Development and utilization of human decidualization reporter cell line uncovers new modulators of female fertility

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Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved August 19, 2019 (received for review May 2, 2019)

Failure of embryo implantation accounts for a significant percentage of female infertility. Exquisitely coordinated molecular programs govern the interaction between the competent blastocyst and the receptive uterus. Decidualization, the rapid proliferation and differentiation of endometrial stromal cells into decidual cells, is required for implantation. Decidualization defects can cause poor placentation, intrauterine growth restriction, and early parturition leading to preterm birth. Decidualization has not yet been systematically studied at the genetic level due to the lack of a suitable high-throughput screening tool. Herein we describe the generation of an immortalized human endometrial stromal cell line that uses yellow fluorescent protein under the control of the prolactin promoter as a quantifiable visual readout of the decidualization response (hESC-PRLY cells). Using this cell line, we performed a genome-wide siRNA library screen, as well as a screen of 910 small molecules, to identify more than 4,000 previously unrecognized genetic and chemical modulators of decidualization. Ontology analysis revealed several groups of decidualization modulators, including many previously unappreciated transcription factors, sensory receptors, growth factors, and kinases. Expression studies of hits revealed that the majority of decidualization modulators are acutely sensitive to ovarian hormone exposure. Gradient treatment of exogenous factors was used to identify EC_{50} values of small-molecule hits, as well as verify several growth factor hits identified by the siRNA screen. The high-throughput decidualization reporter cell line and the findings described herein will aid in the development of patient-specific treatments for decidualization-based recurrent pregnancy loss, subfertility, and infertility.

fertility | decidualization | obstetrics | gynecology

As many as 15% of couples and ∼72 million females worldwide have impaired fertility (1). Additionally, 15% of clinically recognized pregnancies end in miscarriage (2), with approximately half of all women whose embryos implant 11 or more days after ovulation proceeding to miscarry (3). After clinical investigations, many of the causes of impaired female fertility were found to be identifiable pathologies, including improper ovarian function. However, ∼10% remained unexplained (4, 5). It is suspected that in some infertile women, fertilization is successfully attained but pregnancies are immediately lost during the implantation phase (6, 7). These pregnancies are clinically unrecognized, since the hormone used to diagnose pregnancy, human chorionic gonadotropin (hCG), is only detectable after the implantation phase (8).

The ovary, embryo, and endometrium undergo complex changes after ovulation. These changes support the onset of implantation and thus the successful establishment of pregnancy (2, 9, 10). If the ovary fails to produce correct and sufficient levels of signals, or if the uterus fails to respond to those signals, the success of the implantation process and the overall health of any achieved pregnancy can be severely impacted. In particular, the decidualization process, a complex and rapid endometrial remodeling response, is a critical regulator of successful implantation and subsequent placental formation and function (2, 9, 10).

Decidualization involves the rapid proliferation, then differentiation of fibroblast-like endometrial stromal cells into epithelioid-like decidual cells, some of which become large and polyploid or multinuclear. These cells become part of the decidual tissue that surrounds the implanting conceptus (2, 9). The maternal decidual tissue plays a crucial role in the establishment of pregnancy (11, 12). Accompanying the transformation of uterine stromal cells to decidual cells are changes occurring in the endometrium that include extensive extracellular matrix remodeling, vascular remodeling, angiogenesis, and apoptosis. While these are happening, the conceptus enlarges and placental development occurs (2, 9). In addition to implantation defects, experimental evidence suggests that early decidualization defects can lead to defects in placentation, intrauterine fetal growth, and parturition (13). A complex network involving transcription factors, morphogens, cytokines, and their respective signaling pathways is believed to regulate decidualization. Decidualization requires the continued presence of progesterone produced largely by the corpora lutea of the ovary (2, 9) and this response is enhanced by the presence of cyclic adenosine monophosphate (cAMP) (14, 15) and estrogen (16). Although the decidual response is irrefutably dependent on the presence of progesterone, prospective clinical trials of exogenous progesterone supplementation in pregnant women with a history of recurrent pregnancy loss showed little to no effect on the subsequent rate of live births (17). This suggests that hormone supplementation may need to be administered prior to the implantation phase to have any effect, and that loss of clinically recognized pregnancies may be more

Significance

Up to 15% of couples experience infertility. Implantation must occur in order for the pregnancy hormone hCG to be detected, and thus many pregnancies go unrecognized before being lost due to implantation failure. Decidualization is a complex differentiation process during which the uterine lining grows and dramatically changes form in response to ovarian hormone stimulus, and this process is required for successful implantation. By developing and utilizing a new reporter cell line, the present study systematically uncovers more than 4,000 genetic and chemical modulators of the human decidual response in the hopes of catalyzing new treatments for female infertility, subfertility, and recurrent pregnancy loss.

Author contributions: M.H., Y.Y., and L.M. designed research; M.H. and Y.Y. performed research; M.H., Y.Y., and L.M. analyzed data; and M.H., Y.Y., and L.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1907652116/-/DCSupplemental.

First published September 9, 2019.

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dependent on the ability of the uterus to respond to progesterone rather than absolute hormone levels.

Since it is not possible to directly study human endometrial stromal cell decidualization in vivo due to ethical reasons, cell culture models have been developed to study decidualization in vitro. Pure populations of endometrial fibroblasts of menstrual cycling women (human endometrial stromal cells, hESCs) have been isolated and cultured in vitro (18–22). Moreover, an immortalized telomerase-expressing endometrial stromal cell line has been developed (23), which has already been of great use to study human decidualization (24–28). Similar to hESCs, these immortalized cells require progesterone to initiate the decidualization response (29), which can be enhanced by the addition of cAMP analogs (dbcAMP, 8-Br-cAMP), estrogen (16), and prostaglandin E2 (14, 15, 30) as measured by increased gene expression of decidualization markers, such as insulin-like growth factor binding protein 1 (*IGFBP1*), forkhead box protein A1 (*FOXO1A*), and prolactin (*PRL*).

While immortalized hESCs represent an exceptional tool to study decidualization in their own right, the endpoint requires the extractions of RNA or protein from large quantities of cells, and laborious subsequent downstream methods to determine the effects of any modulators on this process. Although RNA sequencing of hormone-treated and -untreated, as well as intentionally mutated or inherently pathologic hESCs, has revealed to the field those genes whose transcripts vary in response to hormone or pathology status (31, 32), the counterpart test to modulate each gene and then use a high-throughput system to determine each gene's effect on the decidual response has remained elusive due to lack of a model amenable to high-throughput screening. The goal of this study is to develop a modified version of immortalized hESCs compatible with high-throughput screening and utilize this new cell line to screen for genetic and chemical modulators of the decidual response. By introducing yellow fluorescent protein (YFP) behind the *PRL* promoter and nucleofecting this construct stably into immortalized hESCs, we have successfully generated a high-throughput screening tool capable of quantifying the human decidual response in vitro and cataloging its sensitivity to any given modulator. Herein we describe the results of a genome-wide small-interfering RNA (siRNA) screen, as well as a screen of 910 small molecules, collectively identifying more than 4,200 genetic and chemical modulators of the human decidual response, and thus demonstrating the power of the hESC-PRLY decidualization reporter cell line as a valuable tool for future studies.

Results

Newly Developed hESC-PRLY Cells Constitute High-Throughput Compatible Decidualization Screening Tool. In order to develop a high-throughput–compatible cell line for the screening of modulators of the decidual response, we utilized immortalized hESCs as a template for further modification. PRL expression has been shown to dramatically increase in hESCs from undetectable to robust levels after 3 to 4 d in culture with a mixture of progesterone, estrogen analog, and cAMP (hereafter called "induction medium" or "hormone mixture"), and this increase in PRL expression is a well-documented and reliable readout for the decidual response in both primary derived and immortalized hESCs (2). In order to turn this PRL induction into a fluorescent readout compatible with high-throughput screening, initial attempts were made to introduce fluorescent proteins behind the endogenous PRL promoter via CRISPR-mediated knockin. However, these attempts were unsuccessful due to exceedingly low locus-targeted rates of homologous recombination in immortalized hESCs. In an effort to increase the yield of successfully modified clones, we opted to utilize an exogenous, previously developed shortened version of the PRL promoter that faithfully recapitulates endogenous expression levels (33, 34), therefore allowing random insertion rather than targeting a specific locus. A construct containing a cytomegalovirus (CMV) constitutively active promoter flanked by loxP sites that sits between YFP and an exogenous shortened version of the hormone-sensitive PRL promoter (Fig. 1*A*) was therefore used. Flanking the entire cassette are insulator sites, boundary elements that prevent position effect variegation (35). This construct allows YFP to be expressed constitutively in any construct-containing cell via the CMV promoter and then, upon treatment with cre-recombinase enzyme, allows excision of the CMV promoter such that YFP is instead under the control of the hormone-sensitive PRL promoter. The targeting construct was thus nucleofected into immortalized hESCs. YFP-expressing, successfully nucleofected cells were then isolated by FACS, briefly propagated and then nucleofected with cre-recombinase–coding plasmid (Fig. 1*B*). The cells thereafter no longer expressing YFP represent the population of immortalized hESCs with YFP under control of the PRL rather than CMV promoter, and these cells were isolated using several subsequent rounds of FACS.

Due to differences in insertion sites and therefore likely differences in hormone responsiveness, these newly derived hESC-PRLY cells were then plated clonally as single cells and allowed to expand. Eighteen clones were successfully established and tested for sensitivity to hormone induction medium. After 3 d in induction medium, hESC-PRLY cells exhibit robust expression of YFP in both nuclear and cytoplasmic compartments (Fig. 1*C*), as shown by fluorescence microscopy. Reassuringly, this response time frame perfectly aligns with the induction of PRL mRNA in parental immortalized hESCs (23) and continues to be detectable and robust through day 4 and into day 5 of hormone treatment. While many reliably hormone-sensitive clones were derived in this way, we selected the hESC-PRLY clone with the reproducibly highest quantified induction response: 10-fold increase in YFP signal as measured by average [YFP intensity × cellular area] (Fig. 1*D*). The decidualization indicator of increased cellular area is included as a factor in the final readout because it is independent of the fluorescence intensity signal and exogenous PRL promoter. In order to ensure that the typical decidualization readouts used in parental hESCs are responding with the same robustness and time course in hESC-PRLY, the transcripts of *IGFBP1*, *PRL*, and *EREG* were compared across the 2 cell lines over the induction time course, and no detectable differences were found (Fig. 1*E*).

While a 10-fold increase in signal is considered confidently robust for smaller experiments, the question remained whether hESC-PRLY cells are compatible with high-throughput screening. To determine this, hESC-PRLY cells were seeded at 150 or 200 cells per well on 384-well plates and treated with control or induction medium for the standard 3 d, then subjected to Hoechst nuclear counterstain, imaging with IN Cell analyzer 2000, and image analysis using IN Cell Developer Toolbox. A common parameter to assess the compatibility of an assay with high-throughput screening is the *Z*-factor, a measure of effect size that takes into account the variation between replicates and the dynamic range of the assay (Fig. 2*A*). An assay with a *Z*-factor between 0.5 and 1 is considered excellent, with a dynamic range compatible with high-throughput screening. The *Z*-factor of hESC-PRLY induction was determined to be between 0.43 and 0.58 for cells seeded at 150 or 200 cells per well in 384-well plates, depending on the final measure of cytoplasmic intensity, nuclear intensity, or [intensity × cellular area]. (Fig. 2*B*). The measure of [intensity × cellular area] had the most reproducibly high *Z*-factor (less dependent on seeded cell number) and was used as the final readout in all downstream applications, referred to hereafter as [$I \times A$].

Whole-Genome siRNA Screen Shows Enrichment of Multiple Ontology Groups. With a high-throughput compatible decidualization reporter cell line in hand, we set out to perform screening to determine genetic and chemical modulators of decidualization.

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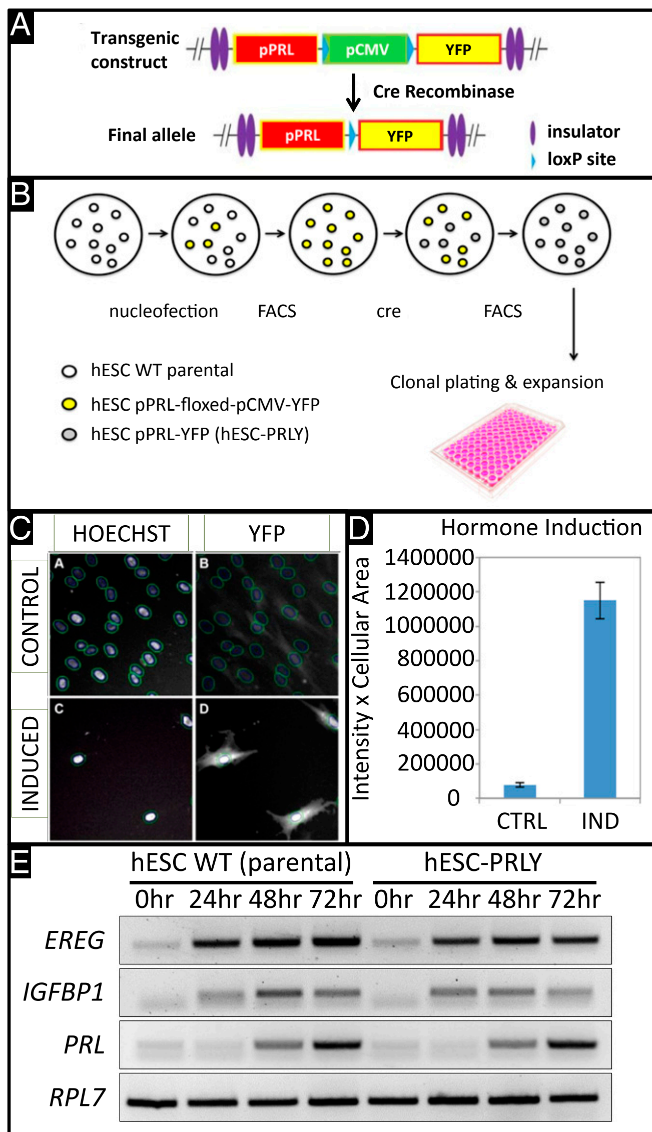

Fig. 1. Generation of hESC-PRLY cell line. A construct consisting of a CMV constitutively active promoter flanked by loxP sites sits between the coding sequence for YFP and the PRL promoter. When the construct encounters cre-recombinase enzyme, the CMV promoter is excised, leaving YFP under the control of the PRL promoter (*A*). Nucleofection was used to introduce the construct into immortalized hESCs, and the yellow fluorescence from CMV-controlled expression of YFP was used as an indicator to isolate construct-containing cells via FACS. Cre-recombinase–expressing plasmid was then nucleofected into the cells, allowing excision of the CMV promoter. Cells were then sorted a second time to isolate the YFP-negative population, which has YFP under the control of the PRL promoter. Finally, this population was plated as single cells and clones were expanded for further testing (*B*). Several clones exhibited robust induction of YFP upon treatment with hormone mixture as visualized by fluorescence microscopy (*C*). (Magnification: 10×.) The most hormone-sensitive clone, showing a quantifiable induction of [$I \times A$] of approximately 10-fold, was selected for use in downstream high-throughput screening (*D*). A panel of typical decidualization readouts comparing parental hESCs and the clonally derived hESC-PRLYs shows no differences (*E*).

Silencing screening libraries readily available to us were the Ambion Silencer Select Human Genome, Druggable Human Genome, and Extended Druggable Human Genome libraries of siRNAs targeting 32,000 genes in probe triplicate. To utilize this library, we set out to optimize the siRNA transfection process in hESC-PRLYs. After assessing a wide panel of transfection reagents and protocols for use in fibroblast-like cells, it was determined that DharmaFECT-4 reagent successfully transfects hESC-PRLYs with >80% efficiency, as measured by nuclear incorporation of siGLO after 7 d in culture (incorporation seen as early as 24 h) (Fig. 2*C*). Higher concentrations of siRNA up to 150 nM show

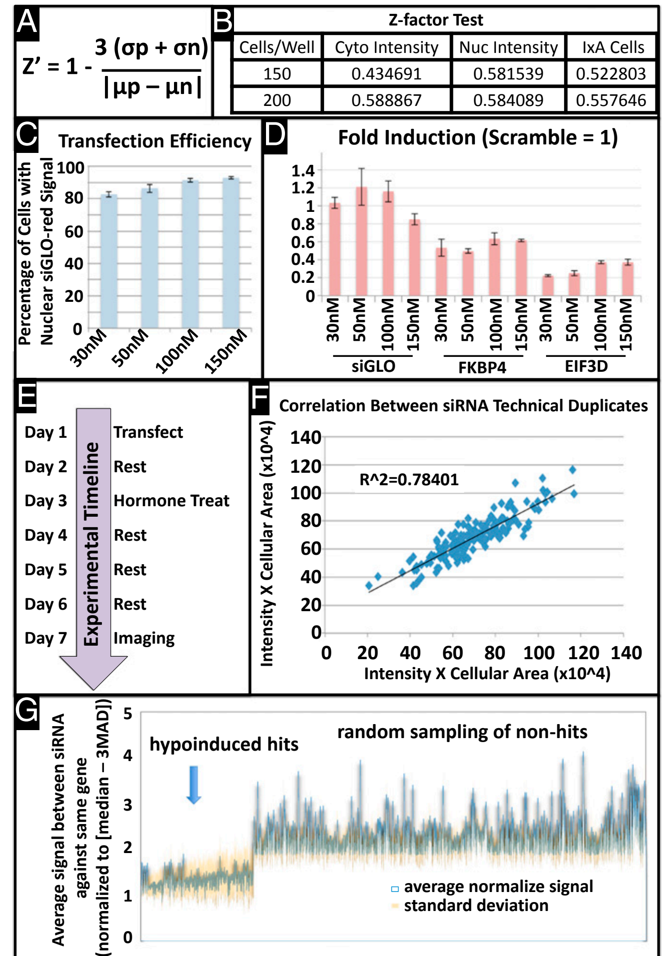

Fig. 2. Optimization of hESC-PRLYs for high-throughput siRNA screening. To determine the compatibility of hESC-PRLYs with high-throughput screening, a *Z*-factor test was performed to determine whether the dynamic range of induction is sufficient. *Z*-factor (*A*) is a measure of effect size and was calculated for the induction response of hESC-PRLYs seeded at 150 or 200 cells per well in 384-well plates (*B*) and determined to be between 0.43 and 0.58 for the parameters of cytoplasmic YFP intensity, nuclear YFP intensity, and [$I \times A$] shown as "I × A Cells." A *Z*-factor between 0.5 and 1 is considered an excellent assay with a dynamic range wide enough to be compatible with high-throughput screening. Using the 150 cells per well seeding density, cells were assayed with a panel of transfection protocols to determine which reagent and protocol resulted in the highest transfection efficiency. As measured by percentage of cells showing nuclear siGLO signal after 7 d in culture, reverse transfection of 30 nM to 150 nM siRNA using DharmaFECT-4 reagent was found to have optimal efficiency at >80% (*C*). To show that siRNA against genes known to be required for the decidual response will inhibit YFP readout, cells were transfected with anti-FKBP4 and anti-EIF3D siRNA, showing an approximate 50% reduction in YFP readout normalized to the fold-induction seen in scramble transfected cells (*D*). A 7-d timeline was used to perform genome-wide siRNA screening of hESC-PRLY cells (*E*) and technical replicates show high correlation at $R^2 = 0.78$ (*F*). Additionally, biological replicates using different siRNA against the same gene show minimal variation in signal in gene hits as compared to siRNAs with no effect on the PRL-YFP readout, which exhibit more variation (*G*).

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higher transfection efficiency; however, this is at the expense of cell viability. Therefore, 30 nM siRNA was used in downstream applications. As a positive control, genes known to be required for full-scale decidualization were knocked down first. Dharmacon SMARTpool siRNA against FKBP4 [required for decidualization (36, 37)] and EIF3D [member of the translation initiation complex (38)] were reverse-transfected into hESC-PRLYs. Cells were allowed to rest for 2 d before hormone treatment for 3 additional days, and then imaged with automated microscopy. Knocking down these positive control genes resulted in ∼50% reduction in the fold-induction response in hESC-PRLYs (Fig. 2*D*), showing that an siRNA screen at a genome-wide level is likely to be successful, although predictably dependent on the efficiency and specificity of each individual siRNA. The final design of the screening assay is 7 d, with reverse transfection on the first day, hormone induction on the third day, and counterstaining and imaging on the seventh day; this allows 2 d for the siRNA treatment to take hold, and the typical 3 to 4 d for the hormone treatment to cause robust YFP production (Fig. 2*E*). To further validate the assay, an initial set of siRNA library plates was run to determine the reproducibility between technical replicates and the variation between readouts of siRNAs targeting the same gene. Technical duplicates show an excellent correlation, particularly for a multistep high-throughput assay, of $R^2 = 0.78$ (Fig. 2*F*). Additionally, although variation between siRNAs against the same gene is somewhat high, particularly for genes with no effect on decidualization (due likely to differences in targeting specificity and efficiency), variation between multiple siRNAs against genes considered hits is comparatively low (Fig. 2*G*). Scramble-transfected or reagent-only–treated control wells (more than 600 wells across the entire screen) exceedingly rarely showed up as hits during analysis, with a calculated false-discovery rate (FDR) of 0.147%.

Using the optimized transfection and induction protocols established for hESC-PRLYs, we performed screening in technical doublets of the Ambion Silencer Select Human Genome, Human Druggable Genome, and Human Extended Druggable Genome Libraries, which contain siRNAs against 32,000 genes at 3 siRNAs per gene. Each assay plate contained technical duplicates of hormone-treated siRNA-transfected cells, as well as negative controls of untreated and scramble-transfected cells to ensure normal induction, siGLO-transfected cells to ensure high efficiency transfection, and DharmaFECT-4 reagent-only–treated cells to ensure minimal reagent toxicity. To call hits, median absolute deviation (MAD) was calculated for each plate, and an siRNA was considered a hit if the average induction value $[I \times A]$ of its technical doublet was 3MAD or more away from the median induction of the plate as a whole. Using these cutoffs, siRNA screening revealed 4,238 genes whose silencing affected YFP induction by 3MAD or more in either direction. Ontology analysis using PANTHER online software revealed that collective hits in both directions are particularly enriched for enzyme modulators, hydrolases, nucleic acid-binding proteins, receptors, transcription factors, transferases, and transporters (Fig. 3*A*). Enzyme modulator hits were enriched for G protein modulators and protease inhibitors (Fig. 3*B*). Receptor hits were enriched for G protein receptors (Fig. 3*C*). Transferase hits were enriched for kinases (Fig. 3*D*). Hydrolase hits were enriched for proteases and phosphatases (Fig. 3*E*). Transcription factor hits were enriched for zinc finger transcription factors (Fig. 3*F*). Transporter hits were enriched for ion channels and cation transporters (Fig. 3*G*). Calculated enrichment *P* values and FDRs can be found in Dataset S1 (molecular function) and Dataset S2 (biological process). A complete list of hits in Dataset S3 shows each gene acronym, full gene name, and normalized hit value.

Perhaps unsurprisingly, homeodomain transcription factors were revealed as hits in both directions, including several factors with history in the literature of involvement in development but

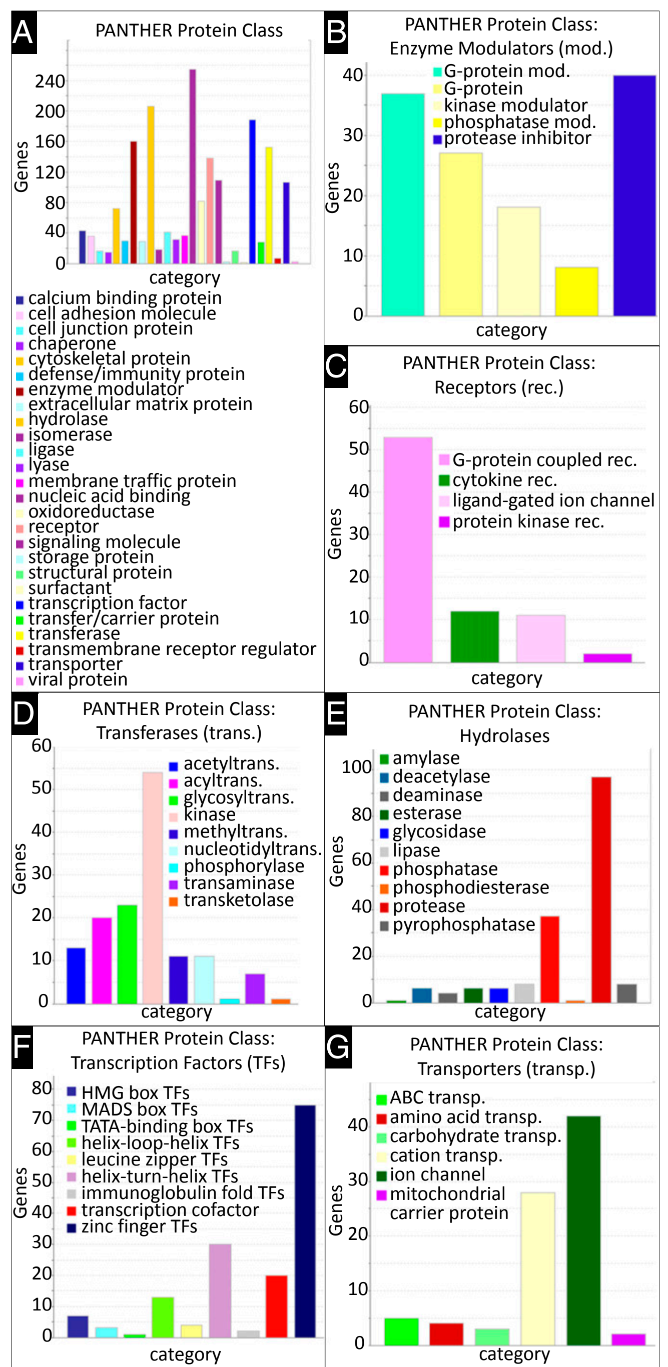

Fig. 3. Ontology analysis of decidualization modulators (siRNA hits). Screening of Ambion Silencer select siRNA libraries revealed 4,238 genes whose silencing affected YFP induction by 3MAD or more in either direction. Ontology analysis revealed these hits (2,892 recognized by the software) to be particularly enriched for enzyme modulators, hydrolases, nucleic acid binding proteins, receptors, transcription factors, transferases, and transporters (*A*). Enzyme modulator hits were enriched for G protein modulators and protease inhibitors (*B*). Receptor hits were enriched for G protein receptors (*C*). Transferase hits were enriched for kinases (*D*). Hydrolase hits were enriched for proteases and phosphatases (*E*). Transcription factor hits were enriched for zinc finger transcription factors (*F*). Transporter hits were enriched for ion channels and cation transporters (*G*).

not yet directly in decidualization (*SI Appendix*, Fig. S1). Additionally, using the highest confidence setting, STRING network analysis of the hypoinduced hits (genes normally facilitating the

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hormone response) revealed several signaling nodes of unexpected decidualization modulators (*SI Appendix*, Fig. S2), including nodes centering around the genes Ubiquitin C (*UBC*), G protein subunit-α I3 (*GNAI3*), G protein subunit-α Q (*GNAQ*), the microtubule organizing protein NudeE neurodevelopment protein 1 (*NDE1*), Ras homolog family member A (*RHOA*), and the serine protease inhibitor Serpin family A member 1 (*SERPINA1*), to name only a few. Unexpectedly, a signaling node for olfactory receptors centering around receptor transporter protein 2 (*RTP2*) was identified during STRING analysis. In fact, 87 olfactory receptors in total were identified as modulators of decidualization by siRNA screening (*SI Appendix*, Fig. S3), suggesting an intimate involvement of G protein-coupled receptors (GPCRs) and their ligands in decidualization. Additionally, 108 genes encoding signaling molecules (as defined by PANTHER ontology grouping) were identified as decidualization modulators by siRNA screening (*SI Appendix*, Fig. S4), including several chemokines, morphogens, and growth factors.

Screening of Multiple Small-Molecule Libraries Implicates New Signaling Pathways in the Decidual Response. In addition to the genome-wide siRNA screen, we performed high-throughput screens with the SCREEN-WELL ICCB-known bioactives library and the Selleckchem kinase inhibitor library, which contain 472 and 438 unique compounds, respectively, in order to identify small molecules that modulate the decidualization process. The collections cover a wide variety of biologically active compounds, including kinase inhibitors, signaling pathway ligands, second messenger modulators, ion channel blockers, and lipid biosynthesis inhibitors. The hESC-PRLYs were seeded in 384-well plates, and treated with hormone mixture along with 500 nM compound for 96 h in duplicates, before $[I \times A]$ was assayed via InCell Analyzer automated microscopy. For the small-molecule screenings, 2.5MAD was used to call hits, where hyperinduced hits measure 2.5MAD or more above the median $[I \times A]$, and hypo-hits measure 2.5MAD or more below. Initial screening uncovered ∼50 compounds that negatively affect decidualization, and 38 that enhance the process (Fig. 4 *A* and *B*). Clustering of the hypo-hits revealed that inhibitors against the cyclin-dependent kinases (CDKs), the tyrosine kinase BCR-ABL, and the mechanistic target of rapamysin are among the small molecules that inhibit decidualization (Fig. 4*C*). On the other hand, inhibitors that target polo-like kinases, glycogen synthase kinase 3 (GSK3), and Janus kinases (JAKs) appeared to enhance the process (Fig. 4*D*). Consistent effects exhibited by various inhibitors against functionally and structurally related family members indicate the effects to be target-specific. In addition, molecules previously reported to promote decidualization, including many prostaglandins, enhanced the decidual response upon exogenous treatment, which further supports the legitimacy of the hESC-PRLY screening model. A comprehensive list of small-molecule hits and their normalized induction values upon exogenous treatment can be found in Dataset S3.

Expression Profiling of Hits from siRNA Screen Reveals Most Hits Are Sensitive to Hormone Treatment at the Transcriptional Level. In order to determine whether hits identified in the siRNA screen as modulators of hormone responsiveness are also sensitive at the transcriptional level to fluctuating levels of ovarian hormones, we tested expression of every transcription factor hit and olfactory receptor hit over the standard induction time course using RT-PCR. Verified human transcription factors as determined by a 2018 review (39), as well as several transcriptional coregulators and putative transcription factors, were tested across the following time points for their response in expression level to hormone treatment: 0, 2, 6, 12, 24, 48, and 72 h. Ultimately, transcription factor hits could be cleanly grouped into the following categories: genes whose expression goes immediately from detectable to undetectable at 2 h posthormone (Fig. 5*A*); genes

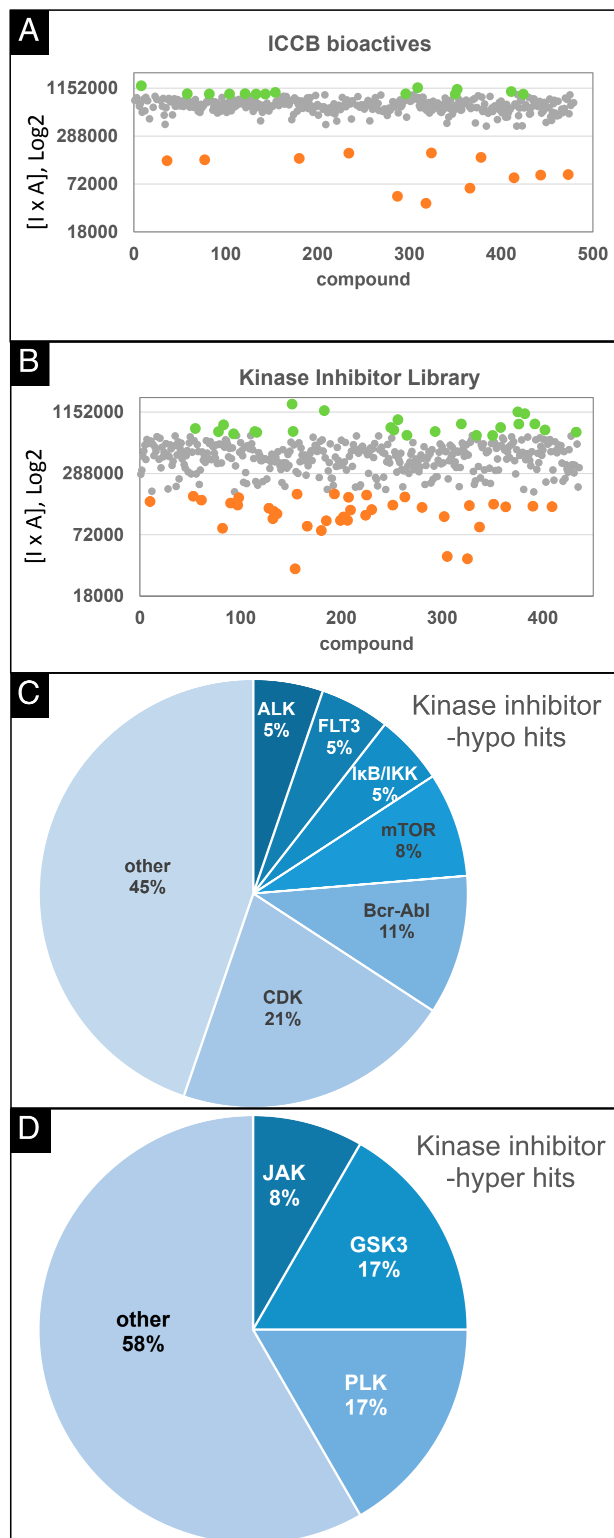

Fig. 4. Small-molecule screenings on hESC-PRLY decidualization. Scatter plots of compounds used for screening from the 2 small-molecule libraries: the SCREEN-WELL ICCB bioactives library (*A*) and the Selleckchem Kinase Inhibitor library (*B*). Gray dots are compounds that showed no effects on decidualization; orange dots are hypo-hits that inhibit decidualization; green dots are hyper-hits that promote decidualization. Hits for the kinase inhibitor library can be clustered by functional groups in both the hypoinduced (*C*) and hyperinduced categories (*D*).

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whose expression drops from detectable to undetectable levels before 24 h (Fig. 5*B*); genes whose expression gradually decreases over the full duration of the treatment schedule (Fig. 5*C*); genes with largely static expression across treatment schedule (Fig. 5*D*); genes exhibiting stochastic expression across the treatment schedule that is seemingly not dependent on hormone levels (Fig. 5*E*); and perhaps most intriguingly, genes that spike at 2 h and subsequently drop to pretreatment levels or lower (Fig. 5*F*). While several genes are acutely up-regulated in response to induction medium, this increase in expression is generally transient and recovers to baseline levels within 24 h. Quantification using ImageJ software (Fig. 5 *G*–*L*) cleanly delineated these 6 groups, which were then analyzed using DiRE (Distant Regulatory Elements of Coregulated Genes) online freeware (https://dire.dcode.org) to identify potential transcriptional regulators of each group (Fig. 5 *M*–*R*). Comprehensive gel images documenting the expression pattern in response to hormone of transcription factor hits, as well as several transcriptional cofactors and modulators, can be found in Dataset S4. Olfactory receptors were likewise tested across the same time points to evaluate their transcription-level response to hormone treatment and can be similarly grouped into 5 distinct expression profiles (*SI Appendix*, Fig. S5). Primer sequences for all tested genes can be found in Dataset S5.

Treatment of hESC-PRLYs with Response Gradients of Exogenous Growth Factors and Small Molecules Corroborates Findings of High-Throughput Screening. A subset of siRNA screening hits were growth factors and cytokines. To evaluate their involvement in decidualization, we first investigated their expression level by RT-PCR in wild-type hESCs at 0, 24, and 96 h postinduction (hpi). The majority of these genes maintained high expression level throughout the induction course (the tumor necrosis factors, ephrins, fibroblast growth factors, growth differentiation factors, interleukins, transforming growth factors, and so forth), or were induced by hormone treatment (chemokine CXCLs, epiregulin, IL1F9, and WNT10B); in contrast, a handful of genes were rapidly suppressed then regained transcription (*ECGF*, *PENK*, and *TRH*) (Fig. 6*A*). By far the rarest category of expression

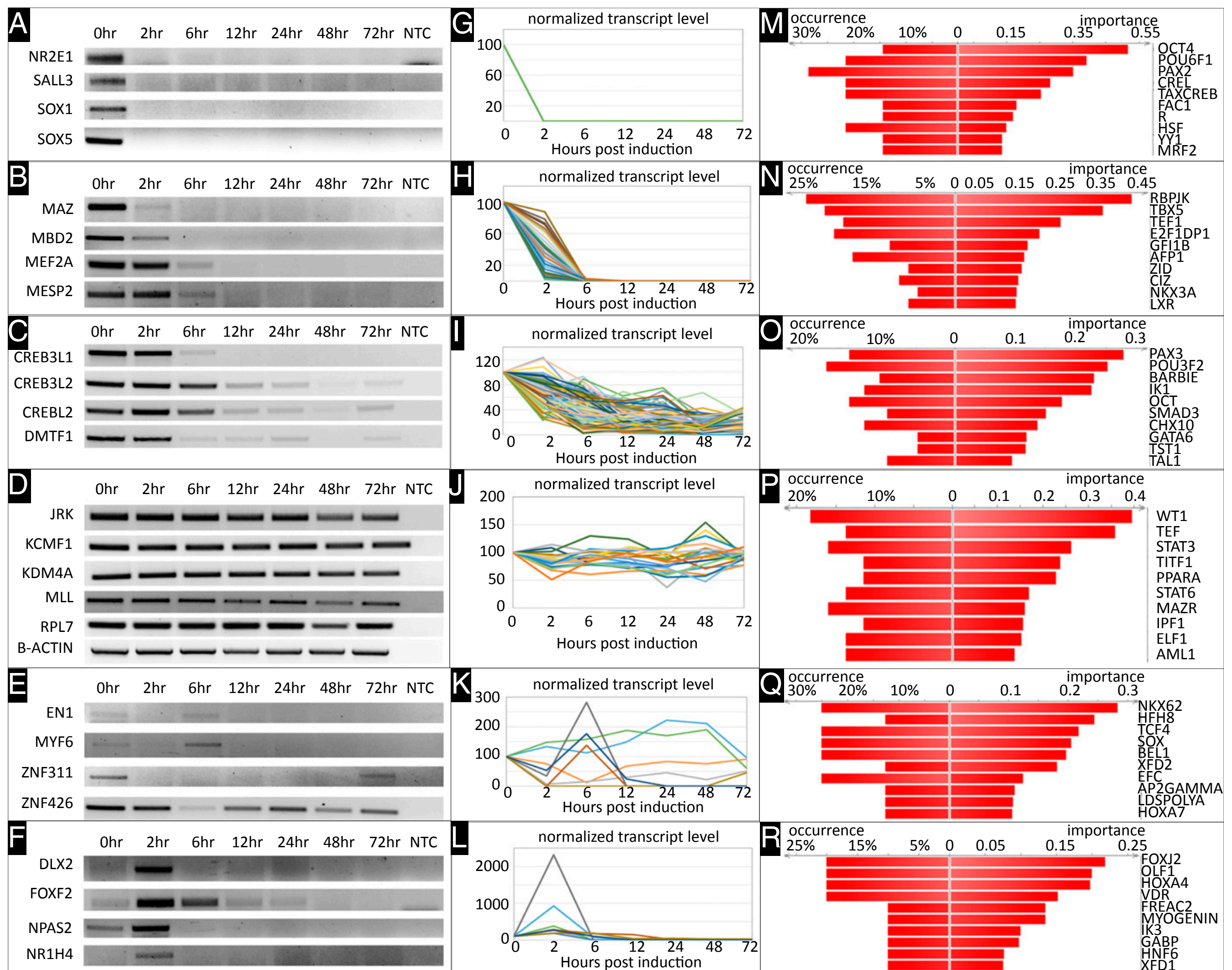

Fig. 5. Expression profiles of transcription factor and transcriptional regulator hits can be grouped into 6 categories: genes whose expression goes immediately from detectable to undetectable at 2 h posthormone (*A*), genes whose expression drops from detectable to undetectable levels before 24 h (*B*), genes whose expression gradually decreases over the full duration of the treatment schedule (*C*), genes with largely static expression across treatment schedule, including loading controls RPL7 and B-actin (*D*), genes exhibiting stochastic expression across treatment schedule (*E*), and genes that spike at 2 h and subsequently drop to pretreatment levels or lower (*F*). ImageJ quantifications (*G*–*L*) cleanly delineated the 6 groups, which were then analyzed using DiRE online freeware to identify potential transcriptional regulators of each group (*M*–*R*).

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profile across all genes were those genes absent in the hormone naïve state which are then immediately induced upon hormone exposure and remain highly expressed across multiple days in culture. In fact, only 1 tested transcript, EREG, fits this expression category.

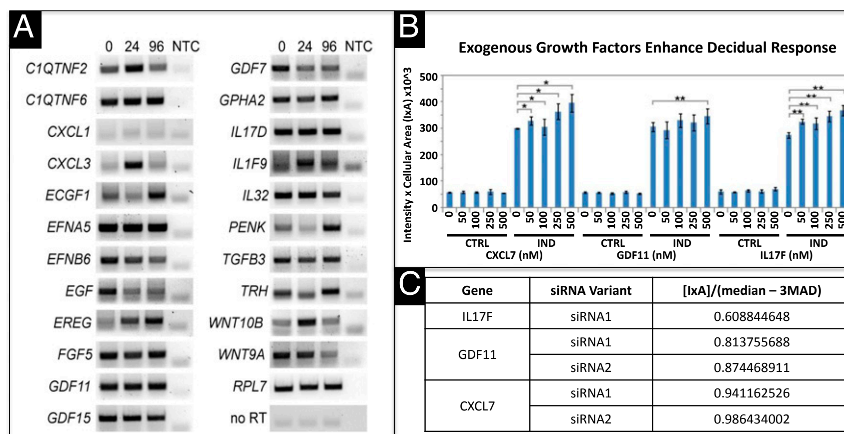
To study the function of growth factors, we examined the effects of exogenous growth factors on decidualization of the hESC-PRLYs. Commercially obtained growth factors were added with or without hormone mixture to hESC-PRLYs, in concentrations ranging from 1 to 500 nM. Both CXCL7 and IL-17F showed a dose-dependent enhancement of decidualization, with significant effect seen at concentrations as low as 50 nM. GDF11 exhibited similar effect at 500 nM (Fig. 6*B*). These results, together with the initial screening data showing that knocking down these 3 genes negatively affects the decidual process (quantified in Fig. 6*C*), strongly implicate these growth factors as having prominent regulatory roles in human decidualization. To further validate the results of the small-molecule screening, titrated response curves were performed in the presence of hormone mixture, validating the effects of 20 kinase inhibitors and 13 bioactive molecules (*SI Appendix*, Fig. S6) on the induction response in hESC-PRLY cells, with many compounds effectively modulating the decidual response within the submicromolar range.

Discussion

Up to 15% of couples experience infertility or subfertility, defined as the inability to conceive after 1 y, according to the World Health Organization (40). Even with timed intercourse the chances of conception are no more than 25% per cycle (41). Couples undergoing in vitro fertilization procedures may subvert certain fertility issues, such as embryonic trisomies and other nondisjunction syndromes, via genetic testing of embryos prior to implantation (42), but the rates of implantation even for perfectly healthy blastocysts are still dependent on the adequately robust decidualization of the uterus, allowing it to be receptive to the embryo, and the synchronized timing of these events (43). It has long been known that uterine decidualization is dependent on, and titrated by, the presence and levels of ovarian hormones (2). It has also long been suspected that an insufficient decidual response may be among the predominant causes of early pregnancy loss of genetically normal embryos, and is also associated with placentation and parturition complications (13). In hopeful attempts to reach each woman's threshold for progesterone, exogenous supplementation is frequently used during early pregnancy, but placebo-controlled trials of progesterone supplementation showed no detectable increases in viable pregnancies (17), suggesting that the issue has more to do with hormone responsiveness in most patients than insufficient progesterone as a molecular signal. Despite this evidence that genetic defects affecting uterine receptivity are key contributors to female infertility, subfertility, early pregnancy loss, placentation, and parturition defects, there have been only a limited number of studies to explore genetic defects preventing or suppressing the uterus from responding appropriately or sufficiently robustly to ovarian hormones. Up to now, in order to test this question, it was necessary to laboriously test only a few candidate genes at a time using hESCs derived from patients, or immortalized hESCs, or alternatively performing genome-wide associative studies on infertile women, which are only correlative. These laborious methods were necessary due to lack of a high-throughput screening tool to prospectively and systematically answer this question. By generating hESC-PRLY cells that respond quantitatively robustly to hormone mixture with a fluorescent readout, we have solved this long-standing issue in the field of female reproductive research, and successfully screened several molecular libraries as verification of the model.

Each assay using hESC-PRLYs needs to be internally optimized and controlled, depending on the treatments involved. While the induction response as measured by $[I \times A]$ is robust with an excellent Z-factor, each treatment, whether genetic or environmental, needs to be individually optimized as its own assay. For the siRNA screen described herein, it was necessary to determine the optimal transfection protocol and timeline and verify its reproducibility using technical duplicate correlation. For small-molecule screening and exogenous recombinant growth factor verification, it was necessary to perform dose–response gradients. This gives an indication of how this model can be used in the future to screen for possible hormone-free contraceptive methods, larger libraries of small molecules, and how best to perform validation and rescue experiments, possibly with the use of a modified hESC-PRLY cell line expressing inducible CAS9.

Collectively, the screens revealed several expected results, such as FKBP4 (36, 44) and NEDD8 (45), showing up as hypo-induced hits (required for decidualization) in the siRNA screen, and prostaglandins (46), showing up as hits in the small-molecule screen (enhance decidualization when added exogenously). While



Gene	siRNA Variant	[IxA]/(median – 3MAD)
IL17F	siRNA1	0.608844648
GDF11	siRNA1	0.813755688
	siRNA2	0.874468911
CXCL7	siRNA1	0.941162526
	siRNA2	0.986434002

Fig. 6. Involvement of growth factors and cytokines during decidualization. RT-PCR gels of a selection of hits from the siRNA screen show various expression patterns (*A*). RPL7 was used as an internal loading control, and mRNA samples without reverse transcription (no RT) were used as template to assess potential genomic DNA contamination. NTC, no template control. Exogenous growth factors at concentrations ranging from 1 to 500 nM were added to hESC-PRLY with or without hormone mixtures to evaluate their effect on decidualization. *$P < 0.05$; **$P < 0.01$; $n = 4$ (*B*). Initial normalized quantified siRNA screening results of the tested growth factor genes (*C*).

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several expected genes did not come through as hits in the siRNA screen, such as ACVR1 (ALK2) (47), this can largely be explained by the unvalidated efficiency of many of the siRNAs and our inability to visually isolate successfully transfected cells, causing the signal to be an average of untransfected and successfully transfected cells. When a separate siRNA against ALK2 was tested (SMARTpool, Dharmacon), the induction signal was suppressed by 50% (*SI Appendix*, Fig. S7). Therefore, while the false-negative rate of the siRNA screen may be high due to dependence on efficiency of each siRNA and less than 100% transfection efficiency, high reproducibility between duplicates and a low FDR provide ample confidence that positive hits are real. In order to assess the concordance rate between the $[I \times A]$ in hESC-PRLY cells as compared to the typical readout of endogenous PRL expression in parental hESCs, 36 of the hypoinduced siRNA double-probe hits were tested via qPCR. We were able to validate knockdown of 15 genes. An additional 15 genes were below the threshold of detection, and therefore knockdown could be neither verified nor disproved. Despite this, these 15 treatments still showed significant down-regulation of PRL; we therefore suspect that the inability to detect the knockdown in these samples is due to endogenously low, but still important, expression being abrogated. The remaining 6 genes were detectable but did not show knockdown. Importantly, of the 15 genes whose knockdown was successfully validated, 13 showed significant reduction in PRL expression upon hormone induction. This concordance rate of 87% is a rough estimate of the reproducibility of our hits across different measures of decidualization. Nine small molecules were also successfully validated in the same manner (*SI Appendix*, Fig. S8).

The most interesting findings using hESC-PRLYs are unique ones. For example, this study suggests that olfactory receptors (ORs) are playing a major role in uterine receptivity, as evidenced by their robust presence in both the hypo- and hyperinduced hit categories upon individual siRNA knockdown. First discovered in the olfactory epithelium (OE), the ORs are a family of GPCR proteins, comprised of about 400 ORF-containing family members in the human genome (48–50). Intriguingly, the expression of many ORs is not limited to the OE, but can be found in a plethora of other tissue types (51). Previous gene-profiling analysis reported that in humans, atrioventricular nodes, skin, and uterus are the top 3 tissue types surveyed that show the highest quantile-based OR expression levels (52). Contrary to the OE olfactory sensory neurons, where each cell expresses 1 and only 1 OR, cells outside the OE tend to express a combination of different ORs (53). Upon binding to extracellular stimuli—including but not limited to odorant molecules, hormones, and transmitters—they transduce the signal internally and activate various signaling pathways in a cell type-dependent manner. In most cell types outside the OE, activation of ORs leads to an increase of intracellular second messenger cAMP and subsequent calcium flux from outside the cells (54–61). Functions of ORs in non-OE tissues vary as well. For example, OR1D2 (hOR17-4) present at the midpiece of human spermatozoa facilitates sperm motility upon activation (62), whereas OR2AT4 expressed in human keratinocytes can sense Sandalore, a synthetic sandalwood odorant and agonist of the receptor, and positively regulate cell proliferation and migration to promote wound healing (54). In our study, 46 ORs identified as hits in the siRNA screen were easily detected in hESC cells, and 90% exhibited variations in expression dependent on ovarian hormones. For example, OR51E2 was highly expressed in hESC under hormone-naïve conditions, but was instantly suppressed upon hormone treatment. Knocking it down via siRNA resulted in enhanced $[I \times A]$ signal, suggesting OR51E2 normally plays an inhibitory role during decidualization. OR51E2 is an evolutionarily older OR with extensive sequence similarity among orthologs, and is among the most universally expressed ORs outside OE in humans (63). It has been reported to be involved in proliferation of prostate cancer cells (64, 65) and retinal pigment epithelial cells (66), as well as melanocyte homeostasis (57). The natural ligands of these ORs, their downstream signaling transduction cascades, and physiological functions during decidualization demand further investigation. The STRING network for the OR signaling node in hESCs centers around *RTP2*, another hypoinduced hit upon siRNA knockdown, whose role is to modulate the targeting and transport of ORs to the cell surface (67). Corroborating the involvement of homeodomain transcription factors in decidualization, *RTP2* expression is regulated by homeodomain transcription factors (genecards.org), including HOXA5 and multiple members of the NKX subfamily. Taken together, these data provide strong evidence that ORs are playing important roles in the decidual response.

Several growth factors were identified in the siRNA screen that were successfully validated using the addition of exogenous ligands, including GDF11, CXCL7, and IL17F. These findings are of particular importance because they are nonhormonal, exogenous modulators of decidualization, and may be useful in the augmentation of the decidual response in those women less sensitive to ovarian hormones. Exogenous modulators are excellent for use in the clinical setting because there is no genetic manipulation required; a patient simply has too much or too little of a particular factor, and this factor needs to be exogenously titrated in order to optimize the decidual response. The findings that signaling molecules and their receptors are so heavily represented in our screening results suggests there is more communication between the receptive uterus and its environment than previously thought.

The finding that a large percentage of siRNA hits were transcription factors is not surprising. However, the finding that the majority of transcription factors that vary in expression across hormone treatment are suppressed upon hormone treatment, or acutely rather than extendedly up-regulated, is an unexpected finding. Additionally, nearly twice as many genes were revealed to increase the decidual response upon knockdown as compared to impairing it, suggesting that a large amount of energy is expended in the uterus to prevent or titrate decidualization. Perhaps then, we must consider that the low-energy state of the endometrium is potentially the decidualized state. We theorize that the uterus actively prevents decidualization when ovarian hormone levels are low, and then when hormone levels exceed a certain threshold, the uterus can rapidly shut down expression of hundreds of genes that are no longer required, including active repressors of the response, which then allows full-scale decidualization to occur. If a system is to be turned on rapidly, as is needed in decidualization when the uterine endometrium encounters an implanting blastocyst, it is more effective to release the brakes on an already running system rather than turning the system from entirely off to entirely on. We hypothesize that the energy required to "switch on" decidualization is low, and that this is a direct result of the high energy expended to keep it effectively suppressed during times of low hormone. This theory largely agrees with Conrad Waddington's longstanding "landscape" model, more recently applied to large-scale transcriptional regulation during embryology and differentiation (68), wherein fate decisions are made by influencing a cell's lowest energy state (69).

One method by which this rapid (within 2 h for many of the tested transcripts) large-scale down-regulation of gene expression may be achieved is through microRNAs. When the transcriptional regulator hits belonging to expression group 1 (completely and immediately repressed by 2 h posthormone) were cross-checked for microRNA binding sites (mirdb.org/mining.html), the genes *IRX3*, *SOX1*, *LHX5*, *ZNF704*, *ZNF510,* and *ZNF566* were found to contain binding sites for uterine-expressed microRNAs known to be similarly sensitive to ovarian hormone exposure (70). Taken together, these data beg the question: How many infertile women exhibit implantation defects or early pregnancy loss due to a decidual response that is actually over- rather than underactive? Our

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data suggest that decidual defects causing either hyper- or hyposensitivity to hormone are both likely negative effectors to overall pregnancy rates.

One notable subgroup of transcriptional regulator hits identified by the siRNA screen are the homeodomain transcription factors, 36 of which were identified as either positive or negative regulators of the decidual response. In fact, many of the homeodomain transcription factor hits were among the most acutely sensitive to hormone relative to other transcription factors and transcriptional coregulators tested in the expression panel. Homeodomain factors have long-established roles in vertebrate development, first identified for their intricate roles in axial patterning and body segmentation across multiple vertebrate model organisms (71–73). Developmental processes are enriched for differentiation reactions and fate decisions, and it is important to remember that the decidual response in the uterine endometrium is at its core a differentiation reaction. It is plausible that homeodomain transcription factors are important upstream regulators of the decidual response, titrating uterine hormone sensitivity and integrating the many incoming environmental signals. Indeed, when our hit list was grouped by protein family type, subsequently grouped by expression profile, and then interrogated for regulatory elements using the DiRE database, many of the binding sites belonged to homeobox transcription factors, including but not limited to POU6F1/3F2/1F1, NKX3A/62, HOXA4/A7, and PAX2/3/6. One of the best candidates among the subgroup of homeodomain factor siRNA hits is Engrailed 1 (EN1) due to the extent of signal repression observed upon its knockdown, and because 2 of the 3 siRNA constructs against this gene were highly effective at inhibiting the decidual response. EN1 has never before been implicated in the decidual response, but has previously reported roles in neuron developmental fate (74) and axon guidance (75). It also plays an intricate role in limb development by controlling proper digit number and patterning by integrating canonical WNT pathway signaling in the apical ectodermal ridge (76), as evidenced by murine models. The potential requirement of EN1 in decidualization warrants further investigation, especially the possibility that it may likewise be integrating WNT signaling in the uterus, which is known to be crucial for successful receptivity and implantation (77).

In order to better refine the list of decidualization modulators generated by this screen, we mined the literature for screens comparing expression profiles of human endometrial tissues. Two major studies were identified. In the first study, 238 genes were shown to be differentially expressed between the prereceptive and receptive uterine phases (78). It would follow that a subset of these differentially expressed genes are functionally required for the endometrial transition to receptivity: That is, represent active modulators rather than being passively differentially expressed. Of the 238 differentially expressed genes in this study, 47 (or 19.7%) were identified as modulators of decidualization in our screen. These 47 genes are listed in Dataset S6. In a somewhat similar study, transcriptome analysis compared endometrial tissues from women experiencing recurrent implantation failure to controls (either spontaneously fertile women or women with at least 1 successful implantation via in vitro fertilization) and identified 82 consistently differentially expressed genes (79). Of the 82 genes identified by this study, 13 (or 15.8%) were identified as modulators of decidualization in our screen (80–96). These findings are in Dataset S7. Finally, we searched through the literature, as well as SNPedia, for any SNP association studies that identified deleterious SNPs in any of our hits and associated them with adverse pregnancy outcomes, including recurrent pregnancy loss and recurrent implantation failure. We identified SNPs associated with these conditions in 36 of our hits. In Dataset S8 can be found each gene, the PMID for the reference, and the associated deleterious SNP or SNPs.

For the small-molecule libraries, clustering of the hypo-hits revealed that inhibitors against the CDKs are among the small molecules that inhibit decidualization. CDKs are notorious regulators of proliferation (97), which is an integral part of the decidual response. While CDK inhibitors are typically used in the treatment of various cancers (98), a more localized and lower concentration treatment might be a viable nonhormonal contraceptive depending on the toxicity. In our studies, treatment at 500 nM inhibited decidualization but did not actually kill the cells. Conversely, inhibitors of GSK3 and JAKs enhanced decidualization. Inhibitors of GSK3 and JAKs are both regularly used to treat immune and inflammatory diseases (99, 100). The decidual response has been previously described as an inflammatory reaction that is enhanced during embryo implantation (101). Perhaps this inflammatory reaction needs to be well-balanced, not too heavily induced nor too lightly, relying on autoregulation wherein an upper threshold of cytokines ensures a similar upper threshold of decidualization.

The overall goal of this study was to develop and initially test hESC-PRLY cells as a screening tool for decidualization modulators. By generating a human decidual cell line that responds robustly and quantifiably to hormone mixture with a quick and reliable visual readout, it is now possible to screen untold numbers of decidual modulators, both genetic and environmental. Follow-up studies, such as the titration curves performed for growth factor and small-molecule hits, are important to validate initial findings. The use of a human cell line is imperative for these studies, as despite many evolutionarily conserved aspects of decidualization, animal models have vastly different estrous cycles and implantation rates as compared to humans, and genetically human samples should be used to test decidualization modulators prior to any prospective clinical trials. The development and initial screening of hESC-PRLY cells described herein is a major milestone in the mapping of the human decidualization network of genetic and environmental factors modulating female fertility. It is our hope that the continued use of these cells in future screening projects will identify additional major players in human decidualization, influencing the development of treatment regimens for subfertile women, infertile women, and women experiencing recurrent pregnancy loss.

Methods

For detailed methods, please refer to *SI Appendix*. The reporter transgene was constructed by inserting the YFP-coding sequence downstream of a 1.1-kb human PRL promoter. Individual single-cell clones were established, genotyped, and tested for hormone sensitivity. The following libraries were screened: Ambion Human Genome Library, Ambion Human Druggable Genome Library, Ambion Human Extended Druggable Genome Library, SCREEN-WELL ICCB, and the Selleckchem kinase inhibitor library. Results were imaged with InCell2000 Analyzer automated microscope at 4 fields per well using the YFP, DAPI, and CY3 filters. To be called a hit, the average of the 2 duplicate siRNA [$I \times A$] values for an siRNA had to be 3 or more MAD away from the plate median. To eliminate the possibility of cell toxicity interfering with readouts, any treatment that resulted in fewer cells at the time of imaging than were originally seeded was excluded from analysis. Ontology analysis was performed using PANTHER online freeware (http://www.pantherdb.org/). Interaction network analysis was performed using STRING network online freeware (https://string-db.org/). To identify regulatory elements for hits grouped by expression profile, the DiRE database was used (https://dire.dcode.org/). For the small-molecule screenings, 2.5MAD was used to call hits. GoTaq RT-PCR expression studies were performed using timepoints 2, 6, 12, 24, 48, 72, and 96 h. Validations with SYBER green qPCR endpoints were performed in a scaled-up manner in 48-well plates using the same concentrations of siRNA, small-molecule, and hormone treatment as was used for screening. Primers were designed using PrimerBLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

ACKNOWLEDGMENTS. We thank Dr. Maxene Ilagen and Dr. Michael Prinsen of the Washington University High Throughput Screening Core for monitoring and assisting our use of the Biomek robotic liquid handler, Incell2000 automated fluorescence microscope, and Developer software; the Washington

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University Genome Technology Access Center for performing and training our use of their nucleofection equipment; and Dr. Brent Bany for providing the pPRL-pGL4.14 plasmid for this project. This work was financially supported by National Institutes of Health Grants HD087973, HD082792, and DK113642, and Clinical and Translational Science Awards Grant UL1 TR002345.

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