


Decidualization of Human Endometrial Stromal Fibroblasts is a Multiphasic Process Involving Distinct Transcriptional Programs

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Abstract

Decidual stromal cells differentiate from endometrial stromal fibroblasts (ESFs) under the influence of progesterone and cyclic adenosine monophosphate (cAMP) and are essential for implantation and the maintenance of pregnancy. They evolved in the stem lineage of placental (eutherian) mammals coincidental with the evolution of implantation. Here we use the well-established in vitro decidualization protocol to compare early (3 days) and late (8 days) gene transcription patterns in immortalized human ESF. We document extensive, dynamic changes in the early and late decidual cell transcriptomes. The data suggest the existence of an early signal transducer and activator of transcription (STAT) pathway dominated state and a later nuclear factor κ B (NFKB) pathway regulated state. Transcription factor expression in both phases is characterized by putative or known progesterone receptor (PGR) target genes, suggesting that both phases are under progesterone control. Decidualization leads to proliferative quiescence, which is reversible by progesterone withdrawal after 3 days but to a lesser extent after 8 days of decidualization. In contrast, progesterone withdrawal induces cell death at comparable levels after short or long exposure to progestins and cAMP. We conclude that decidualization is characterized by a biphasic gene expression dynamic that likely corresponds to different phases in the establishment of the fetal–maternal interface.

Keywords

decidual stromal cells, endometrium, decidualization

Introduction

Cyclic decidualization of the uterine endometrium is crucial for human reproductive success.¹ Decidualization is driven by differentiation of endometrial stromal fibroblasts (ESFs) to decidual stromal cells (DSCs). This process involves considerable transcriptional and cellular remodeling, enabling implantation and placental development as well as menstruation-associated cyclic renewal of endometrium.^{1,2} Several signaling and regulatory pathways are involved in decidualization, including progesterone, prostaglandin E₂, and protein kinase A (PKA)/cyclic adenosine monophosphate (cAMP) signaling pathways.¹ These signals induce a cascade of autocrine and paracrine signaling pathways^{3,4} and expression of transcription factors (TFs), including progesterone receptor (PGR), forkhead box protein O1 (FOXO1), homeobox (HOX), and signal transducer and activator of transcription (STAT) paralogs,^{1,5} that together set in motion the regulatory program for decidualization.

Genome-wide transcriptomic changes in early decidualization have been studied widely.^{3,6-9} These studies have focused on the proximal changes associated with decidualization, uncovering extensive transcriptomic alterations driving the early

stages of this particular cellular differentiation event. Recently, decidualization has been characterized as consisting of an early proinflammatory phase onset (up to 3 days) followed by a late secretory phase starting before 8 days.^{1,10} Further, cytokines in the early-phase decidualization rather than late phase induce a specific secretory response from human blastocysts, suggesting

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that DSCs likely assume distinct states that are important to implantation and later consolidation of the fetal–maternal interface.^{11,12} Other studies have indicated that decidualization is reversible upon withdrawal of steroid hormones,¹³ suggesting that regulatory programs driving this process are plastic. Together, the aforementioned evidence suggests that decidualization occurs in at least 2 phases, but up to now, there are no full transcriptome analyses describing transcriptional programs and TFs influencing the sequential changes during decidualization.

Here we characterize the transcriptomes of 3- and 8-day in vitro DSCs that roughly correspond to the in vivo implantation window and progressed secretory phase, correspondingly.^{10,11} We tease apart the sequential changes in transcriptional control of inflammatory signaling pathways. Further, we study proliferation and viability of DSCs after removal of decidualization stimuli treated for either 3 days or 8 days. Our results suggest that decidualization is, minimally, a biphasic process with an early STAT pathway dominated phase and a later nuclear factor κ B (NF- κ B) pathway regulated phase with corresponding changes in cell–cell signaling.

Materials and Methods

Cell Culture

Human ESFs (T HESC, Mor lab, Yale University, corresponding to ATCC CRL-4003) were grown in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich, D2906), supplemented with 10% charcoal-stripped calf serum (Gemini Bio-Products, 100-119), 1% antibiotic/antimycotic (ABAM; Gibco 15240062), 1 nmol/L sodium pyruvate (Gibco, 11360-070), 0.1% insulin-transferrin-selenium (ITS premix; BD Biosciences, 354350), and 0.12% sodium bicarbonate (Sigma, S5761). Cells were in vitro decidualized for 3 days ($n = 3$) or 8 days ($n = 2$) by addition of 0.5 mM/L 8-bromoadenosine 3',5'-cyclic monophosphate (8-br-cAMP; Sigma, B7880) and 1.0 μ M/L of the synthetic progestin medroxyprogesterone acetate (MPA, Sigma, M1629) in DMEM (Sigma, D8900) supplemented with 2% charcoal-stripped calf serum. Decidualization media was changed every 2 days. Total RNA was extracted with RNeasy Plus Mini or Midi RNA extraction kits (Qiagen, 74134 and 75142) followed by on-column DNase I treatment. Total RNA quality was assayed with a Bioanalyzer 2100 (Agilent), and 500 ng of RNA samples were sequenced with Illumina Genome Analyzer II platform. For analysis of gene expression changes in human primary ESFs, 2 different cell lines were cultured in parallel in DMEM (Sigma, D2906) supplemented with 10% charcoal-stripped fetal bovine serum (FBS) until $\sim 60\%$ confluency, at which point the media were changed to DMEM (Sigma, D8900) supplemented with 2% charcoal-stripped FBS and 0.5 mM/L 8-br-cAMP and 1 μ M/L MPA. On the eighth day of decidualization, RNA was harvested using the RNeasy Plus Mini kit (Qiagen, 74134). Total RNA was converted to complementary DNA (cDNA) with the iScript cDNA synthesis kit (Biorad, 1708891). Qualitative polymerase chain reaction (PCR) analyses were

conducted in triplicate on 20 ng of cDNA per reaction with Taqman Fast Universal PCR master mix (Thermo Fisher, 4352042). The Taqman probes used in this study were as follows: CEBPD (Hs00270931), NF κ B (Hs00153283), PGR (Hs01556702), PRL (Hs00168730), RUNX1 (Hs00231079), RUNX2 (Hs00231692), STAT5A (Hs00234181), STAT6 (Hs00598625), and TBP (4333769).

RNA-seq and Computational Analyses

For each sample, at least 30 million reads were acquired, and basic quality parameters were checked with FASTQC version 0.10.1. Sequence reads were mapped to the human (GRCh37.69) Ensembl cDNA database using Tophat2,¹⁴ and gene counts were calculated with HTSeq¹⁵ and normalized as transcripts per million (TPM).¹⁶ Differential transcription was analyzed using edgeR version 3.16.5 (UQ normalization;¹⁷ false discovery rate [FDR] < 0.05, absolute fold-change [FC] > 2.0, TPM > 2 for at least one condition in a comparison). Gene heatmaps were produced from averages of absolute TPM values or edgeR logFCs in R with pheatmap using clustering distance “euclidian” and method “complete” or “ward.D.” Transcription factor set was constructed by combining genes in ingenuity pathway analysis (IPA, Qiagen, www.qiagen.com/ingenuity) categories “transcription regulator” and “ligand-dependent nuclear receptor.” The TFs subsets were intersected with PGR and FOXO1 targets that were previously studied using small-interfering RNA (siRNA).^{9,18} Data for receptor and ligand coexpression with trophoblasts were obtained from Pavličev et al.³

For gene ontology (GO) and gene set enrichment analysis and heatmaps, gene lists of differentially transcribed genes (FDR < 0.05, FC > 2.0, TPM > 2 for at least one condition in a comparison) were used as input for METASCAPE (metascape.org), and available pathway databases (GO Biological Processes, Reactome Gene Sets, Canonical Pathways, Biocarta Gene Sets, KEGG Pathway and Hallmark Gene Sets) were selected for analysis. This was repeated in IPA using “IPA canonical pathways” tool which, in addition to enrichment analysis, calculates activation z score for the pathways based on the direction of the transcriptional change. In IPA, we used combined cutoff (enrichment $P < .001$ and FC $z > 2.5$) to present the most enriched pathways that are significantly regulated during both phases.

Data Access

Sequencing data from this study have been submitted to the NCBI Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE114296. All data are also available from authors upon request.

Immunocytochemistry

The ESFs were grown on an 8-well slide in growth medium until confluent and subsequently decidualized for 3d and 8d, respectively, by addition of cAMP and MPA, as described

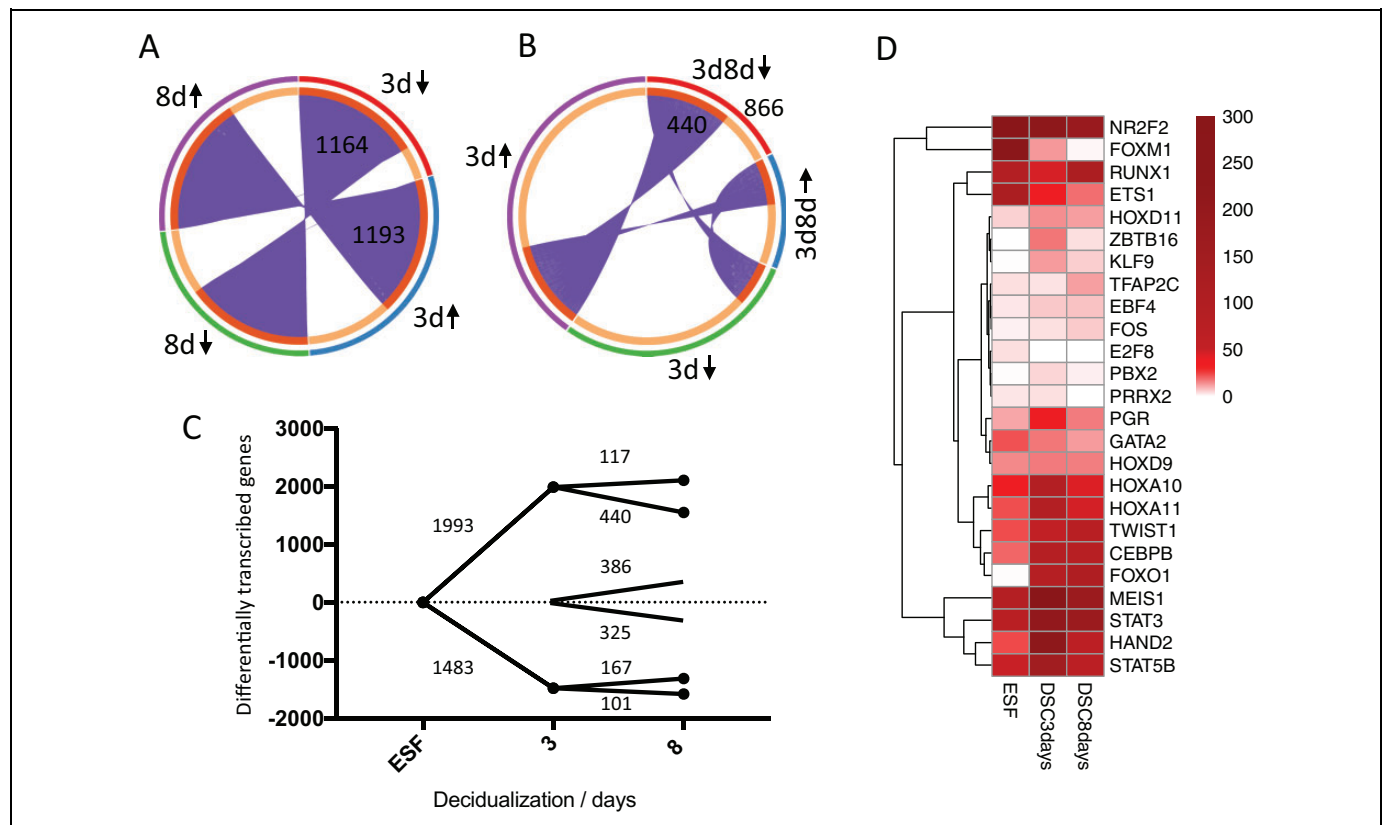


Figure 1. Differential transcription during in vitro decidualization. A, Common and unique differentially transcribed genes ($FC > 2$, $FDR < 0.01$, $TPM > 2$) in pairwise comparison of 3 and 8 days of decidualization treatment to untreated ESF. B, Common and unique differentially transcribed genes ($FC > 2$, $FDR < 0.01$, $TPM > 2$) in pairwise comparison of 3 days to untreated ESF and 8 to 3 days of treatment. C, Subgroups of genes with significantly increased or decreased transcription when followed through 3 to 8 days ($FC > 2$, $FDR < 0.01$, $TPM > 2$). D, Transcription of 25 decidualization TFs based on averages of absolute TPM values and presented using hierarchical clustering (Euclidean, complete), see¹⁹ for the list of TF-specific references. For the purpose of presentation, *MEIS1* was scaled down from 585 to 300 TPM. See Supplemental Table S1 for the gene lists and data. DSCs indicates decidual stromal cells; ESF, endometrial stromal fibroblasts; FC, fold-change; FDR, false discovery rate; TF, transcription factor; TPM, transcripts per million.

earlier.¹⁹ After 3 or 8d in decidualization medium, cells were washed with phosphate-buffered saline (PBS), and the decidualization medium was replaced by growth medium for 24 or 48 hours, respectively. A separate sample of decidualized and nondecidualized cells was included in the experiment. Two replicates of each treatment were included in the experiment, and the complete experiment was repeated twice.

The cells in all treatments were fixed in 100% methanol, permeabilized in 0.1% PBS Triton X-100 solution, blocked by blocking buffer (Abcam), and incubated overnight at 4°C with the KI-67 primary antibody (Abcam, 15580/rabbit, polyclonal). Secondary fluorescent antibody (Abcam, ab150077) was used for subsequent incubation at room temperature for 1 hour. Diamidino-2-phenylindole (DAPI) (Sigma, 10236276001) was used for nuclear staining. Cells were scored for the presence or absence of KI67 fluorescence by utilizing the cell counter plugin in ImageJ (v1.43).

Flow Cytometry

Human ESFs were grown to 75% confluency in 6-well plates and treated with decidualization medium for either 3 days or 8

days. For proliferation experiments, decidualization medium after 3 or 8 days of treatment was replaced with growth medium for 1 or 2 days prior to flow cytometry. Cells were trypsinized (TrypLE Express, Gibco, 12604-013) and centrifuged at 300g for 3 minutes and placed on ice. The medium was removed, and the cell pellet was resuspended with ice-cold BioWhittaker Hank's balanced salt solution without Ca/Mg/Phenol Red (Lonza, 10-547F) and 1 µg/mL propidium iodide (PI; Sigma, P4864). Flow cytometry was conducted on a BD FACSaria. Negative control, untreated cells were used to draw gates for discrimination of healthy and PI-positive cells.

Results

Decidualization is Marked by Distinct Early and Late Phases of Gene Expression

We sequenced RNA from immortalized ESF (ATCC CRL-4003) treated with MPA and 8-br-cAMP for 3 days and 8 days, respectively. These transcriptomes were first compared to that of undifferentiated ESF (Figure 1A, Supplemental Table S1).

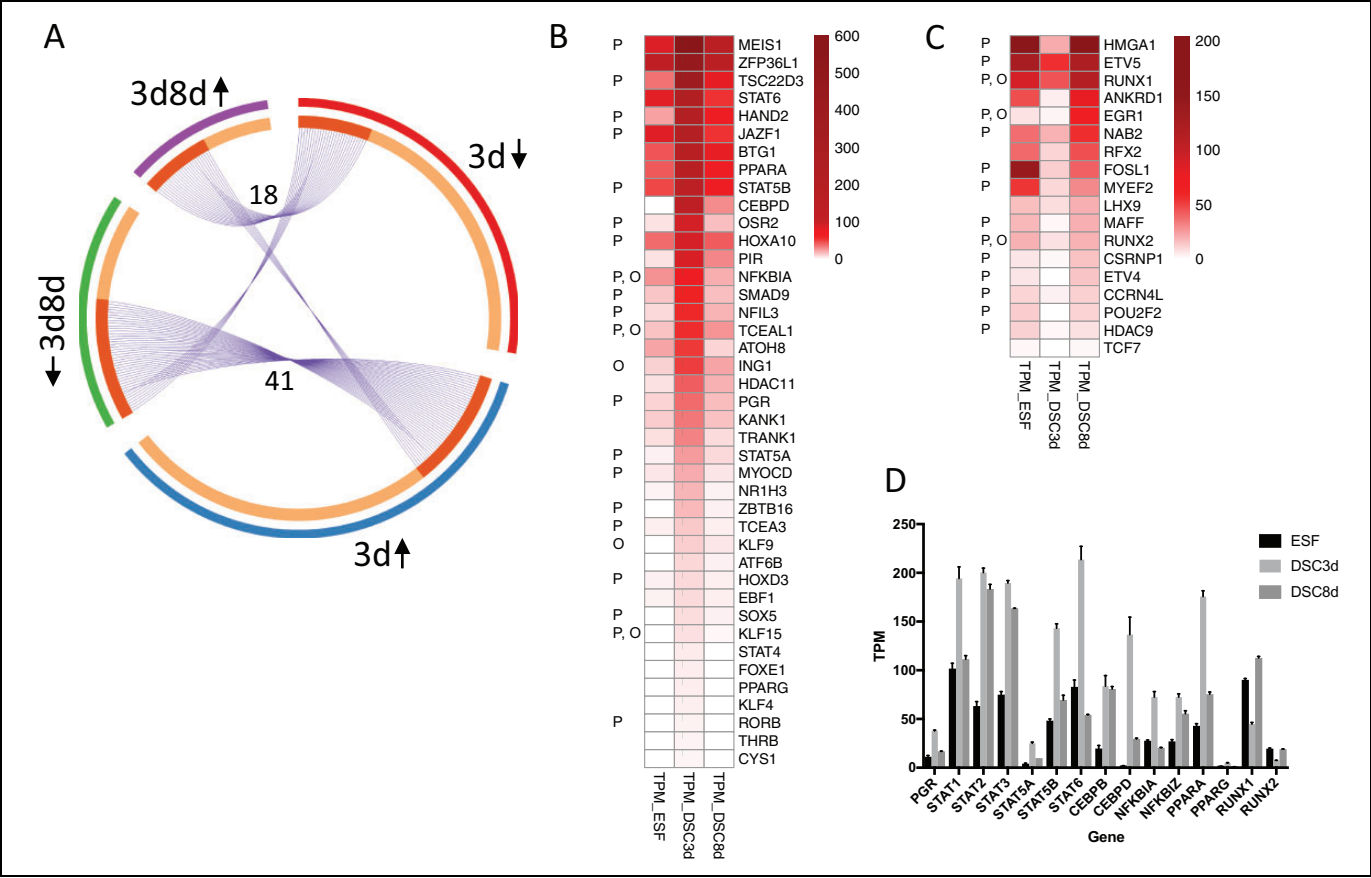


Figure 2. Differential transcription of TFs during in vitro decidualization. A, Common and unique differentially transcribed TFs ($FC > 2$, $FDR < 0.01$, $TPM > 2$) during late and early decidualization. B, TFs that were early upregulated and late downregulated sorted by transcript abundance (TPM) at 3 days. Genes regulated by *PGR* (P) and *FOXO1* (O) siRNA^{9,18} are marked on the left side of the heatmaps. C, TFs that were early downregulated and late upregulated sorted by transcript abundance (TPM) at 8 days. D, 15 TFs that are involved in the regulation of the inflammatory status of the cells and are reciprocally regulated. DSCs indicates decidual stromal cells; ESF, endometrial stromal fibroblasts; FC, fold-change; FDR, false discovery rate; siRNA, small interfering RNA; TF, transcription factor; TPM, transcripts per million.

Of the genes differentially upregulated after 3 days of decidualization ($FC > 2$ and $FDR < 0.01$), 60% (1193/1993) remained upregulated at 8 days (Figure 1A). Similarly, 78% (1164/1483) of downregulated genes after 3 days remained downregulated at 8 days (Figure 1A).

We next determined late-phase (3-8d) regulated genes and compared these to the early-phase (untreated ESF to 8 days) regulated genes (Figure 1B and C; Supplemental Table S1). Of the upregulated genes in the early phase, only 5.9% (117/1993) were further upregulated in the late phase, whereas more than half (51%, 440/866) of the late phase downregulated genes were upregulated in the early phase. As expected, several known decidualization markers (prolactin [*PRL*], Wnt family member [*WNT*] 4, *WNT5A*, matrix metalloproteinase [*MMP*] 7, *MMP11*; Supplemental Table S1) were upregulated in both early and late phases. In contrast, several TFs having a key role in decidualization (*PGR*, *HOXA10*, *STAT5B*, heart and neural crest derivatives expressed 2 [*HAND2*], et al; Figure 1D) exhibited upregulation in the early phase followed by downregulation in the late phase. Of the 1483 genes that were downregulated in the early phase, only 101 (6.8%) were further

downregulated in the late phase (Figure 1B and C). Interestingly, 386 and 325 genes were upregulated and downregulated, respectively, during the late phase only, indicating that there is a second cadre of genes specifically regulated during the late decidualization response (Figure 1C).

Early and Late Transcription of Decidualization TFs

Next, we analyzed the phase-specific differential expression specifically for transcriptional regulators. The proportion of regulated TFs mirrored the results obtained by analyzing all genes. Of the 349 differentially regulated TFs, 41 (12%) exhibited an early upregulation–late downregulation transcriptional pattern, 18 (5.2%) showed an early downregulated–late upregulated pattern, and only a few TFs showed a consistent up- or downregulation of transcription (Figure 2A). In the following analysis, we will specifically concentrate on TFs that have the early upregulation–late downregulation and early downregulation–late upregulation patterns.

Several key decidualization TFs displayed a concerted pattern of early upregulation and late downregulation (Figure 2B;

Supplemental Table S2), these genes include *PGR* and *HOXA10*. Concordantly, the same pattern was observed for several of their known transcriptional coregulators. The TFs most highly expressed at 3 days sharing this pattern was myeloid ecotropic viral integration site 1 (*MEIS1*; Figure 2B), which is a known cofactor of *HOXA10* in decidualization.²⁰ Antiproliferative effects of progesterone are mediated via interactions of *PGR* with HAND2, zinc-finger and BTB domain containing 16 (*ZBTB16/PLZF*), and Kruppel-like factor (*KLF*) 9 and *KLF15*,^{21–24} all of which share the early upregulated and late downregulated transcriptional pattern (Figure 2B). On the other hand, of the known core decidual regulators, *FOXO1* was upregulated early and subsequently maintained at this level later in decidualization (Figure 1D).

To gain insight into how the phase-specific TF networks are governed by two known core decidualization regulators *PGR* and *FOXO1*, we next intersected the early upregulation–late downregulation and early downregulation–late upregulation groups with the set of *PGR* and *FOXO1* target genes that were previously studied using siRNA^{9,18} (Figure 2B and C, Table 2). This intersection revealed that half (20/41) of the early upregulated–late downregulated TFs were *PGR* targets, whereas only few (5/41) were regulated by *FOXO1* (Figure 2B). Similarly, in the early upregulated–late downregulated TF group, *PGR* regulated 14 of 18 genes and *FOXO1* only 3 of 18 genes (Figure 2C). One potential confounding factor in these analyses is that the present study was conducted with a slightly different decidualization protocol than the siRNA studies mentioned earlier. In the present study, ESFs were treated with 8-br-cAMP and MPA for 3 days and 8 days, respectively, whereas in the siRNA studies, ESFs were treated over 2 days with 8-br-cAMP, MPA, and estrogen (estradiol). Therefore, we caution that the addition of estrogen to the decidualization medium may result in differences in gene expression compared to our data. Additionally, in the study focused on the effect of siRNA-mediated *FOXO1* knockdown, we noticed the knockdown efficiency was less than 50%, suggesting that *FOXO1* protein was likely still present and able to regulate gene expression. However, we include the *FOXO1* analysis here in order to present a more complete picture. With these caveats in mind, we conclude here that *PGR*, which is itself an early upregulated/late downregulated gene, still exhibits a significant overlap of target genes to be considered a major regulatory input during phase-specific regulation of decidualization.

Early and Late Regulation of TFs Involved in Inflammation

We observed marked phase-specific transcriptional regulation of both STAT and NF- κ B pathway components, constituting major regulators of inflammatory mediators (Figure 2D, Supplemental Figure 1). Of 7 STAT paralogs, 5 shared a pattern of early upregulated–late downregulated transcription, suggesting that several STATs were similarly regulated and may define a phase-specific inflammatory status. Specifically, *STAT1*, *STAT5B*, and *STAT6* were highly transcribed and showed a pattern of early upregulated–late downregulated transcription.

These factors are known to be involved in decidualization and fetal–maternal T-helper cell (Th) 1/Th2 balance.^{25–27} *STAT5B* is known to be a crucial regulator for human decidualization.²⁶ In ruminants, *STAT1* is a central regulator of pregnancy,²⁷ but in human DSCs, the role of *STAT1* is only superficially known. However, it has been shown to repress *PRL* transcription.²⁸ The role of *STAT6* has not been studied in DSCs, but in lymphocytes at the feto–maternal interface, it mediates shift in Th1/Th2 balance toward Th2 type.²⁵

Interestingly, of the NF- κ B pathway genes, only the inhibitory factors such as NF- κ B inhibitor α (*NF κ B1 α*), glucocorticoid-induced leucine zipper protein (*GILZ/TSC22D3*), and ZFP36 ring finger protein like-1 (*ZFP36L1*) were early upregulated–late downregulated (Figure 2B). The canonical NF- κ B RelA (p65) pathway inhibitor *NF κ B1 α* showed an early upregulated–late downregulated transcriptional pattern in both immortalized cells and primary ESF cell lines (Figure 2B, Supplemental Figure 1). Likewise, *GILZ*, a known and potent NF- κ RelA (p65) transrepressor,²⁹ and *ZFP36L1*, a member of the ZFP36 (Tristetrapolin) family of cytokine suppressors³⁰ and crucial regulator of placentation,³¹ exhibited a similar pattern. Notably, *GILZ* is highly transcribed at 3 days (TPM; ESF: 36, 3 days: 371, 8 days: 80). Considering the different known NF- κ B subpathways, the constitutive transcription of transforming growth factor β -activated kinase 1 (*TAK1/MAP3K7*; TPM; ESF: 17, 3 days: 23, 8 days: 18) versus the very low transcription of NF- κ B inducing kinase (*NIK/MAP3K14*; TPM; ESF 2.1, 3 days: 1.4, 8 days: 1.0) suggest that canonical RelA (p65) NF- κ B pathway is more active in decidualizing cells than NIK-mediated noncanonical NF- κ B pathway.³² In summary, these transcriptional patterns suggest that the early decidualization inflammatory state is dominated by STAT signaling. In the late phase, however, STATs are downregulated and the transcription of NF- κ B repressors decreases, possibly leading to NF- κ B pathway activation.

Early Downregulated and Late Upregulated TFs Include Known Regulators of Differentiation, Immunity, Morphogenesis, and Vascularization

Genes of functional pathways involved in differentiation, immunity, morphogenesis, and vasculature exhibited an early downregulated–late upregulated transcriptional pattern (Figure 2C). A majority (14/18) of these TFs are upregulated at 3 days in *PGR* knockdown,¹⁸ suggesting that their early downregulation is mediated by progesterone signaling. These *PGR* targets include runt-related transcription factor 1 (*RUNX1*) and *RUNX2* (Figure 2D; Supplemental Figure 1) and TFs that are reported to have negative interactions with STATs^{33,34} and in our analysis have reciprocal transcription patterns. The RUNXs are differentiation regulators in hematopoiesis, osteoblasts, and immune cells.^{35,36} The ETS variants *ETV4* and *ETV5*, which are involved in branching morphogenesis,^{37,38} together with the genes detected in the GO term “anatomical structure morphogenesis” (Supplemental Table S1), likely regulate

morphological transformation associated with decidualization. Ankyrin repeat domain 1 (*ANKRD1/CARP*) and early growth response 1 (*EGR1*) are involved in circulatory system development by regulating genes such as vascular endothelial growth factor (*VEGF*) and interleukin (*IL*) 8^{39,40} that in our data were upregulated in the late phase of decidualization. Further, *EGR1* is known to be early downregulated by ZBTB16, which is a PGR target,²¹ and is reciprocally regulated early upregulated–late downregulated, connecting the *EGR1* pattern to a PGR transcriptional program.

Pathway and Gene-Set Enrichment Analysis of Early and Late Decidualization

We conducted GO enrichment analysis to determine whether functionally related sets of genes also show differential early and late transcription profiles. Hierarchical clustering of differentially expressed GO and other gene-set terms revealed that genes involved in cell cycle, mitosis, and DNA metabolism were downregulated in the early phase (Figure 3A and Supplemental Table S3). Functional terms of the late downregulated genes clustered with upregulated genes of both phases, further suggesting phase-specific regulation of functionally enriched pathways important to decidualization (Figure 3A). Of the specific functional categories, “P53 pathway” and “tumor necrosis factor- α (TNF α) signaling via NF- κ B” are upregulated in both early and late decidualization, whereas “interferon γ (IFN γ) response” and “cholesterol homeostasis” are first upregulated and then downregulated. “Anatomical structure morphogenesis” and “circulatory system development” are specifically upregulated later in decidualization (Figure 3A and Supplemental Table S3).

In late decidualization, “IFN γ response” is the most enriched downregulated functional category term, and “TNF α signaling via NF- κ B” is the most enriched upregulated term (Figure 3B). Inspecting the genes in these inflammatory pathway categories suggests a highly concerted downregulation of IFN γ signaling (40 downregulated and 12 upregulated; Supplemental Table S3). In contrast, genes in the “TNF α signaling via NF- κ B” pathway exhibited more upregulated genes in the late phase (36 upregulated and 24 downregulated; Supplemental Table S3). In the IFN γ group, several classic interferon-induced (IFI) genes, such as *IFI44L*, *IFIT1*, *IFIT27*, *IFI30*, *IFIH1*, *IFIT3*, 2'-5'-oligoadenylate synthetase (*OAS*) 2, *OAS3*, and MX dynamin like GTPase 1 (*MX1*), were only transcribed during the early phase of decidualization (Figure 3C). Other genes of IFN γ group that were upregulated specifically in the early phase included *NF κ B1 α* and *IL15* as well as *caspase4* that promotes IL1B release⁴¹ (Figure 3C).

TNF/NF- κ B upregulated genes (Figure 3D) constitute a functionally wider array of genes, including secreted signaling molecules, such as bone morphogenetic protein 2 (*BMP2*), inhibin subunit β A (*INH β A*), cysteine-rich angiogenic inducer 61 (*CYR61*), and *VEGF*; receptors such as F2R like trypsin receptor 1 (*F2RL1/PAR2*) that mediates proliferation and

inflammation in ESFs;⁴² and TFs such as *EGR1*, *EGR3*, nuclear receptor 4A1 (*NR4A1*), *NR4A2*, and FOS like 1, AP-1 transcription factor subunit (*FOSL1*). Additional NF- κ B targets upregulated late in decidualization include *IL8*⁴³ (TPM; ESF: 2.1, 3d: 2.5, 8d: 52) and *IL11*,⁴⁴ the latter of which is a known signal specifically active in late decidualization.⁴⁵

We next conducted canonical pathways analysis (IPA) that, in addition to enrichment, also takes into account directionality of transcription. Nuclear factor erythroid 2-related factor 2 (NRF2/NFE2L2)-mediated oxidative stress response was the canonical pathway exhibiting the strongest directional upregulation signal in early decidualization. Interestingly, this pathway was strongly downregulated in late decidualization, suggesting distinct phases of oxidative stress response in these cells as decidualization progresses (Figure 4A). The NRF2 signaling is a general inducer of antioxidant and oxidative stress responses involved in response to injury and inflammation⁴⁶ and has been implicated in the evolution of the DSCs.¹⁹ At the crux of NRF2 signaling is NRF2 itself, the transcript of which is highly expressed in ESF cells and was differentially upregulated in both early and late decidualization (TPM; ESF: 117, 3d: 195, 8d: 208; early FC FDR = 3.2E–06). The NRF2 protein is posttranslationally regulated by the Kelch-like ECH-associated protein 1 Cullin 3 (KEAP1-CUL3) E3 ligase complex and directly controls the transcription of genes responsible for oxidative detoxification and glutathione synthesis, including glutamate–cysteine ligase modifier (*GCLM*) and catalytic (*GCLC*) subunits; and glutathione S-transferases (GSTs).⁴⁶ In line with previous observations of decidual cells and cellular redox stress,⁴⁷ our early decidualization data set exhibits a marked upregulation of oxidative stress response genes known to be directly controlled by NRF2, included among these are *GCLM*, *GSTA4*, *GSTM2*, *GSTM3*, *GSTM4*, *heme oxygenase 1* (*HMOX1*), and *thioredoxin reductase 1* (*TXNRD1*) (Figure 4B).

In addition to the NRF2 pathway, prolactin signaling and interferon signaling pathways show the pattern of early upregulation and late downregulation (Figure 4A, C–E). Further inspection of genes that constitute these pathways reveal coregulation of genes involved in Janus kinase (JAK)/STAT signaling (Figure 4D; Supplemental Figure 2), members of which are known decidual regulators such as STAT3 and STAT5.⁵ Prolactin signaling genes shared more (16/23) JAK/STAT signaling components than interferon signaling (4/23; Supplemental Figure 2, Supplemental Table S4), suggesting that prolactin signaling likely constitutes a major input for JAK/STAT-dependent transcription with interferon signaling playing a minor role. Interestingly, 14 of the 16 shared prolactin and JAK/STAT signaling genes clustered as early upregulated–late downregulated, whereas both PRL itself and its receptor PRLR were further upregulated in late decidualization (Figure 4C and D). These observations suggest that negative regulation of prolactin signaling may be constituted by intracellular signaling components, while extracellular mediators are upregulated and, generally, that differential regulation of STAT paralogs is central for modulating prolactin signaling.

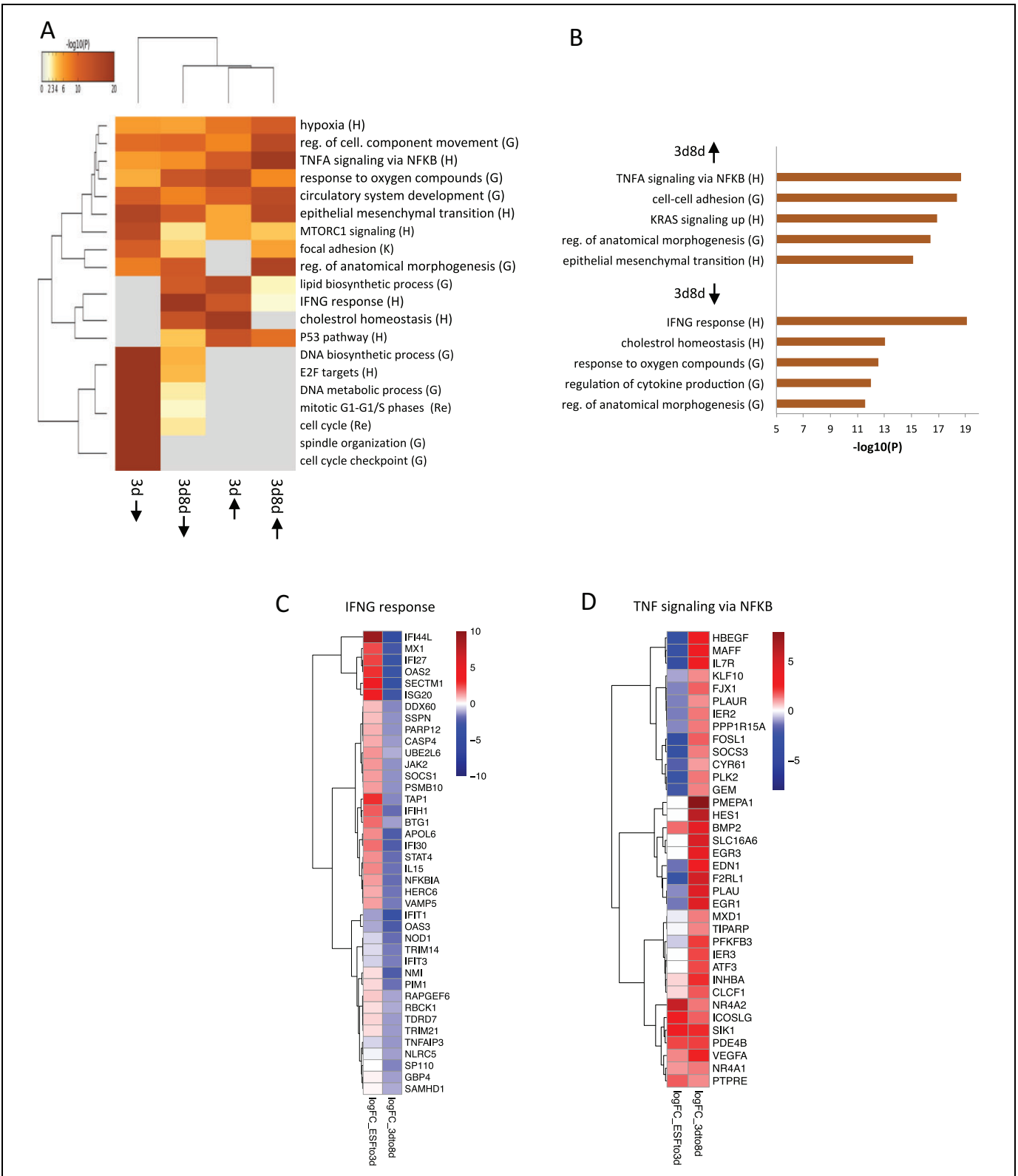


Figure 3. Functional enrichment analysis of the differentially transcribed genes during early (ESFto3d) and late (3dto8d) decidualization. A, Hierarchical clustering of the functional enrichment categories from METASCAPE (P values, default; see Supplemental Table S3). Used categories are from GO Biological Processes (G), Hallmark gene sets (H), Reactome (Re), and KEGG (K). Genes with increased and decreased transcription were analyzed separately. B, Top enriched functional terms for the late phase of decidualization. (C) Late-phase downregulated genes in "interferon gamma response" and (D) late-phase upregulated genes in "TNF α signaling via NF- κ B" presented with hierarchical clustering (Euclidean, complete) of the fold-change (logFC) of transcription. See Supplemental Table S3 for details. GO indicates gene ontology.

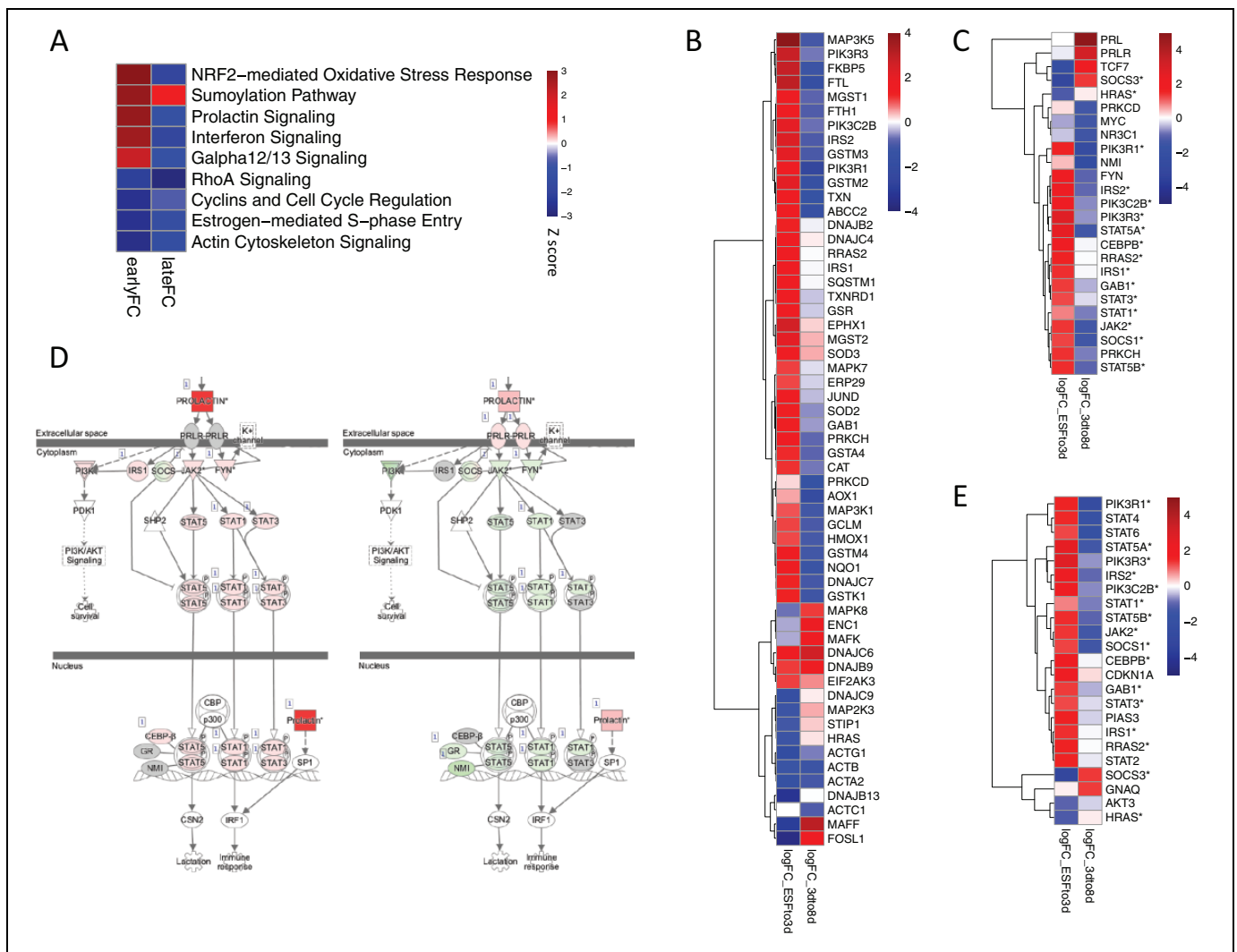


Figure 4. Canonical pathway (IPA) analysis of the early and late differentially transcribed genes. **A**, Top enriched and regulated canonical pathways using combined cutoff (enrichment $P < .001$, fold-change $z > 2.5$) for both early and late regulated genes. See Supplemental Table S4 for the gene list of all top categories. **(B)** “NRF2-mediated oxidative stress response” pathway genes and **(C)** “prolactin signaling” pathway genes for the early and late phases presented with hierarchical clustering (Euclidean, ward.D [B]/complete [C]) of the logFC of transcription. **D**, “Prolactin signaling” canonical pathway diagram for the early (on left) and late (on right) decidualization. Fold-change (z score) values were overlaid on the symbols (red = increased, green = decreased). The symbols refer to single genes or complexes (eg, $SOCS = SOCS1 + SOCS3$, $PI3K = PI3KR1 + PI3KR3 + PI3KC2B$). **E**, “JAK/STAT Signaling” pathway genes presented with hierarchical clustering (Euclidean, complete) of the logFC of transcription. Common genes in **(C)** and **(E)** are marked with *. IPA indicates ingenuity pathway analysis; JAK, Janus kinase; NRF2, nuclear factor erythroid 2-related factor 2; STAT, signal transducer and activator of transcription.

Early and Late Regulated Receptor and Ligand Genes That are Coexpressed in Trophoblast Cells

To investigate the potential phase-dependent changes in the interactions of DSCs with invading fetal cells (extravillous trophoblast [EVT]/syncytial trophoblast [SYN]), we took a set of previously characterized 74 receptor and ligand genes that are expressed in both cultured DSC and EVT/SYN cells³ and found that 46 of these were differentially transcribed during early (36) or late (27) phases (Supplemental Figure 3). Notably, we detected several Ephrin receptors (EPH) and ligands

(EFN),⁴⁸ which are both membrane proteins mediating cell-cell interactions. Among others, Ephrin receptors *EPHB1*, *EPHB2*, and *EPHA2* were early downregulated, whereas Ephrin ligands *EFNB1*, *EFNA4*, and *EFNA5* were early upregulated (Supplemental Figure 3). Ephrins have been reported to mediate implantation⁴⁹ and spiral artery remodeling.^{50,51} Among the ligands, several fibroblast growth factors (FGF) were early downregulated (*FGF1*, *FGF2*, and *FGF5*) with the exception of *FGF7* (keratinocyte growth factor [*KGF*]) that was highly transcribed during both early and late phases (TPM; ESF: 39, 3d: 443, 8d: 334). *FGF7* binds to several collagens,⁵²

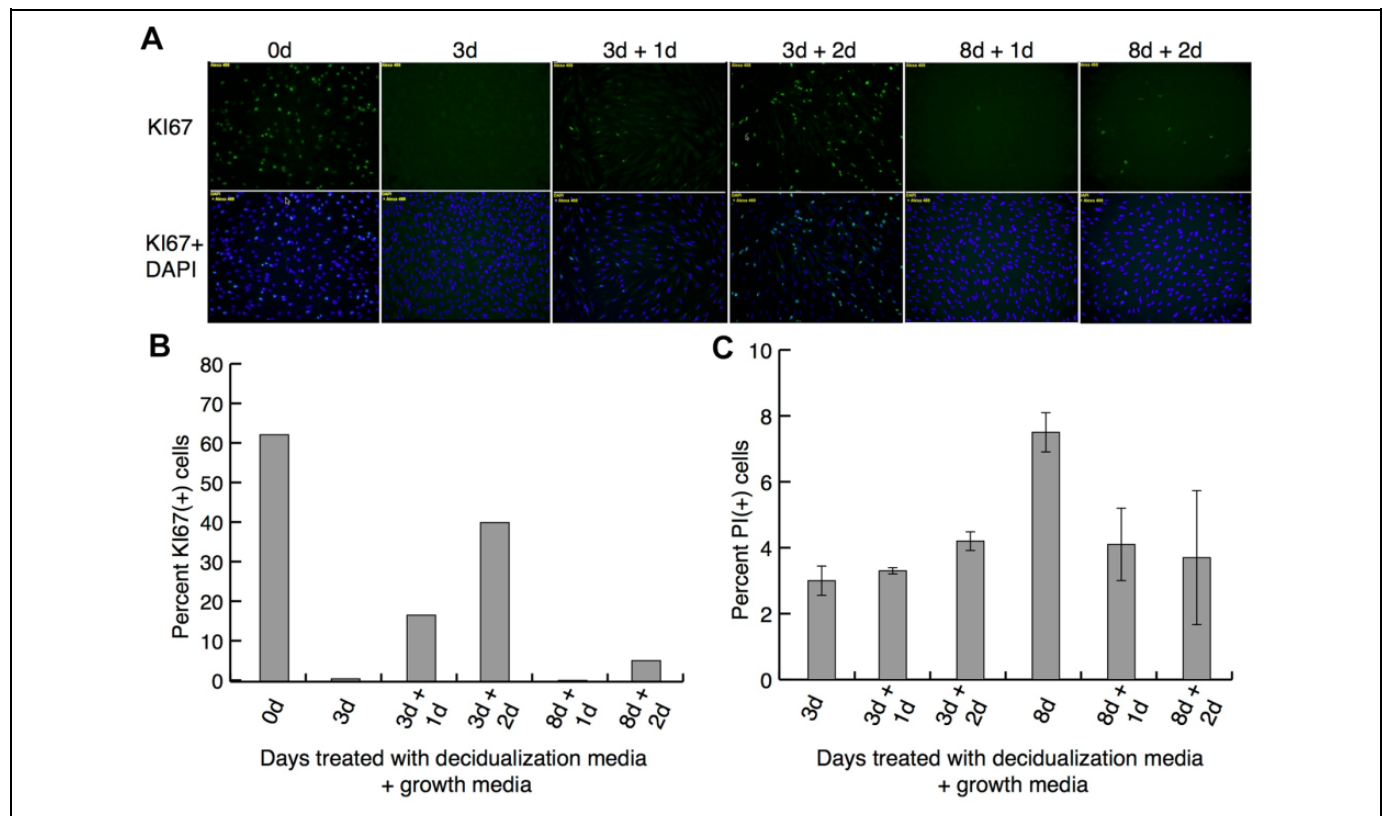


Figure 5. Proliferation and viability of DSCs at early and late phases of decidualization. **A**, Immunofluorescence staining in ESF (0d) and DSCs (3d and 8d) against fluorescent antibody for KI67, a marker for cellular proliferation. The vast majority of ESFs are proliferating prior to exposure to decidualizing stimuli (at 0d). After 3 days exposure to decidualization stimuli, only background signal is observed for KI67. However, the signal can be rescued by removing decidualization stimuli from the media (3d + 1d and 3d + 2d). This reversal of proliferative quiescence observed after 3d of decidualization is greatly reduced after 8d of decidualization (8d + 1d and 8d + 2d). **B**, Percent cells in each of the treatment groups in (A) that were scored positive for a KI67 signal as deduced with the ImageJ software program. **C**, Percent cells stained positive for propidium iodide by flow cytometry in each of the treatment groups above, as well as for 8d decidualization. Error bars represent standard deviation of 2 replicates. DSCs indicates Decidual stromal cells; ESF, endometrial stromal fibroblasts. D indicates days

promotes branching morphogenesis,⁵³ promotes decidualization, and its levels are higher in the first-trimester abortion decidua than in nonpregnant endometrium.⁵⁴

Several members of the tumor necrosis factor receptor superfamily (TNFRSF) were differentially transcribed during the early and late phases, including those that are modulators of NF κ B pathway (Supplemental Figure 3B). Specifically, *TNFRSF11B*, *TNFRSF10A*, and *TNFRSF10C* were upregulated in both phases and *TNFRSF10D* in the late phase. *TNFRSF10A* is also called TNF-related apoptosis-inducing ligand receptor (TRAILR1, death receptor 5), whereas *TNFRSF10C* and *TNFRSF10D* are apoptosis-repressing decoy receptors.⁵⁵ The late 18-fold upregulated *TNFRSF11B* (osteoprotegerin) is a secreted decoy receptor that represses TRAIL-induced NF- κ B activation. In pregnancy, *TNFRSF11B* has been suggested to protect against TRAIL/NF- κ B-induced proapoptotic effects.⁵⁶ Additionally, TRAIL activation in DSCs has been reported to protect lymphocytes from apoptosis.⁵⁷ The late upregulation TNFRSF decoy receptors constitutes an opposite pattern to predicted intracellular release the NF κ B pathway repression (*PGR*, *NF κ B1 α* , and *GILZ*) and thus may

constitute a distinct NF κ B-related regulatory loop to counteract proapoptotic effects of inflammation that is directly amenable for extracellular modification from other cell types such as EVT/SYN.

Analysis of Proliferation and Viability of DSC at Early and Late Decidualization

Data presented thus far suggest that DSC exhibits transcriptional regulatory programs that are temporally distinct and likely involve dynamic regulation of genes associated with proliferation and inflammatory cellular stress. The early downregulation of GO terms associated with cell cycle, mitosis, and DNA replication suggest that DSCs commence a proliferative quiescent state. To determine whether proliferative quiescence is reversible during decidualization, we first assayed DSC for the presence or absence of the proliferation marker KI67 (MKI67) at both early and late stages (Figure 5A and B). Immunostaining with an antibody against KI67 of DSC decidualized for 3 days showed a marked decrease in KI67-positive cells (Figure 5A and B) relative to ESF, supporting our

transcriptomic analyses showing a strong downregulation of genes associated with cellular proliferation. Strikingly, withdrawal of decidualization stimuli at 3 days resulted in the re-emergence of this proliferation marker (Figure 5A and B), showing an increase in both intensity of the signal and the absolute number of KI67-positive cells. In contrast, in late-stage decidualization, fewer cells were positive for KI67, and signal intensity was markedly lower after both 1 and 2 days of stimuli withdrawal (Figure 5A and B), suggesting that late-stage decidualization is less amenable to reversal from proliferative quiescence. Furthermore, given enrichment of GO terms associated with cellular inflammation with decreased transcription of genes in oxidative stress protection in later stage decidualization, we hypothesized that early and late phases may be marked by differential cellular death. We assayed for PI staining by flow cytometry at 3 days, 8 days, and following withdrawal of decidualization stimuli (Figure 5C). Our data showed an increase in the number of PI-positive cells after 8 days of decidualization, suggesting that late-stage decidualization is marked by an increase in cellular death. However, the removal of decidual stimulus resulted in similar level of cellular death for both 3 days and 8 days decidualized cells, suggesting that apoptotic pathways have distinct phase-specific dynamics compared to proliferation pathways. Taken together, these results experimentally support our transcriptomic analyses and support our hypothesis in which decidualization is characterized by at least 2 phases and distinct transcriptional regulatory programs.

Discussion

Decidualization is at Least Biphasic With Distinct Transcriptional Gene Sets

In this study, we presented RNA-seq from ESF decidualized with MPA and 8-br-cAMP for 3 days and 8 days and compared these to undifferentiated ESF. Concordant with previous reports based on cytokine data,¹⁰ our results provide genome-wide transcriptomic support for the hypothesis that decidualization involves at least 2 distinct regulatory phases. Almost 25% of genes upregulated in the early phase were downregulated in the late phase, suggesting that decidualization may be characterized by wave-like transcriptional subprograms, perhaps similar to those described in unrelated cellular differentiation processes.^{58–60} The intersection of our results with previous PGR knockdown data⁹ suggests that PGR is a main driver for the early upregulated–late downregulated transcriptional program, as numerous TFs exhibiting this pattern are putative or known PGR targets.^{21,22,24,61,62} Several known regulators of decidualization displayed the early upregulated–late downregulated pattern, including *PGR*, *HOXA10*, *HAND2*, and *STAT* paralogs. In contrast, core regulators of differentiation in other cell types, such as *RUNX1* (hematopoiesis) and *RUNX2* (osteogenesis),^{63,64} show the opposite pattern. This strongly suggests that the early regulatory program includes components that function to exclude

other differentiation programs that may interfere with the decidual differentiation program.

Previous transcriptomic studies^{3,6–9} of decidualization have concentrated on the early phase, and our data justify this approach as a means to understand the initiation of decidualization. Also, previous studies comparing the early 3-day to late 8-day decidualization phases suggest that *in vitro* 3-day treatment roughly corresponds to the *in vivo* implantation window.^{10,11} However, concentrating solely on the early phase does not afford a window on the transition of transcriptional programs to the 2-week secretory phase of the menstrual cycle.

Transition in Inflammatory Regulatory Programs From Early to Late Decidualization

Our observations suggest a transition between the dominance of two well-defined inflammatory transcriptional programs, from STAT dominated signaling previously linked to early decidualization⁵ to a later stage of increased NF- κ B signaling, which was previously linked to menstruation.^{65,66} STAT proteins are also known to induce differentiation of other cell types, for example, specific Th and regulatory T-cell subsets,⁵⁹ and importantly are known to control a wide array of inflammatory cytokine signaling.

We suggest that STAT signaling is likely a core transducer of classical markers of decidualization as well as prolactin signaling. This conclusion is based on the observation that many *STAT* paralogs exhibit a concerted transcriptional pattern of early upregulation and late downregulation. On the other hand, inhibitory NF- κ B TFs, such as, *NF κ B1 α* and *GILZ*, display a similar pattern, whereas activatory NF- κ TFs, such as NF- κ B p65, are not differentially regulated during the decidual phases. The NF- κ B pathway is particularly relevant for decidualization in the light of the previous observation that NF- κ B p65 and PGR are mutual repressors.⁶⁷ A recent study suggested that repression of PGR by NF- κ B p65 may mediate functional progesterone withdrawal in a mouse model.⁶⁸ Strikingly, in human DSCs a co-immunoprecipitation time series revealed that NF- κ B p65 and PGR interaction is strongest during late decidualization.⁶⁸ Thus, in the early phase, PGR-induced *NF κ B1 α* and *GILZ* may act as a sink and exhaust a considerable proportion of free NF- κ B p65. As the levels of *NF κ B1 α* and *GILZ* decrease in progressed DSCs, this repression is subsequently relieved, and free NF- κ B p65 may repress PGR and prime functional progesterone withdrawal. In our experiments, the decidualization media containing MPA was replaced every second day, and thus progesterone withdrawal from the media cannot explain that pattern of transcription of NF- κ B inhibitors. Rather, these results suggest that there might be a cell autonomous NF- κ B-mediated anticipation of progesterone withdrawal toward the end of the menstrual cycle.

Of secreted cytokines, we observed robust early upregulation and late downregulation of anti-inflammatory *IL15*, which is known to be transcriptionally regulated by *HOXA10*⁶⁹ and STAT signaling as well as late upregulation of proinflammatory *IL8* and *IL11*, observations of which are consistent with

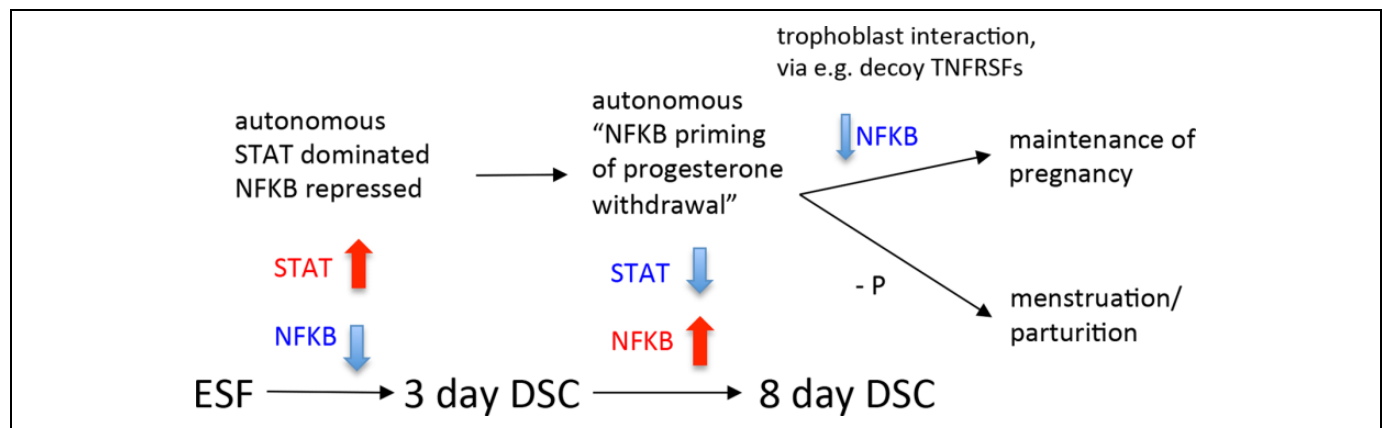


Figure 6. Illustration of the transcriptional changes of selected inflammatory TF pathway components during decidualization that represent the proposed STAT-to-NF-κB transition. Activatory STATs and repressory NF-κB pathway TFs are upregulated in the early phase and downregulated in the late phase. The late-phase release of NF-κB repression suggests autonomous NF-κB-mediated anticipation of progesterone withdrawal toward the end of the menstrual cycle. Late-phase upregulation of repressory NF-κB pathway decoy receptors (such as *TNFRSFs*) may constitute a distinct regulatory loop to counteract proapoptotic effects of inflammation. This late-phase effect is directly amenable for extracellular modification from other cell types such as trophoblasts and thus may be linked to maintenance of pregnancy. NF-κB indicates nuclear factor κB; STAT, signal transducer and activator of transcription; TF, transcription factor.

their being known NF-κB targets.^{43,44} Notably, IL8 has been linked to progesterone withdrawal-induced and NF-κB-related inflammation⁶⁵ as well as being a target of the core decidualization gene *FOXO1*.⁷⁰ Taken together, we suggest a hypothesis, whereby decidualization is marked by an STAT-to-NF-κB transition that controls a physiological inflammation reaction and anticipates the progesterone withdrawal toward the end of the menstrual cycle (Figure 6). Further, the analysis of genes that are coexpressed in DSCs and in EVT and SYN suggests that the autonomous NF-κB upregulation may be counteracted by cell-to-cell interactions. The NF-κB pathway decoy receptors osteoprotegerin (*TNFRSF11B*), *TNFRSF10C* and *TNFRSF10D*, are upregulated in the late phase of decidualization and among other receptor pathways, hypothetically, could mediate NF-κB repression that contributes to the maintenance of pregnancy.

Proliferation, Quiescence, and Cell Survival During Early and Late Decidualization

Decidualization is accompanied by cellular stress, including oxidative stress.⁴⁷ PGR signaling induces gene responses that counteract stress, with FOXO1 playing the function of sentinel and having the ability to either induce or counteract cell death at the cellular level.^{47,70,71} NRF2 (NFE2L2) signaling is a general inducer of antioxidant and oxidative stress responses involved in response to injury and inflammation.⁴⁶ We found that NRF2-mediated protective oxidative stress responses were induced specifically in the early phase and are subsequently downregulated in the late phase, as suggested by concerted regulation of numerous “redox genes.” This indicates an early burst of reactive oxygen species (ROS) and oxidative stress that are subsequently regulated by cellular redox mechanisms. While to our knowledge the functional relationship between

FOXO1 and NRF2 and their capacities to regulate ROS in DSC have not been studied, there is ample data to suggest that a coordinated response is likely.⁷² If this interaction does occur, our data indicate that it would most likely be phase specific.

Overall, our analysis indicates that DSCs exhibit transcriptional regulatory programs that have temporally distinct profiles involved in proliferation as well as inflammation and oxidative stress. The early downregulation of GO terms associated with cell cycle, mitosis, and DNA replication is consistent with the transition of DSCs to a quiescent state. We tested the reversibility of this quiescent state and observed that for cells cultured in decidualization media for 3 days, withdrawal of decidualization treatment restored proliferation, whereas after 8 days, we observed restoration of proliferation in a minority of cells. We further observed elevated PI staining in cells cultured for 8 days compared to 3 days, further supporting the hypothesis that DSCs become senescent with time.⁷⁰ However, removal of the decidual stimulus resulted in similar levels of cell death for both phases. This observation suggests that apoptotic pathways are poised from early decidualization onward.

Decidualization Phases in an Evolutionary Context

In a previous article, we provided evidence that the gene regulatory network of decidualization is derived from a cellular stress response that likely existed in the therian ancestor of eutherian mammals.¹⁹ In this previous study focusing on ESF from the opossum (*Monodelphis domestica*), treatment with cAMP and MPA induced a cellular stress response that led to apoptosis. This stress response was accompanied by elevated levels of ROS and upregulation of TRAIL ligands and receptors, which ultimately led to increased cellular death. Our data and analyses presented here in the current study have direct

implications for the evolutionary hypothesis regarding the origin of DSC. Here, in human ESF, we see a distinct stress response during the early phase of decidualization that subsequently wanes into a distinct late-phase decidualization transcriptional program. Although we do not offer a formal comparison of the gene expression datasets between opossum and human ESF stimulated with cAMP and MPA, multiple lines of evidence discussed here regarding NRF2-related oxidative stress and apoptosis-related TRAIL signaling are strikingly similar to data observed in opossum ESF stimulated with cAMP/MPA, adding further support to the hypotheses regarding the evolutionary origin of DSC put forward in Erkenbrack et al.¹⁹ Furthermore, we suggest that the gene regulatory network driving the second phase of decidualization described here is evolutionarily derived within the placental-mammalian clade and likely was appended onto the decidualization regulatory program in order to restrain the observed earlier stress response. Thus, the ancestral mammalian lineage leading to extant eutherian mammals likely co-opted this stress response to cope with invasive placentation and menstruation, grafting a novel developmental regulatory program onto a more ancient program along the way.

Authors' Note

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Supplemental Material

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