genes were gated for further analysis using absolute fold change (IFCI) > 1.5 and FDR < 0.05. Gene ontology enrichment analysis was performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery) functional annotation clustering tool [16].

2.5. Chromatin immunoprecipitation-sequencing and quantitative (q)PCR

Chromatin was isolated from $0.8\text{--}2 \times 10^7$ T-HESC cells fixed in formaldehyde. Chromatin was immunoprecipitated by Active Motif (Carlsbad, CA) using ChIP grade antibodies against either EGR1 (Cell Signaling Technology (Danvers, MA (4154BF)) or acetylated lysine 27 of histone H3 ((H3K27ac) (Active Motif, 39133)). For ChIP sequencing, input and immunoprecipitated DNA was sequenced on a NextSeq 500 platform with single-end 75 bp read. Sequencing reads were aligned to GRCh37/hg19. Following normalization by down-sampling, peaks were called with the MACS 2.1.0 algorithm with p-value cutoffs of 10^{-7} for narrow peaks and 10^{-1} for broad peaks. Primers used for ChIP-qPCR are listed in Supplemental Table ST2.

2.6. Western blot analysis

Protein was extracted from cells with NP40 buffer with proteinase inhibitors (cOmplete Mini, Roche, Basel, Switzerland) and phosphatase inhibitors (Sigma Aldrich) and quantified with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein (15 µg) was resolved by SDS-PAGE in 4–15% polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membranes. Following incubation of PVDF membranes in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST), the following antibodies were used for antigen detection: anti-EGR1 (Cell Signaling Technology (4154); diluted 1:2000) and anti-β-actin (Sigma-Aldrich (A1978); diluted 1:50000). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Peroxidase activity was detected with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

2.7. Mouse husbandry

Care and handling of mice occurred according to an animal protocol previously approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine (BCM) and to guidelines described in the Guide for the Care and Use of Laboratory Animals

(National Research Council (Eighth Edition 2011)). Mice were housed at an AAALAC-accredited *vivarium* with a 12h light-12h dark cycle at the Center for Comparative Medicine at BCM and were fed and watered *ad libitum*. For timed pregnancy experiments using CD1 proven stud males, the morning of detecting a vaginal plug in CD1 females was designated as gestational day 1. Following euthanasia by approved methods, uterine tissue was dissected and processed for histology at a predetermined gestation day. Two hours prior to euthanasia, mice received a dose of 1 mg per 20 g of body weight of 5-bromo-2'-deoxyuridine (BrdU; BD Biosciences, San Jose, CA) *via* intraperitoneal injection.

2.8. Immunohistochemical analysis

Mouse uterine tissues were fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS) before paraffin embedding and sectioning onto slides for immunohistochemistry [17]. The EGR1 transcription factor was detected with a primary rabbit monoclonal antibody (Cell Signaling Technology Inc. (4153); diluted 1:100) followed by a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA (P-1000); diluted 1:200). The peroxidase activity was detected with VECTASTAIN Elite ABC-HRP Kit (Vector Laboratories, Burlingame, CA). Following immunostaining, tissue sections were counterstained with hematoxylin prior to applying permount and affixing coverslips.

2.9. Immunofluorescent analysis

Cells were plated on coverslips (acid-etched and uncoated) in 24-well plates at 1.25×10^5 cells per well. Following hormone treatment, cells were washed with ice-cold Dulbecco's PBS containing Ca^{2+} and Mg^{2+} and fixed with ice-cold 4% paraformaldehyde in Pipes-EGTA- MgCl_2 (PEM) buffer. Autofluorescence was quenched by incubating in 0.1 M ammonium chloride in PEM for 10 min. Cells were permeabilized with 0.5% Triton X-100 in PEM for 30 min. Following incubation in 5% milk in TBST, EGR1 was detected with a rabbit monoclonal primary antibody (Cell Signaling Technology (4153); diluted 1:500) followed by secondary antibody, a goat anti-rabbit Alexa Fluor 594-conjugated antibody (ThermoFisher Scientific (R37117)). Following another round of fixation and autofluorescence quenching, cells were stained with DAPI and coverslips were mounted with Prolong Gold Antifade Mountant (ThermoFisher Scientific). Cells were imaged using GE Healthcare Inverted Deconvolution/Image Restoration Microscope System at the Baylor College of Medicine Integrated Microscopy Core. Intensity of EGR1 staining was measured using ImageJ's (<https://imagej.nih.gov/ij/>) Analyze Particles tool on 16-bit images and expressed as the nuclear mean grey value using DAPI images for nuclear demarcation. Samples without the inclusion of primary EGR1 antibody were used for background subtraction in the analysis.

2.10 Statistical analyses

Results are presented as averages $\pm$ standard deviation. Analyses were performed using R (version 3.3.1) in R Studio (R Studio Inc., Boston, MA). Quantile comparison plots were generated to inspect normality of data. Statistical analyses were performed with two sample t-test or Wilcoxon rank sum test; or with ANOVA or Kruskal-Wallis rank sum test with *post hoc* analyses performed with either Tukey's range test or Wilcoxon rank sum test.

Differences with p-values < 0.05 were considered significant with the following designations: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Specific tests used were annotated in the Figure Legends.

2.11. Analysis of EGR1 expression in the endometrium of women with recurrent implantation failure

Data to evaluate the levels of *EGR1* transcript in the endometrium of women with recurrent implantation failure and in control endometrium were exported from a published and publicly available dataset in the Gene Expression Omnibus (GEO) database: GSE58144 [9]. These data were extracted using GEO's GEO2R portal. The full accession ID number of the EGR1 expression data is GSE58144/GPL15789/17940. Wilcoxon rank sum test was used to compare the control and the recurrent implantation failure groups.

3. Results

3.1. Perturbation of EGR1 levels is associated with human endometrial disorders linked to implantation failure

Our recent hESC studies indicate that tight control of EGR1 expression levels is required for these cells to decidualize [8], suggesting that perturbation of EGR1 levels may be associated with endometrial disorders linked to early embryo implantation failure. Support for this supposition comes from our analysis of a recently published transcriptome dataset from van Koot *et al.* [9]. The dataset comprised microarray-profiled transcriptome information (GEO accession: GSE58144) from human endometrial tissues biopsied during the mid-luteal stage of the ovarian cycle (*i.e.* the receptivity (or implantation) window at days 5 to 8 after luteinizing hormone (LH) surge). Biopsies were obtained from two patient groups: (1) women who successfully became pregnant within two cycles after *in vitro* fertilization/ intracytoplasmic sperm injection (IVF/ICSI) and therefore their infertility was inferred to be of non-endometrial origin; and (2) women with recurrent implantation failure (RIF) despite undergoing IVF/ICSI. Our analysis revealed that relative *EGR1* mRNA levels are significantly lower in the endometrium of women with RIF as compared with women who were able to become pregnant (Fig. 1). This is the first result to reveal a connection between deregulation of normal EGR1 levels and an endometrial disorder linked to human implantation failure. This finding also underscores the importance of delineating the normal functional contribution of EGR1 in the pre-decidual stromal cell to human endometrial decidualization, an essential cellular process in uterine periimplantation biology.

3.2. Expression of EGR1 protein in pre-decidual and decidualized hESCs

In agreement with our previous findings at the mRNA level [8], EGR1 protein levels significantly decrease in hESCs upon EPC exposure within 3 days (Fig. 2A). The decrease in EGR1 protein levels is rapid, with most of the decrease occurring within 4–5 hours of EPC exposure (Fig. 2B; note: *EGR1* mRNA levels decline even more rapidly during this period (Supplemental Fig. 1A); siRNA mediated knockdown of EGR1 confirms specificity of the EGR1 antibody used (Supplemental Fig. 1B)). Before EPC treatment, EGR1 is predominantly located in the nuclei and exhibits a broad spectrum of expression levels per cell (Fig. 2C and Supplemental Fig. 1C); ~80% of cells are positive for EGR1 expression at

this time-point. The *EGR1* expression levels decrease globally within 6 hours of EPC treatment. A minor sub-population of cells re-express at high levels *EGR1* at a later time-point (24 h) despite prolonged EPC exposure (cells with a nuclear mean grey value (NMGV) > 1000; Fig. 2C and Supplemental Fig. 1C).

Through ChIP-qPCR analysis, we demonstrated that the rapid reduction of *EGR1* levels with EPC treatment is not accompanied by a decrease in acetylation of the lysine 27 of histone H3 (H3K27Ac) in the promoter region upstream of the annotated *EGR1* gene (Supplemental Fig. 2A B). These results show that the *EGR1* gene in hESCs is not regulated by changes in this epigenetic mark and suggest that this gene may be maintained in a “poised state” [18] under EPC treatment. In contrast, the *EGR2* gene in hESCs exhibits significant downregulation of H3K27Ac at a putative intronic enhancer [18] within 3 days of EPC treatment (Supplemental Fig. 2A B), which is coincident with the decrease in *EGR2* expression levels during hESC decidualization [11].

The striking decrease in *EGR1* levels during hESC decidualization is also observed in the early pregnant mouse (Supplemental Fig. S3). Many murine endometrial stromal cells clearly express *EGR1* protein during gestational days 4–5 (the window of implantation); however, the majority of these cells no longer express *EGR1* at gestational day 6. Only a few stromal cells remain that express *EGR1*, which are found closely surrounding the implanting embryo (Supplemental Fig. S3).

3.3. Early growth response 1 in pre-decidual hESCs is required for decidualization

To address whether *EGR1* expressed in pre-decidual hESCs is required for these cells to decidualize in response to EPC, siRNA-mediated knockdown of *EGR1* was performed on hESCs forty-eight hours prior to administration of EPC cocktail. Following effective *EGR1* knockdown (Fig. 3A), hESCs failed to decidualize as evidenced by significant attenuation in the induction of the decidual markers, insulin-like growth factor binding protein 1 (*IGFBP1*) and prolactin (*PRL*) [19] (Fig. 3A). This decidual molecular defect was reflected at the cellular level by an inability of hESCs with diminished *EGR1* levels to transform from a fibroblastic spindle to a polygonal epithelioid morphology when exposed to EPC for six days (Fig. 3B–E). Together, our results strongly support a critical role for *EGR1* in pre-decidual hESCs in the decidualization of these cells when exposed to a decidualogenic stimulus.

3.4. Early growth response 1 transcriptionally primes pre-decidual hESCs for decidualization

Because *EGR1* is a transcription factor, the *EGR1*-regulated transcriptome was profiled in pre-decidual T-HESC cells by ChIP-seq and RNA-seq. Analysis of the ChIP-seq dataset with the Galaxy Project’s Cis-regulatory Element Annotation System Tool revealed that *EGR1* primarily binds within the 5’ untranslated region (UTR) or promoter of target genes (Supplemental Fig. S4A). The Galaxy/Cistrome SeqPos Motif Tool uncovered that the top enriched cluster of motifs found in the regions bound by *EGR1* was comprised of *EGR* family G-C-rich motifs, including the *EGR1* binding motif (motif ID: MC00320; Supplemental Fig. S4B). Integration of the *EGR1* cistrome and transcriptome datasets with

the list of genes whose expression is significantly changed during EPC-induced decidualization [11] revealed that EGR1 controls a remarkable number of genes that are involved in decidualization (Fig. 4A). Significantly, 40% of genes that are transcriptionally altered during EPC treatment (the decidual transcriptome) are also bound by EGR1 within 10 kb upstream to 10 kb downstream of their annotated gene location. Moreover, 19% of EPC-regulated genes are bound by EGR1 within 1 kb upstream to 1 kb downstream of their annotated gene location (Supplemental Fig. S4C). Remarkably, the number of genes bound by EGR1 at day 0 of EPC treatment is on par with the number of genes bound by the progesterone receptor (PGR, the master-regulator of decidualization) at day 3 of EPC treatment [20] (Supplemental Fig. S4D). Although the number of genes that are regulated by EGR1 at the transcriptional level (either directly or indirectly) may seem small compared to the decidual transcriptome, it still amounts to 651 genes. Out of these, 47% of genes are bound by EGR1 (Fig. 4A). The majority of the EGR1 target genes are positively regulated by EGR1 (*i.e.* knockdown of EGR1 leads to their downregulation) while genes negatively regulated by EGR1 are primarily indirect secondary targets of EGR1 (Fig. 4B). Not surprisingly, the direct targets of EGR1 have a more robust response to EGR1 knockdown as emphasized by higher enrichment of gene expression changes with lower false discovery rates (FDR) (Fig. 4C and Supplemental Fig. S5). When performing Gene Ontology analysis of direct and indirect targets of EGR1 that are also involved in decidualization (the 140 and 169 genes depicted in the Venn diagram in Fig. 4A), it is clear that direct and indirect target genes execute markedly different biological programs (Fig. 4D). While both direct and indirect targets are involved in cell cycle regulation and cell signaling, direct targets exhibit a strong enrichment for genes encoding transcriptional regulators.

Because our results above underscored an important functional role for EGR1 in priming pre-decidual hESCs for later decidualization (Fig. 3), we next focused on all genes regulated by EGR1, irrespective of whether the expression of these genes changed with decidualization. Using the aforementioned approach, we again found direct and indirect genes of EGR1 controlled strikingly different biological programs (Fig. 5A and B). Specifically, genes directly regulated by EGR1 were primarily involved in regulation of transcriptional processes while indirect EGR1-target genes were involved in cell signaling. Both direct and indirect targets of EGR1 were enriched for genes involved in cell cycle regulation. The expression changes induced by EGR1 knockdown of some of the targets involved in these processes were confirmed by quantitative RT-PCR (Fig. 6). Collectively, our global cistromic and transcriptomic analyses indicate that EGR1 is essential for transcriptionally priming the pre-decidual hESC for imminent decidualization. Our analyses also reveal that many of the genes directly controlled by EGR1 encode proteins involved in harnessing the transcriptional machinery for cell division while others are key to cell signaling, cellular properties predicted to be acquired by a hESC that is primed for decidualization.

4. Discussion

When embryonic aneuploidy, chromosomal abnormalities, maternal thrombophilic disorders, and obvious anomalies in uterine anatomy are excluded as etiologic factors, implantation failure intrinsic to the uterus is commonly implicated in recurrent pregnancy

loss [21]. Endometrial dysfunction also compromises the full potential of assisted reproductive technologies [22–24]. Superimposed on the above, approximately a third of pregnancies in healthy women – detected early by the human chorionic gonadotropin (hCG) assay – fail soon after hCG detection, highlighting failure of implantation in the uterus as a significant causal factor for preclinical pregnancy loss even for healthy couples [25–27]. That said, the progression of a programmed sequence of uterine cellular changes is known to underpin the establishment of pregnancy [28], with endometrial decidualization following embryo implantation representing a crucial cellular transformation step that is essential not only for the formation but also the progressive development of the fetomaternal interface [29]. Therefore, expanding our understanding of decidualization at the molecular level is crucial if we are to gain new mechanistic perspectives on this cellular process. Such insights may also uncover novel molecular targets that may contribute to new clinical solutions to address adverse fertility issues arising from a dysfunctional uterus.

Extending from our previous investigations [8], we report in this study that EGR1 protein is significantly expressed in pre-decidual hESCs; however, the levels of this protein are rapidly reduced with the onset of the decidual response. These results provide translational support for observations made here and those of others [5, 7] that show EGR1 is highly expressed in the subluminal stromal compartment of the murine endometrium during the window of receptivity; however, this expression significantly declines following embryo implantation. Results from these studies raised the obvious question: Do pre-decidual hESCs require EGR1 to decidualize? With the use of cultured hESCs, together with a siRNA-mediated knockdown approach, we clearly show that EGR1 protein expression in pre-decidual hESCs is essential for their subsequent decidualization. These findings support functional studies in the murine uterus, which show a compromised decidual response in the absence of EGR1 [7]. In addition, our results offer functional support for the proposal that perturbation of uterine EGR1 levels may be linked to endometrial pathologies associated with recurrent implantation failure.

The inability of the pre-decidual hESC to respond to the decidualogenic signal without the EGR1 transcription factor was pretext to molecularly phenotype this response impairment at the cistrome and transcriptome level. Analysis of the EGR1 cistrome dataset in pre-decidual hESCs disclosed that EGR1 binds within 10 kb upstream to 10 kb downstream of over 8000 genes; approximately 50% of these genes are bound within 1 kb upstream to 1 kb downstream. Integration with our previously published hESC decidual transcriptome dataset [11] showed that over 40% of the 8000 EGR1-bound genes exhibit changes in expression during hESC decidualization. This result may explain why abnormal persistent EGR1 expression blocks hESC decidualization following administration of EPC [8]. Normal expression of these genes during decidualization may be perturbed due to inappropriate binding of EGR1 to the regulatory elements of these genes. Interestingly, examination of the EGR1 transcriptomic data from pre-decidual hESCs revealed that EGR1 directly or indirectly regulates the expression of 65 genes prior to exposure of the decidualogenic signal. The biological processes primarily governed by these genes include transcriptional regulation, cell proliferation and signaling. In particular, controlling a wide array of other transcriptional regulators suggests that EGR1 can essentially prime the entire transcriptional milieu of the pre-decidual hESC prior to decidualization.

Collectively, our studies address an understudied area of hESC peri-implantation biology. We demonstrate that the EGR1 transcription factor is indispensable for the pre-decidual hESC to decidualize in response to requisite hormone signals. Genome-wide analysis supports that EGR1 transcriptionally primes the pre-decidual hESC for decidualization by controlling the expression of genes primarily involved in transcriptional regulation, cellular proliferation, and signaling. Future studies will establish the functional importance of these genes to hESC decidualization as well as identify the transcriptional and/or post-transcriptional and post-translational mechanisms that control EGR1 levels in hESCs following the onset of the decidual response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Decidualization of the endometrium is critical for pregnancy establishment
- Endometrial EGR1 levels are decreased in women with recurrent implantation failure
- Priming of endometrial cells for decidualization requires EGR1 function
- The EGR1 transcriptome is predicted to prime endometrial cells to decidualize

Figure 1. Transcript levels of *EGR1* are significantly decreased in the endometrium of women with recurrent implantation failure

Expression data was retrieved from the Gene Expression Omnibus dataset GSE58144 [9].

Human endometrial tissue, which was biopsied during the mid-luteal phase of the menstrual cycle, was the RNA source. Control samples were derived from women who successfully conceived following IVF/ICSI whereas recurrent implantation failure samples were obtained from subjects who failed to get pregnant. Statistical analysis performed with Wilcoxon rank sum test.

Figure 2. Early growth response 1 protein expression is rapidly downregulated with EPC administration

Western analysis of EGR1 protein expression in T-HESC cells under EPC exposure for: **(A)** 0 to 6 days (shown in quadruplicate) and **(B)** 0 to 360 minutes; for each β -actin is a loading control. **(C)** Boxplots representing the nuclear mean grey value (NMGV) of EGR1 immunofluorescent staining at 0, 240, and 360 min and 24 h of EPC treatment. Percentage of cells with NMGV > 1000 for each time-point is noted above plot. *** denotes p-values calculated for comparisons with the 0 min of EPC treatment data point. Examples images of EGR1 immunofluorescent detection are shown in Supplemental Fig. S1B. Statistical analysis performed with Kruskal-Wallis rank sum test with *post hoc* pairwise Wilcoxon rank sum tests.

Figure 3. Maintenance of *EGR1* levels in pre-decidual primary hESCs is required for decidualization

(A) Expression of *EGR1*, *IGFBP1*, and *PRL* mRNA measured by quantitative RT-PCR following non-targeting (NT) or *EGR1* siRNA transfection and EPC exposure for 0, 3, or 6 days in primary hESCs. Statistical analysis performed with ANOVA with *post hoc* Tukey's range test. Cell morphology of hESCs at day 0 of EPC treatment after (B) NT siRNA or (C) *EGR1* siRNA transfection and at day 6 of EPC treatment (D and E).

Figure 4. The transcriptome regulated by EGR1 in T-HESC cells

(A) Number of genes within and overlaps between the following datasets: 1) Genes bound by EGR1 (within 10 kb upstream to 10 kb downstream of annotated gene), 2) transcriptome controlled by EGR1 at day 0 of EPC treatment, and 3) transcriptome of genes altered by 3 days of EPC treatment [11]. (B) Percentage of all genes up- and downregulated by *EGR1* knockdown which are either direct or indirect targets of EGR1. (C) Distribution of $-\log_{10}(\text{FDR})$ of all genes altered by *EGR1* knockdown directly or indirectly. (D) Summaries of DAVID Functional Annotation Clustering analysis of genes which are altered during decidualization (3 days of EPC treatment) [11] and which are also either direct or indirect targets of EGR1 (the 140 and 169 genes shown in (4A)).

A Direct EGR1 targets

	Enrichment Score	# of genes	Summary
1	3.15	26	Transcription from RNA polymerase II promoter
2	2.83	114	Transcription regulation
3	1.71	4	Cation/H+ exchanger, proton antiporter activity
4	1.59	17	Glycosyltransferase, signal-anchor
5	1.52	8	Leucine-zipper
6	1.35	10	Transcription factor, fork head
7	1.18	10	Component of endoplasmic reticulum membrane, lipid metabolism
8	1.13	24	Cancer
9	1.08	8	Cell Cycle: G1/S Check Point
10	0.97	65	Zinc finger region:C2H2-type

ABL1, ADAM19, AMPD2, ANKMY1, ANTXR2, AOX1, ARHGAP45, ATAD2B, B3GALT4, BATF3, BIRC3, BPTF, BRCA1, CALU, CAMKK2, CD3EAP, CDKN1A, CDKN2B, CDT1, CHAF1B, CHPF, CHST11, CHST2, COLGALT1, CREB5, CRIP2, CSNK1G3, CYGB, DCP1A, DCP2, DDIT3, DENND4A, DGKG, DTL, DUSP2, E2F3, ECE1, EGR1, EGR3, EID2B, ELOVL5, ENO2, ETV1, EXTL2, EXTL3, FAM20C, FGF5, FLII, FOSB, FOXC2, FOXF1, FOXL1, FOXP1, GALNT18, GAS7, GINS1, GLI4, GM2A, GPC6, GRB2, ID1, ID4, IGF2, INCENP, ITGB8, ITPK1, KAT6A, KDM4B, KLF13, KLF5, LASP1, LFNG, LOXL2, LYRM4, MAK16, MAMLD1, MAPK1, MDC1, MED21, MKI67, MLLT1, MOK, MYB, NAB2, NAT10, NAV2, NFATC2IP, NFATC4, NFKBID, NPTX1, NR2F1, NR4A2, NRGN, ORC1, OSBP1, OSR2, PDPK1, PEG10, PGAP1, PIGZ, PKIB, PLA2G12A, PRRX2, PRUNE2, PTGER2, PTPN22, R3HDM1, RBBP5, REV3L, RFC3, RFX2, RGCC, RIBC2, RIPK1, RNF168, RNF44, SALL2, SAMD11, SAPCD2, SATB2, SDC4, SDR39U1, SERTAD4, SGM52, SGPL1, SKI, SLC27A2, SLC2A4RG, SLC36A1, SLC37A2, SLC9A1, SLC9A3, SLC9B1, SMARCD2, SPCS3, ST3GAL2, STAT4, SUD5, SYNPO, TAF5L, TBX2, TCF4, TCF7, TFAM, TJP1, TM7SF2, TRABD2A, TRIB2, TRIM41, TRNP1, UHRF1, USP1, VASH1, VEGFA, WDR62, WIZ, XXYL1, ZBTB20, ZBTB46, ZBTB7B, ZNF114, ZNF521, ZNF575, ZNF597, ZNF608, ZNF702P, ZNF837

B Indirect EGR1 targets

	Enrichment Score	# of genes	Summary
1	3.02	126	Glycoprotein, signal peptide, secreted
2	2.47	176	Glycoprotein, transmembrane, receptor
3	2.17	15	Cell cycle, DNA replication, MCM, helicase
4	1.96	22	Lipoprotein, palmitate
5	1.96	8	Neuroblastoma breakpoint family
6	1.7	14	Fibronectin type-III
7	1.57	64	Serine/threonine-protein kinase
8	1.41	4	Coagulation factor 5/8 C-terminal type domain, Galactose-binding domain-like
9	1.29	9	Carboxypeptidase, metalloprotease
10	1.29	8	Response to progesterone, TGFβ receptor signaling pathway, rheumatoid arthritis

AAAS, ABCA13, ABCA6, ABCA9, ACE, ACKR4, ACSBG1, ACVR1C, ADAMTS12, ADAMTS15, ADCYAP1, ADORA2B, ADRA1B, ADRA2C, ADSS, ADTRP, AFP, AMZ1, ANKAR, ANKFY1, ANO7, AP1M1, AP1S2, APCDD1, APCDD1L, APOL1, ARSE, BCL2, BDKRB2, BFP51, BRI3BP, C1QTNF4, C7orf57, C9orf135, CAMK2B, CAMK4, CAPN2, CCDC69, CCDC74A, CCL2, CD163L1, CD36, CDC14B, CGN, CHST15, CHSY1, CIT, CLDN1, COLEC10, COX16, CPA4, CPM, CPXM1, CSGALNACT1, CTRL, CTSS, CTSS, CXCL12, DCLRE1B, DCSTAMP, DLK2, DLL4, DOK7, DPP4, DPY19L2P2, DPY19L3, E2F2, EDA2R, EFHD1, EMILIN3, ENAM, ENTDP1, ESM1, EVI2B, F8A1, FAM171A1, FAM20A, FAM24B, FMN1, FOS, FRMD3, FZD8, GABRE, GALT, GALNT15, GAS1, GBP4, GCCC, GDF5, GHR, GK5, GNAQ, GOLGA8A, GPD1L, GPR3, GPR68, GREB1L, GRIA4, GRK3, GYS1, HCL1, HTR1D, HYAL3, ICOSLG, IGFBP1, IL17RB, IL27RA, INA, INHBB, KANK1, KCNH1, KCNJ8, KCNS2, KCNU1, KIF14, KIF26B, KPNAS, L1CAM, LAT51, LCTL, LHFPL2, LRRC17, LRRC32, LRTOMT, MAP3K13, MAP3K14, MAPK9, MAST4, MCM2, MCM3, MCM4, MCM5, MGAT4C, MREG, MTOR, NAGS, NALCN, NABP, NBP11, NBP15, NBP18, NBP20, NBP21, NBP22, NBP23, NBP24, NBP25, NBP26, NBP27, NBP28, NBP29, NBP30, NBP31, NBP32, NBP33, NBP34, NBP35, NBP36, NBP37, NBP38, NBP39, NBP40, NBP41, NBP42, NBP43, NBP44, NBP45, NBP46, NBP47, NBP48, NBP49, NBP50, NBP51, NBP52, NBP53, NBP54, NBP55, NBP56, NBP57, NBP58, NBP59, NBP60, NBP61, NBP62, NBP63, NBP64, NBP65, NBP66, NBP67, NBP68, NBP69, NBP70, NBP71, NBP72, NBP73, NBP74, NBP75, NBP76, NBP77, NBP78, NBP79, NBP80, NBP81, NBP82, NBP83, NBP84, NBP85, NBP86, NBP87, NBP88, NBP89, NBP90, NBP91, NBP92, NBP93, NBP94, NBP95, NBP96, NBP97, NBP98, NBP99, NBP100, NBP101, NBP102, NBP103, NBP104, NBP105, NBP106, NBP107, NBP108, NBP109, NBP110, NBP111, NBP112, NBP113, NBP114, NBP115, NBP116, NBP117, NBP118, NBP119, NBP120, NBP121, NBP122, NBP123, NBP124, NBP125, NBP126, NBP127, NBP128, NBP129, NBP130, NBP131, NBP132, NBP133, NBP134, NBP135, NBP136, NBP137, NBP138, NBP139, NBP140, NBP141, NBP142, NBP143, NBP144, NBP145, NBP146, NBP147, NBP148, NBP149, NBP150, NBP151, NBP152, 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Figure 6. Validation of gene expression changes induced by EGR1 knockdown

Quantitative RT-PCR of direct and indirect targets of EGR1 following siRNA-mediated knockdown of EGR1 before EPC treatment. *CD163L1* – CD163 molecule like 1; *FOXL1* – forkhead box L1; *GFRA1* – GDNF family receptor alpha 1; *GREB1L* – GREB1 like retinoic acid receptor coactivator; *ID1* – inhibitor of DNA binding 1, HLH protein; *ID4* – inhibitor of DNA binding 4, HLH protein; *POLQ* – DNA polymerase theta; *SLC16A6* – solute carrier family 16 member 6; *SYT7* – synaptotagmin 7; *UHRF1* – ubiquitin like with PHD and ring finger domains 1. Statistical analysis performed with two-sample t-test.