



# Defective decidualization during and after severe preeclampsia reveals a possible maternal contribution to the etiology

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**In preeclampsia (PE), cytotrophoblast (CTB) invasion of the uterus and spiral arteries is often shallow. Thus, the placenta's role has been a focus. In this study, we tested the hypothesis that decidual defects are an important determinant of the placental phenotype. We isolated human endometrial stromal cells from nonpregnant donors with a previous pregnancy that was complicated by severe PE (sPE). Compared with control cells, they failed to decidualize in vitro as demonstrated by morphological criteria and the analysis of stage-specific antigens (i.e., IGFBP1, PRL). These results were bolstered by global transcriptional profiling data that showed they were transcriptionally inert. Additionally, we used laser microdissection to isolate the decidua from tissue sections of the maternal-fetal interface in sPE. Global transcriptional profiling revealed defects in gene expression. Also, decidual cells from patients with sPE, which dedifferentiated in vitro, failed to redecidualize in culture. Conditioned medium from these cells failed to support CTB invasion. To mimic aspects of the uterine environment in normal pregnancy, we added PRL and IGFBP1, which enhanced invasion. These data suggested that failed decidualization is an important contributor to down-regulated CTB invasion in sPE. Future studies will be aimed at determining whether this discovery has translational potential with regard to assessing a woman's risk of developing this pregnancy complication.**

in studying the CTB subpopulation that invades the uterine wall in the context of this syndrome. Targeted analyses of particular molecular families, such as the vascular-type adhesion molecules that are up-regulated as the extravillous CTBs enter the uterine wall, revealed focal defects in differentiation (8). These results were confirmed and augmented by global transcriptional profiling of CTBs isolated from the placentas of affected pregnancies as they differentiated along the invasive pathway over a period of 48 h in culture (9). The surprising finding that the abnormal pattern of gene expression autocorrected to control levels by the end of the culture period pointed to a potentially important role for paracrine effectors. In this regard, the decidua, which supports placental growth and function, is a prime candidate.

In humans, formation of the decidua does not depend on the presence of a conceptus (10). This progressive process, which involves hormonally regulated differentiation of human endometrial stromal cells (hESCs), begins during the midsecretory phase of the menstrual cycle (11). The transformation is initiated in areas that are immediately adjacent to the uterine spiral arteries, ultimately spreading throughout the entire endometrium (12). In vivo and in vitro, this process is driven by increasing levels of progesterone and local cAMP production (13–15), which

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**P**reeclampsia (PE), which affects ~8% of first-time pregnancies, impacts 8 million mother–infant pairs worldwide each year (1, 2). This complication, which is specific to human pregnancy, is characterized by the new onset of hypertension, proteinuria, and other signs of maternal vascular damage such as edema (3). Severe PE (sPE) is diagnosed based on a further elevation of blood pressure (systolic pressure  $\geq 160$  mm Hg or diastolic pressure  $\geq 110$  mm Hg) or any of the following: thrombocytopenia, impaired liver function, progressive renal insufficiency, pulmonary edema, and the new onset of cerebral or visual disturbances (4). Currently, the only definitive cure is delivery of the placenta and therefore the infant. As a result, PE accounts for 15% of preterm births in the United States. Despite decades of research, a full understanding of PE pathogenesis remains elusive, which compounds the difficulties involved in the identification of predictive biomarkers and the development of targeted therapeutic strategies.

It is widely believed that the placenta plays a central role, with deficient cytotrophoblast (CTB) invasion of uterine spiral arterioles being a casual factor (5, 6). Currently, the pathogenesis of PE is conceptualized in a two-stage model, with the placental defect precipitating an abnormal maternal response that manifests as the signs (7). Accordingly, there has been a great deal of interest

## Significance

**We provide evidence of a decidualization defect in the endometrium of women with severe preeclampsia (PE) that was detected at the time of delivery and persisted years after the affected pregnancy. We went on to link this defect to impaired cytotrophoblast invasion. The transcriptional signature of the defect could enable its detection before (or after) conception, which would aid the development of therapies focused on improving decidualization and perhaps preventing severe PE.**

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stimulate the synthesis of a complex network of intracellular and secreted proteins that regulate decidualization (16).

Morphologically, this process is characterized by the transformation of elongated fibroblast-like cells into an enlarged polygonal- or round-shaped population, a process that involves complex cytoskeletal rearrangements (17). Actin, which is concentrated in the cortex of decidual cells, most often in a filamentous form (i.e., F-actin), regulates the intracellular reorganization and resulting shape changes (18–20). Decidualization can also be viewed as a process whereby the cells acquire a secretory phenotype. Decidualized hESCs secrete specific products such as prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1). Thus, these proteins have been widely used as markers of decidualized cells (21). They also play an important role in endometrial differentiation and control of CTB invasion (22, 23). Overall decidualization helps regulate embryo implantation, and subsequently, CTB interactions with the uterus, making this process an essential component of establishing the maternal–fetal interface during normal pregnancy (24). Conversely, suboptimal decidualization can lead to aberrations in placentation and adverse pregnancy outcomes (25).

The uterine contribution to the etiology of sPE has received little attention compared with the placenta's role. Only recently, global transcriptional profiling of chorionic villus samples pointed to insufficient or defective decidualization in pregnancies that were later complicated by sPE (26). In the present study, we hypothesized that aberrant decidualization could be an important contributor to the phenotypic alterations in placentation that are associated with this pregnancy complication. To test this theory, we used two approaches. First, we isolated hESCs from women with previous sPE pregnancies and decidualized the cells in vitro. In parallel, we used laser microdissection to isolate decidual cells from the maternal–fetal interface of affected pregnancies. In both cases, global transcriptional profiling revealed profound sPE-associated decidualization defects compared with controls. Furthermore, conditioned medium (CM) from freshly isolated decidual cells of women who experienced sPE failed to support CTB invasion in vitro. To mimic the decidual microenvironment during normal pregnancy, we added two secreted biomarkers of decidual cells (PRL and IGFBP1), which improved CTB invasion. Together, the results suggested that a hESC decidualization defect, which can be detected at the time of delivery for sPE, is evident months to years after the affected pregnancy. Whether detection is possible before the conception cycle of an sPE pregnancy remains to be determined. We propose that the gene signature that is associated with defective decidualization in sPE could be used to explore this question in a high-risk population. Additionally, our findings suggested that therapies designed to improve decidualization could be a novel strategy in preventing this pregnancy complication.

## Results

**Failure of hESCs from Women with a Previous sPE Pregnancy to Decidualize in Vitro.** We assessed decidualization of hESCs isolated from endometrial biopsies of patients in whom sPE developed in a previous pregnancy ( $n = 13$ ) compared with control patients who had normal obstetric outcomes ( $n = 13$ ). The maternal and neonatal characteristics of the participants are summarized in *SI Appendix, Table S1*. hESCs were decidualized by treatment with cAMP and medroxyprogesterone acetate (MPA) for 5 d. As experimental controls, cells from the same donor were cultured in parallel without these additives. Localization of F-actin in decidualized cells from women with uncomplicated pregnancies showed the expected cytoskeletal reorganization and shape changes that were consistent with transformation from a fibroblast to a decidual phenotype (Fig. 1*A*). In contrast, hESCs from women who had sPE failed to undergo these changes (Fig. 1*B*). In nondecidualized hESCs, PRL (Fig. 1*C–E*) and IGFBP1 (Fig. 1*F–H*) levels detected in CM were low and not statistically different between the two groups. As expected, secretion of both molecules greatly increased upon decidualization of most of the control cultures, but hESCs from patients with former sPE failed to show this dramatic increase (Fig. 1*D, E, G, and H*). This

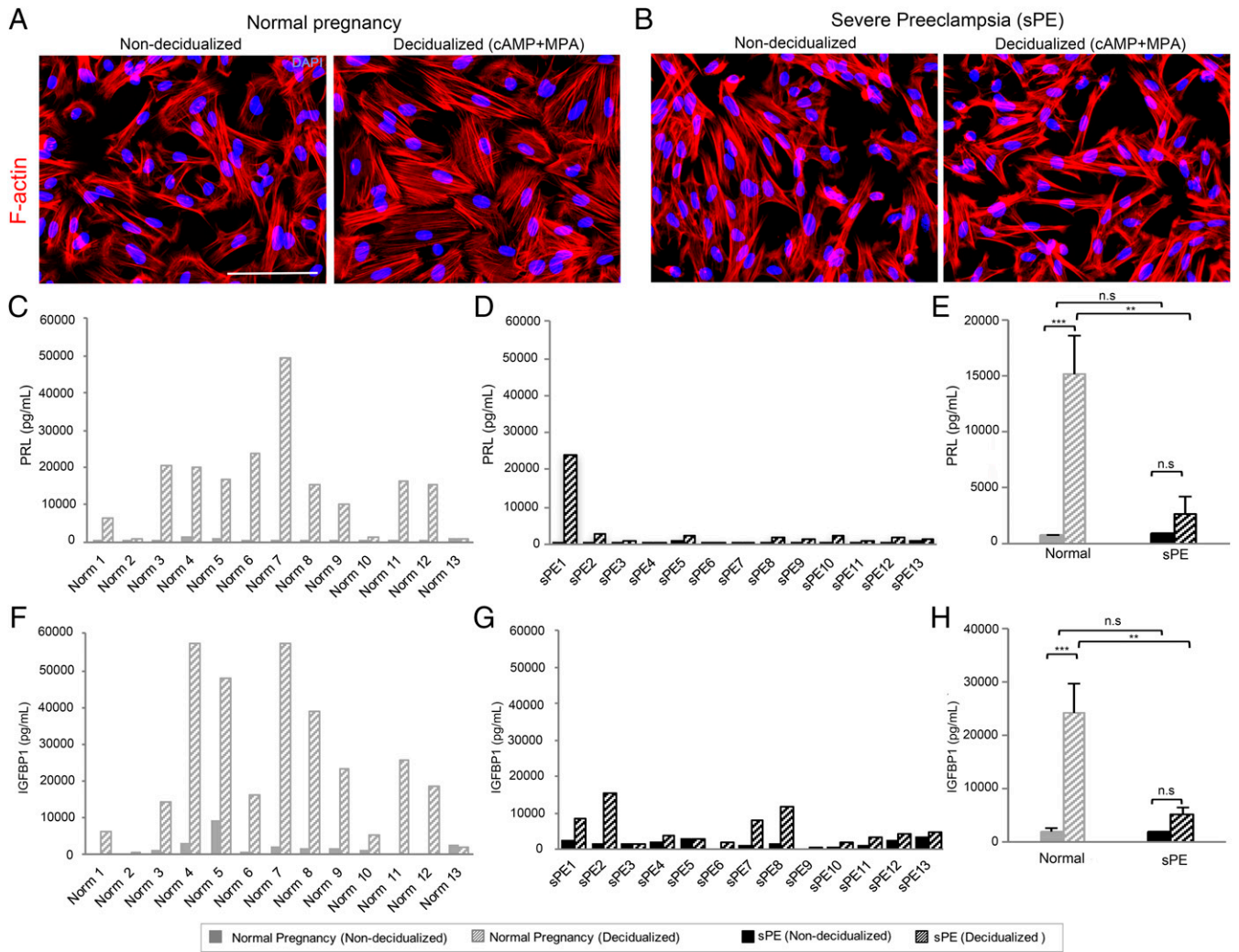
finding suggested that in vitro decidualization was impaired in hESCs obtained from patients with former sPE compared with controls.

**Alterations in the Global Transcriptional Profiles of Decidualized hESCs from Patients with Former sPE.** Next, we used a microarray strategy to identify the molecular changes underlying the functional decidualization defect found in hESCs from women who had experienced sPE. Specifically, we carried out a transcriptomic analysis of hESCs that were established from the normal pregnancy and sPE groups described earlier, nondecidualized and decidualized in vitro (Fig. 2*A*). The clinical characteristics of the endometrial donors are shown in *SI Appendix, Table S2*. An overview of the results is presented in Fig. 2*B*. In the nondecidualized state, only five statistically significant genes were differentially expressed between the control and the sPE samples, and the fold differences were modest (Fig. 2*C*). Thus, in a basal state, the hESCs from patients with former sPE were very similar to those from control women.

During decidualization of the samples from control donors, the expression of 74 genes was significantly regulated based on a false discovery rate (FDR)  $<0.05$  and at least twofold differentially expressed (Fig. 2*D*; complete list provided in *SI Appendix, Table S3*). They included genes known to be involved in decidualization. Some were up-regulated [e.g., CANNABINOID RECEPTOR 1 (*CNRI*), INSULIN RECEPTOR SUBSTRATE 2 (*IRS2*), and MONOAMINE OXIDASE A (*MAOA*)] and others were down-regulated [e.g., COCHLIN (*COCH*), LIM DOMAIN 2 (*LMO2*), and ACTIN FILAMENT ASSOCIATED PROTEIN 1 (*AFAP1L2*)] (16, 27–31). At the pathway level, processes that are relevant to decidualization, including regulation of oxygen responses, insulin secretion, and proliferation, were up-regulated (*SI Appendix, Fig. S1A*). No pathways were significantly down-regulated.

Consistent with the results shown in Fig. 1, the comparison between nondecidualized and decidualized hESCs isolated from patients with former sPE failed to detect the modulated expression of any genes [i.e., zero differentially expressed genes (DEGs); Fig. 2*B*]. In contrast, comparing the transcriptomes of the decidualized cells in the two groups revealed 129 misexpressed genes (at least twofold; Fig. 2*E*; complete list in *SI Appendix, Table S4*). mRNAs with expression patterns that were validated by quantitative RT-PCR (qRT-PCR) analyses (*SI Appendix, Fig. S2*) are denoted with asterisks in Fig. 2*E* and *SI Appendix, Table S4*. The DEG included the up-regulation of mRNAs encoding molecules that are involved in hormone conversion (*HSD17B2*), extracellular structure organization (*LAMA5*, *SULF1*, and *ITGA11*), vascular development (*ANGPT2*, *EGRI*, and *RELAXIN2*), and response to peptides (*KLF2*, *SSTR1*, and *IGFBP5*; *SI Appendix, Fig. S1B*). The down-regulated category included genes that play important roles in decidualization (e.g., *IGFBP1*, *CNRI*, and *IL-1B*). The latter group functioned in numerous pathways such as cytokine–receptor interactions; response to wounding, inflammation, and estrogen (late); and TFG- $\beta$  signaling (*SI Appendix, Fig. S1B*).

Finally, we analyzed the overlap between the DEGs in the nondecidualized vs. decidualized hESC comparison from women who had normal pregnancies (Fig. 2*D*) and the sPE vs. normal pregnancy group following decidualization (Fig. 2*E*). Fifteen genes were up-regulated during normal hESC decidualization and down-regulated during decidualization of hESC from women with sPE. They included signaling molecules such as CANNABINOID RECEPTOR 1 (*CNRI*), INSULIN RECEPTOR SUBSTRATE 2 (*IRS2*), and LY SOPHOSPHATIDIC ACID RECEPTOR 1 (*LPAR1*); an ACTIN BINDING LIM PROTEIN FAMILY, MEMBER 2 (*ABLIM2*); and A LATENT TRANSFORMING GROWTH FACTOR BETA BINDING PROTEIN 1 (*LTPBP1*) with important functions during decidualization (*SI Appendix, Fig. S1C*). In contrast, seven genes were down-regulated during normal decidualization and up-regulated in the equivalent samples from patients with former sPE (*SI Appendix, Fig. S1D*). They included molecules such as COAGULATION FACTOR C HOMOLOG, COCHLIN (*COCH*), and CORNICHON FAMILY AMPA RECEPTOR AUXILIARY PROTEIN 3 (*CNIH3*). Thus, we concluded



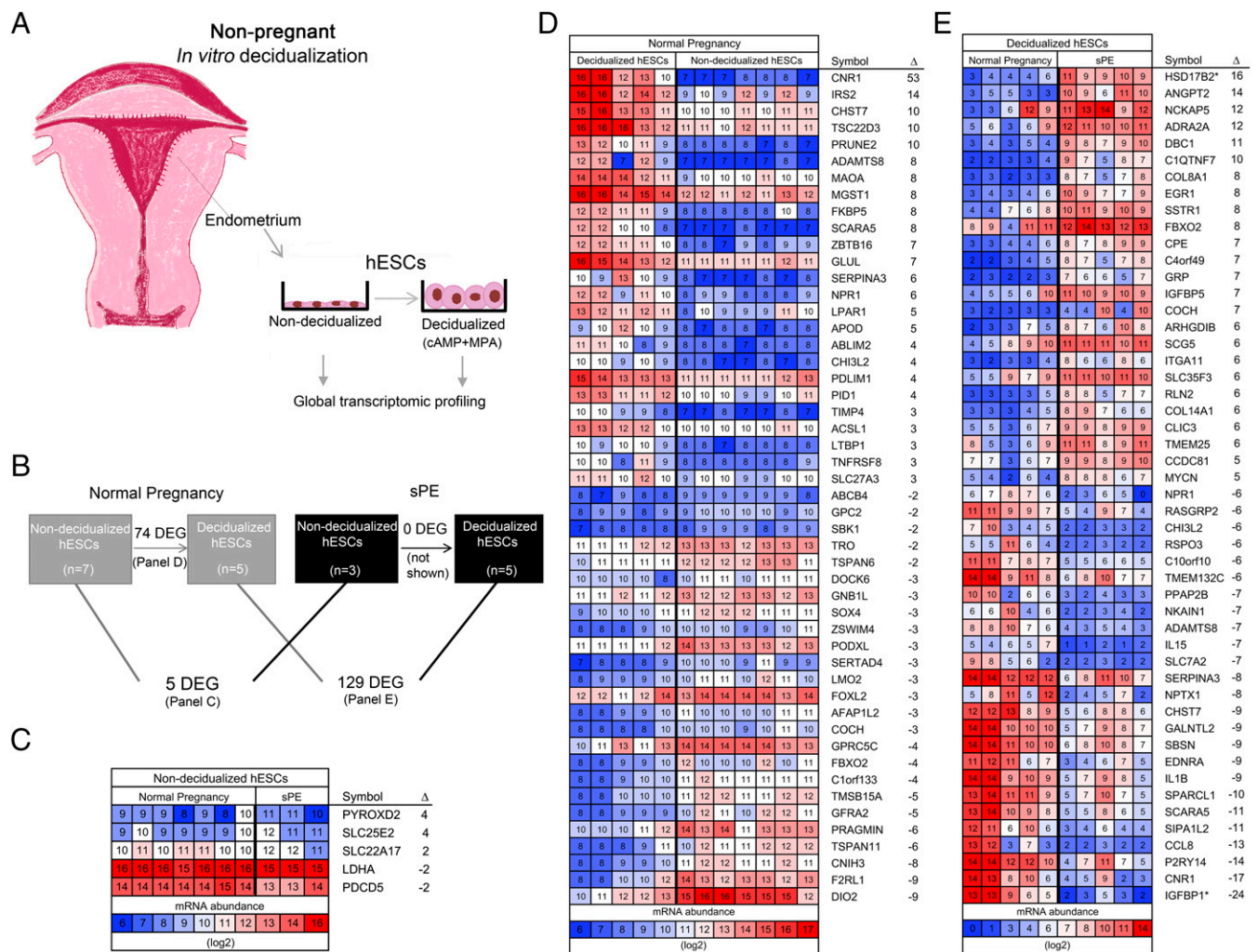
**Fig. 1.** In vitro decidualization of hESCs was impaired in patients with previous pregnancies that were complicated by sPE. hESCs were obtained from nonpregnant women, patients with former sPE, or subjects who had a normal (norm) pregnancy outcome. The cells were decidualized by culturing for 5 d in the presence of cAMP and MPA. Nondecidualized cultures were maintained in the same medium without additives. (A) Localization of F-actin by rhodamine-phalloidin staining of hESCs from women with uncomplicated pregnancies showed the expected cytoskeletal reorganization and shape changes that were consistent with transformation from a fibroblast to a decidual phenotype. (B) hESCs from women who had sPE failed to undergo these changes. (C and D) PRL and (E and F) IGFBP1 secretion. (G and H) Summary of the ELISA data. Low levels of both molecules were detected in the CM of hESCs from the two donor groups before decidualization (solid bars). PRL and IGFBP1 levels greatly increased in most cultures of decidualized hESCs from donors who had normal pregnancy outcomes (gray hashed bars). In contrast, hESCs from patients with former sPE failed to show the decidualization-related increases in secretion of these molecules (black hashed bars;  $**P < 0.01$  and  $***P < 0.005$ ; n.s., nonsignificant). (Scale bar: 100  $\mu\text{m}$ .)

that the genes that had discordant expression patterns in decidualized hESCs from the two groups could play important roles in the failed in vitro transformation of cells from patients with former sPE.

**Molecular Defects in Situ of Decidua Basalis or Parietalis from Control vs. sPE Pregnancies.** We used a laser-microdissection approach to isolate portions of the decidua basalis (DB) or decidua parietalis (DP). Cells were captured from tissue sections of biopsy specimens from cases of women with sPE vs. control women [i.e., gestational age-matched samples from women who had a spontaneous preterm birth with no signs of infection (nPTB); Fig. 3A]. The clinical characteristics of the participants are summarized in *SI Appendix, Table S5*. An overview of the results is shown in Fig. 3B. In the DB, 79 genes were significantly differentially expressed in sPE vs. nPTB with modest fold changes (Fig. 3C; full list in *SI Appendix, Table S6*). They included the up-regulation of mRNAs encoding molecules involved in RNA processing. Down-regulated genes included *DEFB1* (MICROBICIDAL AND CYTOTOXIC PEPTIDE), *CP* (COPPER-CARRYING PROTEIN

IN THE BLOOD), *OGN* (PROTEOGLYCAN), and *COL8A1* (COLLAGEN VIII; major component of membrane corneal endothelium). Given the functions of defensins, we surmised that these genes were up-regulated in nPTB rather than down-regulated in sPE. The up-regulation of a select natural antimicrobial agent in the nPTB samples is likely attributable to the start of an infectious process that has not yet manifested in terms of the clinical signs (32, 33). The low yield of DEGs in this dataset was likely the result of the numerous cell types that comprise the DB and the challenges of capturing decidual cells from this complex mixture. No pathways were significantly up- or down-regulated.

In contrast, the clear boundary between the smooth chorion and the DP enabled efficient laser microdissection of the latter cells. Comparison of heat maps of the mRNA samples that were isolated from sPE cases vs. nPTB controls revealed 227 genes that were differentially expressed in sPE by at least twofold (Fig. 3D; complete list in *SI Appendix, Table S7*). The up-regulated genes encoded molecules with immune functions such as *PRG2* and *KLRF1*. Other genes in this category included *RNASE2*,



**Fig. 2.** Global transcriptional profiling confirmed failed decidualization of hESCs obtained from nonpregnant women with a previous pregnancy complicated by sPE. (A) Schematic drawing of the study design. hESCs were isolated from endometrial biopsies and decidualized in vitro. The donors were non-pregnant women with previous normal pregnancy outcomes or patients with former sPE. (B) Summary of the LIMMA paired comparisons showing the number of DEGs by FDR <0.05 and at least twofold between the groups. (C) Before decidualization, there were five DEGs between hESCs from the normal pregnancy outcome group vs. patients with former sPE. (D) Heat map showing the 50 most highly DEGs (total = 74; *SI Appendix, Table S3*) that were modulated during decidualization of hESCs from donors who had normal pregnancy outcomes. (E) Heat map showing the 50 most highly DEGs (total = 129; *SI Appendix, Table S4*) that were misexpressed following decidualization of hESCs from donors with a former sPE pregnancy compared with those with normal pregnancies (\*mRNA expression patterns validated by qRT-PCR; Δ, fold change).

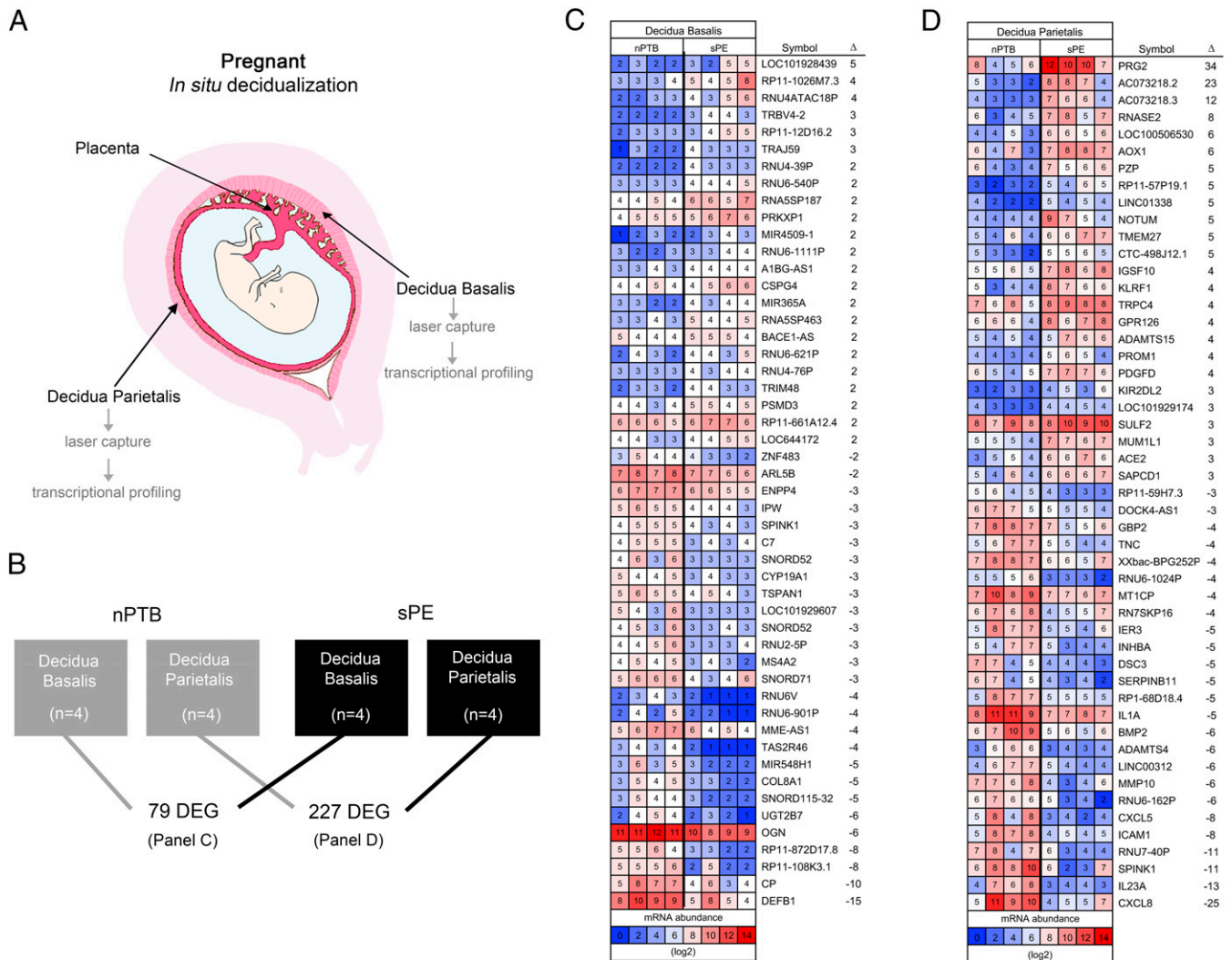
*PZP*, *PDGFD*, a Wnt inhibitor (*NOTUM*), and *PROM1*, which plays a role in the maintenance of adult stem cells. *AOX1*, which catalyzes the formation of superoxide and NO, was also up-regulated, a possible sign of oxidative stress. At a pathway level, regulation of cell communication, several metabolic processes, and transmembrane receptor protein tyrosine kinase signaling (among others) were significantly up-regulated (*SI Appendix, Fig. S3*).

The down-regulated mRNAs included ILs (*CXCL8*, *IL23A*, *IL1A*), *CXCL5*, as well as proteinases and their inhibitors (*SPINK1*, *ADAMTS4*, and *MMP10*) that play important roles during decidualization. At a pathway level, regulation of cell adhesion, locomotion/migration, morphogenesis, extracellular structure, and immune processes were impacted (*SI Appendix, Fig. S3*). However, neither PRL nor IGFBP1 were identified as misexpressed at the mRNA level. This suggested that a posttranslational mechanism could be involved in repressing their expression at the protein level (34).

We validated the microarray results at the protein level for three DEG (*PEG1/MEST* and *PRG2*, up-regulated in sPE; *BMP2*, down-regulated in sPE). In these experiments, we used an immunolocalization approach applied to tissue sections of the fetal membranes

with the adjacent DP. In all cases, the protein-level results confirmed the expression patterns that were suggested by the transcriptomic data (*SI Appendix, Fig. S4*).

**Absence of Decidualization Markers in sPE Pregnancies.** To analyze decidualization in situ, we assessed PRL (Fig. 4 A and B) and IGFBP1 (Fig. 4 C and D) expression in tissue sections of the DB and the DP in sPE ( $n = 5$ ) compared with nPTB ( $n = 4$ ). The clinical characteristics of the pregnancies are summarized in *SI Appendix, Table S8*. CTB identity was confirmed by anti-cytokeratin 7 (CK7; Fig. 4 A–F) immunoreactivity, and stromal cells were visualized with anti-vimentin (VIM; Fig. 4 E and F). The results showed that PRL and IGFBP1 were broadly expressed by decidualized stromal cells (DB and DP) in control nPTB samples (Fig. 4 A and C). In contrast, expression of both decidualization markers was greatly reduced and, in many instances, absent in the sPE samples (Fig. 4 B and D). These data were additional evidence that sPE is associated with widespread defects in decidualization that were evident in samples obtained immediately after delivery.

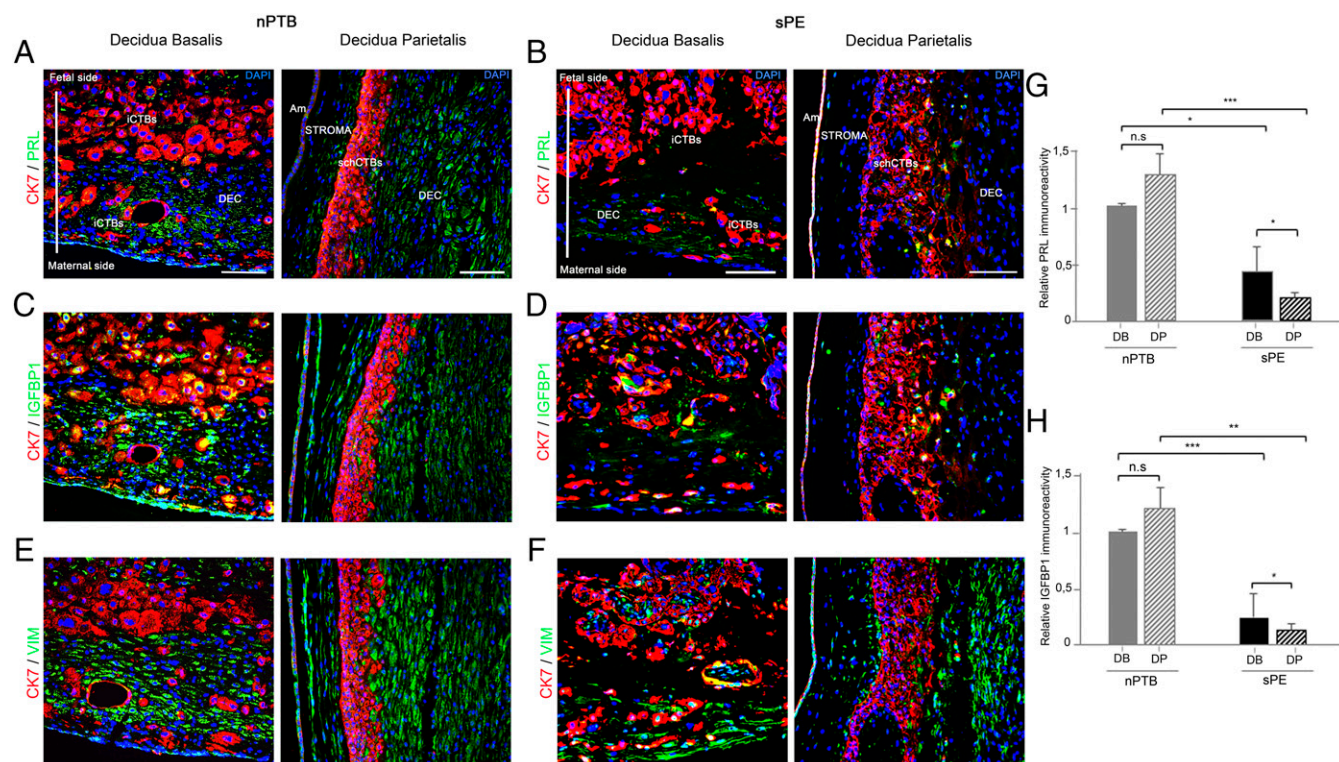


**Fig. 3.** Global transcriptional profiling of the DB and the DP revealed the DEGs in sPE vs. control pregnancies. (A) Schematic drawing of the study design. Laser microdissection enabled isolation of portions of the DB from the basal plate and DP, which was adjacent to the fetal membranes. (B) Summary of the LIMMA paired comparisons showing the number of DEGs between equivalent decidual compartments in sPE vs. nPTB (FDR < 0.05 and fold change  $\geq 2$ ). (C) Heat map showing the 50 most highly DEGs (total = 79; *SI Appendix, Table S6*) in the DB of nPTB vs. sPE patients. (D) Heat map showing the 50 most highly DEGs (total = 227; *SI Appendix, Table S7*) in the DP of nPTB vs. sPE patients.

**Failure of Decidualization Marker Expression in Freshly Isolated Stromal Cells from sPE Decidual Biopsies.** Next, we isolated decidual cells from sPE ( $n = 5$ ) or nPTB cases ( $n = 4$ ) with the goal of determining their status in terms of expressing stage-specific antigens that are typically associated with these cells. Freshly isolated stromal cells that were cultured overnight did not react with antibodies that were specific for markers of endothelial or hematopoietic cells, including macrophages. Immediately after plating, rhodamine-phalloidin immunostaining showed the expected pattern of F-actin distribution in polygonal/round cells that were isolated from the DB or DP from control nPTB samples (Fig. 5A). In contrast, stromal cells from decidual biopsy specimens of patients with sPE had an elongated morphology with a fibroblast-like F-actin organization (Fig. 5B). Immunostaining with anti-PRL (Fig. 5C and D) or anti-IGFBP1 (Fig. 5E and F) showed that VIM-positive stromal cells from sPE deciduas (Fig. 5G and H) had much lower antibody reactivity than was observed in the control nPTB samples. Finally, we quantified the cell secretion of PRL (Fig. 5I) and IGFBP1 (Fig. 5J) after overnight culture. In nPTB, production of both molecules was higher by cells isolated from the DP compared with the DB. In comparison, sPE was associated with a dramatic reduction in PRL and IGFBP1 secretion by cells isolated from both compartments.

These results constituted additional evidence of defective decidualization in sPE.

**Failure of Stromal Cells Isolated from Decidual Biopsy Specimens Obtained at Delivery from Patients with sPE to Redecidualize *In Vitro*.** Next, we asked whether the isolated stromal cells that were cultured for three to five passages could rededicalize *in vitro*. To answer this question, we monitored morphological changes and secreted biomarkers (PRL and IGFBP1) of this process after 5 d of hormone treatment. In cells from nPTB control patients, regardless of their compartment of origin, decidualization was associated with a characteristic polygonal/round phenotype as demonstrated by rhodamine-phalloidin immunostaining (Fig. 6A). In contrast, stromal cells from patients with sPE failed to display morphological changes during rededicalization (Fig. 6B). In control cells following decidualization, secretion of PRL (Fig. 6C) and IGFBP1 increased (Fig. 6D). In contrast, the equivalent cells isolated and cultured from patients with sPE failed to increase secretion of either molecule (Fig. 6C and D) in response to MPA and cAMP treatment. Thus, isolation and culture did not rescue defective decidualization of stromal cells from patients with sPE.



**Fig. 4.** sPE is associated with a striking down-regulation of PRL and IGFBP1 expression in the decidua (DEC). Tissue sections of the maternal/fetal interface that contained portions of the DB or the DP were coimmunostained with an antibody against CK7, which enabled visualization of CTBs, and decidual markers PRL (A and B) and IGFBP1 (C and D). (E and F) Adjacent sections were stained with anti-VIM, which labeled DEC cells. Nuclei were visualized with DAPI. In nPTB samples, anti-PRL and anti-IGFBP1 gave strong signals in the DB and DP (CK7-negative, VIM-positive). In contrast, the VIM-positive DEC cells in sPE samples failed to react with anti-PRL and anti-IGFBP1 or stained weakly for these markers. (G and H) The immunolocalization results shown in A–D were semi-quantified in ImageJ. Representative areas ( $n = 3$  per sample) of the DB and DP from nPTB and sPE cases were subjected to ImageJ analyses. The results (intensity/area [ $0.3 \text{ mm}^2$ ]) were expressed relative to the data from the control/nPTB DB samples. Data are expressed as the mean  $\pm$  SEM (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , and \*\*\* $P \leq 0.001$ ). Three or four representative areas of each sample were analyzed (sPE,  $n = 5$  cases; nPTB,  $n = 4$  cases). Am, amnion; iCTBs, invasive CTBs; schCTBs, smooth chorion CTBs. (Scale bars:  $100 \mu\text{m}$ .)

**Failure of CM from sPE Decidual Cells to Promote CTB Invasion.** To understand the functional impact of the sPE-associated decidualization defect, we asked whether this phenomenon was mechanistically related to reduced CTB invasion in this pregnancy complication. As our approach, we isolated stromal cells from samples of the DB and the DP of sPE ( $n = 4$ ) or control nPTB ( $n = 3$ ) cases. They were cultured overnight. Then, the CM was collected, and an sPE-associated down-regulation of PRL and IGFBP1 secretion in sPE compared with nPTB cultures was confirmed (SI Appendix, Fig. S5). Accordingly, experimental or control CM was added to second-trimester CTBs ( $n = 10$  placentas), which were cultured on a Matrigel substrate. Invasion was assayed by counting the number of CTBs or their cellular processes that reached the underside of the filters (Fig. 7A). In the presence of the control (i.e., nPTB) CM, robust invasion was observed, which was not statistically different between the CTBs that were cultured in the DB or the DP samples (Fig. 7B). In contrast, the CM from the equivalent stromal-cell populations of sPE cases did not support CTB invasion.

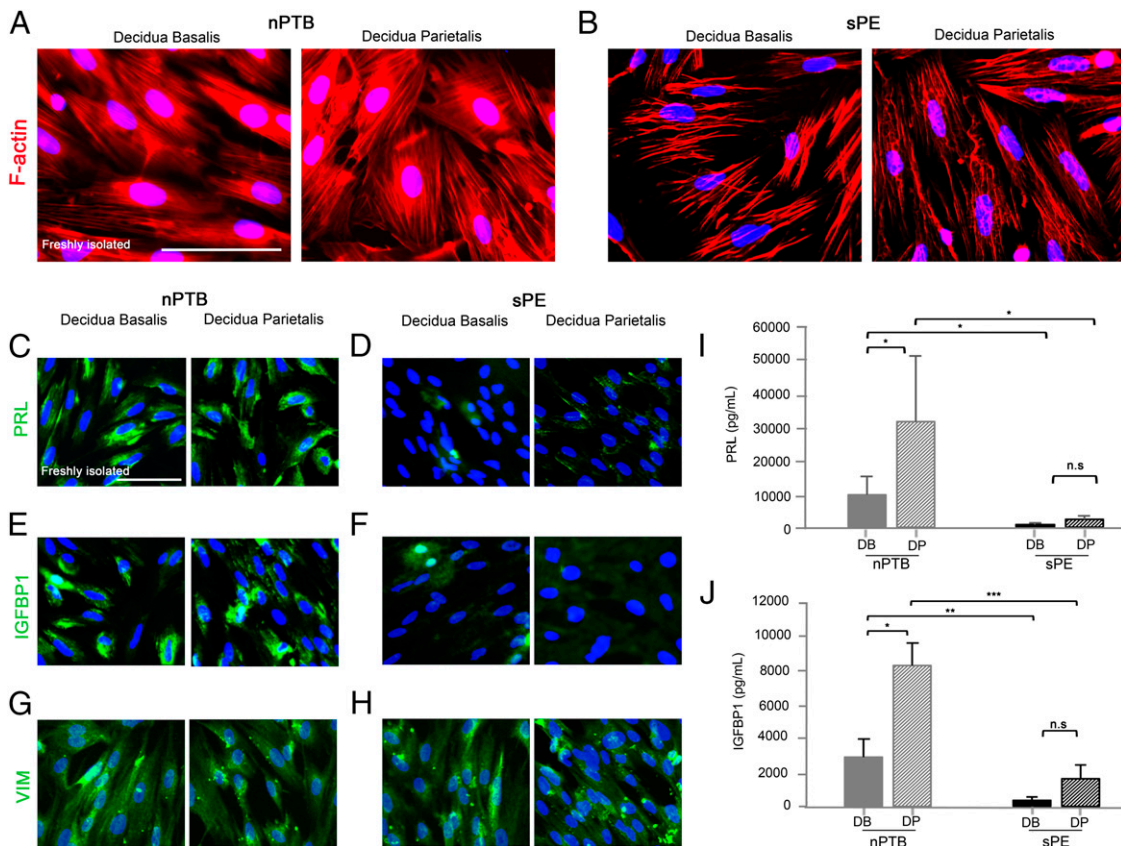
Finally, we sought to establish a link between reduced PRL and IGFBP1 secretion by decidualized stromal cells in the setting of sPE and the inability of CM from these cells to stimulate CTB invasion. When the cells were cultured in fresh medium rather than CM, few CTBs reached the filter undersides (Fig. 7C). This suggested that the nPTB decidual cells released factors that promoted this process. The addition of PRL or IGFBP1 (10 ng/mL) to fresh medium had little effect. However, acting together, these two factors significantly increased invasion. Thus, the intrinsic decidual stromal defects that were observed in sPE had paracrine effects on CTBs that were consistent with the reduced invasion and

shallow placentation that is frequently observed in this pregnancy complication. In vitro, CTB invasion could be significantly increased by adding two secreted proteins that are highly expressed in normal decidua and significantly reduced in sPE, evidence of their effects on placental cells.

## Discussion

Together, the data presented here suggested that hESCs from patients with sPE, in the nonpregnant state and at the end of pregnancy, failed to decidualize. Initial experiments used an in vitro approach. Before decidualization, hESCs isolated from patients in whom sPE developed, or from control women with a previous normal pregnancy, had similar morphological features and patterns of PRL and IGFBP1 secretion. In keeping with these observations, they had nearly identical transcriptomes. However, they differed in their ability to decidualize in culture. By morphological criteria and marker expression (PRL and IGFBP1), the cells in the group that formerly experienced sPE resisted decidualization, which was confirmed by microarray analyses that showed that they were transcriptionally inert. Global transcriptional profiling identified 129 genes as differentially expressed. Approximately 20% (22 of 129) were inversely regulated, suggesting that they might play particularly important roles in normal decidualization. The mis-regulated genes functioned in many biological processes, including extracellular structure organization, tissue development, and responses to wounding, oxygen, and inflammation.

This finding prompted us to ask whether there was evidence of defective decidualization at the end of pregnancies that were complicated by sPE, which necessitated delivery in the preterm period. As gestation-matched controls, we analyzed equivalent



**Fig. 5.** Freshly isolated stromal cells from decidual biopsy specimens of patients with sPE displayed decidualization defects in culture. Cells were isolated from the DB or DP and analyzed at p0. Donors were women whose pregnancies were complicated by nPTB ( $n = 4$ ) or sPE ( $n = 5$ ). (A and B) The F-actin cytoskeleton of the cells was visualized by rhodamine-phalloidin staining, and nuclei were imaged with DAPI. In nPTB pregnancies, cells from either decidual compartment had a polygonal shape with a complex, well-developed network of actin filaments. (B) In contrast, the cells from sPE pregnancies were flattened with a much less well-developed actin cytoskeleton. Immunolocalization of PRL (C and D), IGFBP1 (E and F), and VIM (G and H). (C, E, and G) VIM-positive cells from the nPTB donors gave strong signals for the decidualization markers that were evaluated. (D, F, and H) In contrast, few of the VIM-positive cells from patients with sPE reacted with anti-PRL or anti-IGFBP1. (I and J) Secretion of PRL and IGFBP1 mirrored the immunostaining results. Data are the mean  $\pm$  SEM of each sample, which was analyzed in triplicate ( $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ ). (Scale bars: 100  $\mu$ m.)

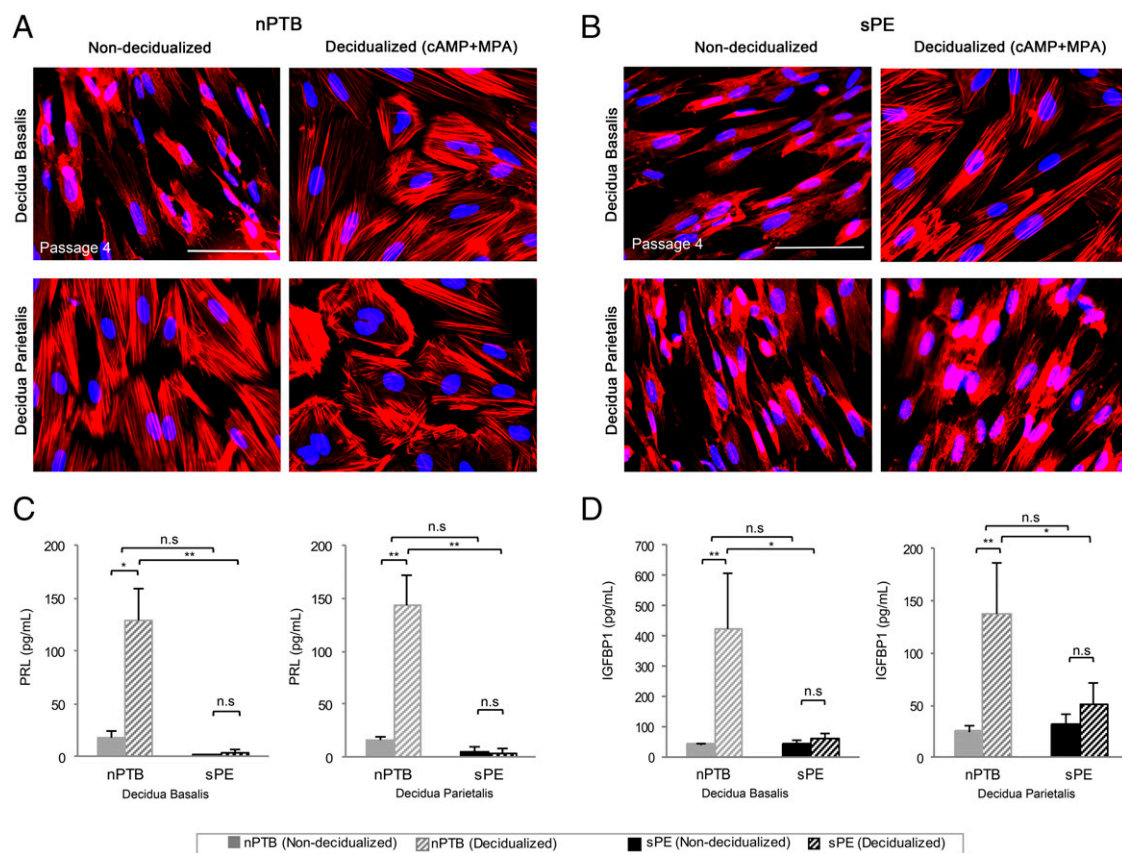
samples from women who had an nPTB, a study design that has enabled us to pinpoint changes in gene expression that are specific to sPE (9, 35). Our goal was to use laser microdissection to isolate cells from both areas of the decidua. Attempts to obtain a pure population from the DB, which contains a mixture of maternal and fetal cells, was relatively unsuccessful, as shown by the fact that a substantial number of the differentially expressed genes encoded known CTB proteins. In contrast, as a result of the clear demarcation between the CTB layer of the smooth chorion and the DP, the laser-capture approach was successful in isolating the latter cells. In all, 227 genes were differentially expressed in sPE vs. nPTB. These results suggested a striking sPE-associated dysregulation of gene expression in the DP. In accordance with this finding, immunoanalyses showed that cells of the DB and DP failed to express PRL and IGFBP1. Following isolation, passage in culture, and subsequent dedifferentiation, they also failed to redecidualize.

Finally, we wondered whether the sPE-associated decidual defect could impact CTB invasion, as is frequently observed in the basal plates of sPE pregnancies. In these experiments, CM from cultured cells established from the DP or DB following delivery as a result of sPE markedly reduced CTB invasion compared with the equivalent nPTB samples (Fig. 7B). The combined addition of PRL and IGFBP1 increased CTB invasion (Fig. 7C). Thus, we linked a potential cause of sPE—failed decidualization and a suboptimal uterine microenvironment—to a potential downstream effect: inhibition of CTB invasion.

Nevertheless, it is important to note that CTB invasion and remodeling of the spiral arteries largely occurs in the first half of

pregnancy, making this the period in which dysregulated decidual gene expression could play a role in sPE-associated defects in these processes. In this regard, a previous study analyzed gene expression by chorionic villous samples that were obtained near the end of the first trimester from pregnancies in which the women went on to develop sPE (26). The comparator was equivalent samples from women who had normal obstetric outcomes. The genes that were dysregulated in the sPE group were decidual rather than trophoblast in origin, overlapping with those that are modulated during normal decidualization in mid/late-secretory endometrium from a nonconception cycle and endometrium from tubal ectopic pregnancies. The majority (73%) changed in the opposite direction of their regulation during normal endometrial maturation. They included IGFBP1 and PRL, which we also showed were down-regulated at the protein level. Analysis of the overlap with the dysregulated genes identified in the present study (in vitro and in situ) identified nine commonalities. They included *EGRI*, *IGFBP1*, *IL15*, *IL1B*, and *NTN4* (*NETRIN*).

Additionally, Rabaglino et al. reported evidence of natural killer (NK) cell dysfunction (26). Likewise, we found differential expression of immune cell genes in microdissected decidual samples from patients with sPE, evidence that maternal leukocytes are found within the uterine lining. NK cell receptors (*KIR2DL2*) and T-cell receptors (*TRDJ3*, *TRAJ5*, and *TRAJ59*) were misexpressed in our datasets. Thus, concordance between the data presented by Rabaglino et al. (26) and in the present paper bolstered the conclusion that sPE is associated



**Fig. 6.** Cultured hESCs from decidual biopsies of patients with sPE failed to redecidualize in vitro. Cells were isolated from the DB or DP and analyzed at p3–p5, i.e., after they lost expression of the decidualization markers PRL and IGFBP1. Donors were women whose pregnancies were complicated by nPTB ( $n = 3$ ) or sPE ( $n = 4$ ). Redecidualization was induced by treatment with cAMP (0.5  $\mu$ M) and MPA (1  $\mu$ M). CM and cells were analyzed after 5 d. (A and B) Visualization of the F-actin cytoskeleton via rhodamine-phalloidin staining. Nuclei were imaged with DAPI. When hESCs from donors who gave birth with nPTB were redecidualized, they enlarged, becoming polygonal/round with a complex actin cytoskeleton. In contrast, hESCs from donors with sPE failed to undergo the expected shape changes, often exhibiting disorganized actin cytoskeletons. (C and D) PRL and IGFBP1 secretion, measured by ELISA, were consistent with failed decidualization of hESCs from patients with sPE. Data are the mean  $\pm$  SEM of each sample, which was analyzed in triplicate ( $*P < 0.05$  and  $**P < 0.01$ ; n.s., not significant). (Scale bar: 100  $\mu$ m.)

with a defect in decidualization, which is evident early in pregnancy when CTBs are remodeling the spiral arteries, and at birth. Also, both studies found evidence of differential gene expression by maternal immune cells that populate the decidua.

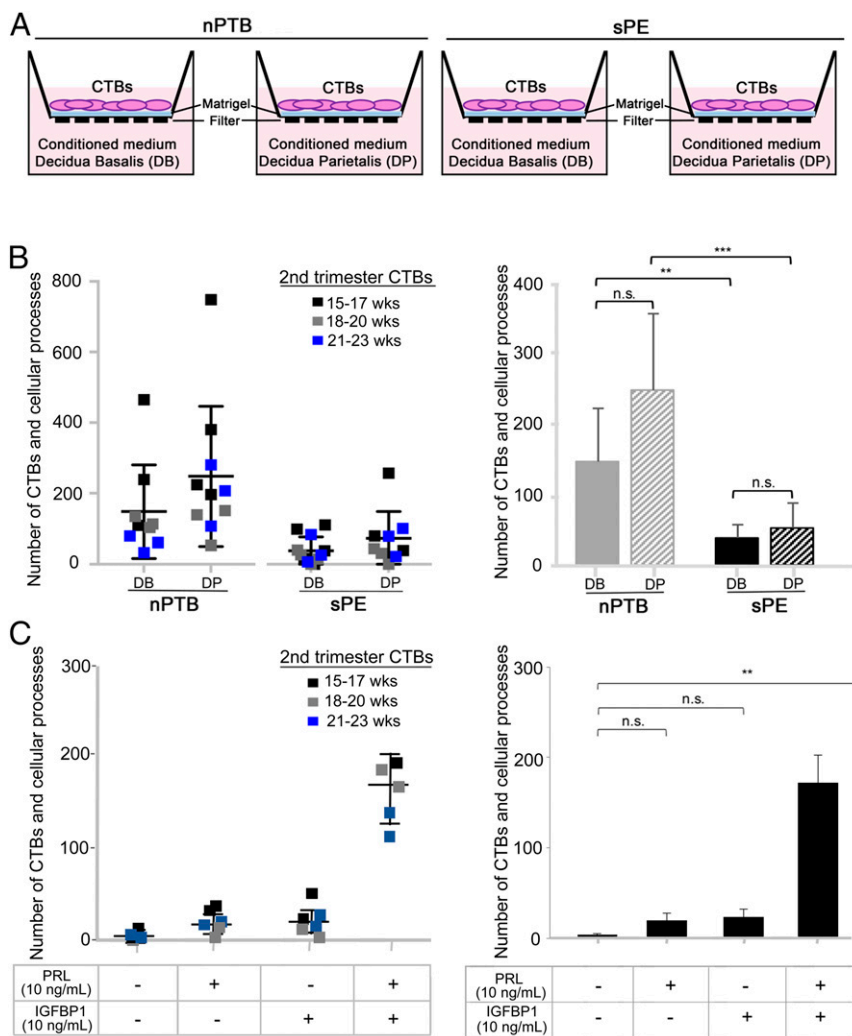
Spontaneous endometrial decidualization followed by progesterone withdrawal, which “triggers” shedding, is a major feature of the endometrium in menstruating species (e.g., humans, old-world primates, elephant shrews, and fruit bats). In contrast, the endometrium of nonmenstruating mammals decidualizes only if there is contact between an embryo and the uterus, i.e., upon implantation. Thus, in humans, the decidua controls conception and the course of pregnancy. In nonmenstruating species, the embryo controls this process as exemplified by delayed implantation in mice (36). Considered in this context, our in vitro and in situ data suggested that sPE is associated with a marked decidualization defect that the embryo is sometimes able to overcome, thereby establishing pregnancy. In this scenario, the extravillous CTB subpopulation penetrates the uterine wall and spiral arteries to the degree that is required to sustain pregnancy for varying periods of time, but short of the duration of normal pregnancy. This may be a result of the highly invasive nature of these placental cells (37), which enables them to anchor not only to the uterus, but also to ectopic sites that they penetrate in search of a blood supply (38). Thus, we concluded that failed decidualization restricts the depth of CTB invasion such that pregnancy is maintained but ends in sPE.

Several other types of data support this theory. A decidual role in PE has been suggested by epidemiological studies. For example, a maternal history of PE is associated with a 24–163%

increased risk of developing this syndrome, whereas a paternal history has no effect (39). Twin studies suggest the role of maternal and fetal gene interactions (40). Whether this is evidence of an inherited decidualization defect remains to be determined. Regarding the link between sPE in a first pregnancy and a subsequent reduction in infertility, a definitive answer has been elusive because women who experience the severe form of this pregnancy complication are less likely to attempt another pregnancy (41). However, the association of recurrent PE with a longer interpregnancy interval may be evidence of subfertility in this group (42, 43). Interestingly, some patients with normal first-time deliveries go on to develop sPE in subsequent pregnancies. We speculate that the reasons could include an age-related decline in the ability of endometrial stromal fibroblasts to decidualize. This could be a primary cause or occur secondarily as the result of a previous Cesarean section (44) or obesity (45). Finally, in women who experience sPE, subsequent normal pregnancies usually outnumber cases of recurrent PE. Nevertheless, recurrence rates as high as 65% have been reported (46). We speculate that some women in this group might regenerate a more normal endometrium during the extensive remodeling of the uterine lining that takes place after delivery. Alternatively, these may be pregnancies that are associated with faulty placentation rather than abnormal decidualization.

With regard to clinical practice, the beneficial effects of removing the placenta in an sPE pregnancy are well known, but removing the decidua in the immediate postpartum period via uterine curettage also aids in resolution of the signs, increasing the rate at which mean arterial pressure decreases and platelet counts increase (47, 48).





**Fig. 7.** CM from decidual cells of patients with sPE inhibited CTB invasion in vitro. (A) Diagram of the experimental design. Decidual cells were isolated from the DB or DP and cultured overnight, and the CM was then isolated. The donors were women whose pregnancies were complicated by nPTB ( $n = 3$ ) or by sPE ( $n = 4$ ). CTBs were isolated from second-trimester placentas (15–17 wk,  $n = 4$ ; 18–20 wk,  $n = 3$ ; 21–23 wk,  $n = 3$ ). They were cultured (72 h) on Matrigel-coated Transwell filters in medium conditioned by the nPTB or sPE decidual cells. CTBs and cellular processes that reached the undersides of the filters were counted. (B) Compared with the equivalent nPTB samples, CM from the cells of sPE donors significantly inhibited CTB invasion regardless of whether they were isolated from the DB or DP. (C) The addition of PRL and IGFBP1 (10 ng/mL each) to fresh medium increased CTB invasion to the levels that were observed when the cells were incubated in CM from nPTB cultures. Data are expressed as the mean  $\pm$  SEM of duplicate wells (\*\* $P < 0.01$  and \*\*\* $P < 0.001$ ; n.s., not significant).

Likewise, curettage exacerbates the decrease in circulating levels of sFlt-1 (49), a biomarker that has been mechanistically linked to the signs of this syndrome (50–52). In this regard, overexpression of sFlt-1 by decidual cells produces a PE-like syndrome in mice (53). Finally, a decidualization defect could help explain our previous finding that isolation and culture of CTBs from sPE placentas reversed the gene dysregulation that was initially observed (9). In light of the data presented here, this may be evidence that a suboptimal uterine environment lies upstream of failed CTB differentiation and invasion. This conclusion is also supported by our finding that CM from the faulty decidualized stroma in sPE restricted CTB invasion (Fig. 7B), which could be stimulated by the addition of two factors that decidual cells normally secrete (Fig. 7C).

The findings presented here show a decidualization defect that is evident at the time of delivery as a result of sPE and lingers for years afterward. Our work challenges the concept that sPE is primarily a disorder of first pregnancies and provides an explanation of why women with sPE are more prone to a recurrence in subsequent pregnancies. Accordingly, the results of this study could have interesting translational potential. It is possible that failure to decidualize could be detected during a nonconception

cycle via an endometrial biopsy, thus enabling estimation of PE risk in a subsequent pregnancy. In this regard, our results suggested that IGFBP1 and PRL assays at the protein level could be useful. Other dysregulated mRNAs may also be biomarkers, suggesting the possibility of the development of a gene-expression signature for the prospective diagnosis of a decidualization defect. Such a test would open the door to possible therapeutic interventions, which could involve pharmacological or other treatments to restore a normal decidual phenotype before pregnancy. Given that failed decidualization is likely associated with a spectrum of reproductive defects—including failed implantation and miscarriage—it could be important to determine whether a subset of patients with these conditions also benefit from the restoration of normal decidualization. Also, women with endometriosis who exhibit decidualization resistance would be an interesting population to evaluate prospectively for possible treatment of infertility. Thus, the results of the work presented here suggest new directions for research into the causes of PE and possible strategies for the development of clinically useful tests that enable prospective risk evaluation and possible treatment.

## Materials and Methods

hESCs were isolated and cultured from endometrial biopsies. Their ability to decidualize in vitro was profiled by monitoring the expression of stage-specific antigens and by global transcriptional profiling. Laser microdissection enabled the enrichment of decidual cells from the maternal-fetal interface. The cells' transcriptome was profiled immediately after isolation and following decidualization in vitro. The effect of soluble factors released from freshly isolated decidual cells on cytotrophoblast invasion was determined by assaying the cells' ability to penetrate Matrigel-coated Transwell filters. See *SI Materials and Methods* for detailed information.

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