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The Androgen and Progesterone Receptors Regulate Distinct Gene Networks and Cellular Functions in Decidualizing Endometrium

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Progesterone is indispensable for differentiation of human endometrial stromal cells (HESCs) into decidual cells, a process that critically controls embryo implantation. We now show an important role for androgen receptor (AR) signaling in this differentiation process. Decreased posttranslational modification of the AR by small ubiquitin-like modifier (SUMO)-1 in decidualizing cells accounted for increased responsiveness to androgen. By combining small interfering RNA technology with genome-wide expression profiling, we found that AR and progesterone receptor (PR) regulate the expression of distinct decidual gene networks. Ingenuity pathway analysis implicated a preponderance of AR-induced genes in cytoskeletal organization and cell motility, whereas analysis of AR-repressed genes suggested involvement in cell cycle regulation. Functionally, AR depletion prevented differentiation-dependent stress fiber formation and promoted motility and proliferation of decidualizing cells. In comparison, PR depletion perturbed the expression of many more genes, underscoring the importance of this nuclear receptor in diverse cellular functions. However, several PR-dependent genes encode for signaling intermediates, and knockdown of PR, but not AR, compromised activation of WNT/β-catenin, TGFβ/SMAD, and signal transducer and activator of transcription (STAT) pathways in decidualizing cells. Thus, the nonredundant function of the AR in decidualizing HESCs, centered on cytoskeletal organization and cell cycle regulation, implies an important role for androgens in modulating fetal-maternal interactions. Moreover, we show that PR regulates HESC differentiation, at least in part, by reprogramming growth factor and cytokine signal transduction. (*Endocrinology* 149: 4462–4474, 2008)

PROGESTERONE IS A pleiotropic hormone that regulates all aspects of female reproduction, from ovulation and embryo implantation to parturition. The actions of progesterone (P4) on reproductive target tissues are mediated predominantly by its cognate nuclear receptors, P4 receptor (PR)-A and PR-B, members of the superfamily of ligand-dependent transcription factors. In the uterus, the postovulatory rise in P4 levels induces differentiation of the endometrial mucosa in preparation for pregnancy (1). A cardinal event in this remodeling process is the transformation of endometrial stromal fibroblast into secretory, epithelioid-like decidual cells (2–4). In addition to the morphological changes, decidualization bestows some unique functional properties on human endometrial stromal cells (HESCs), including the ability to modulate local immune cells, to resist environmental stress signals, and to modulate trophoblast invasion (2–4). Mice deficient in PR fail to mount a decidual response and are sterile (1). However, activation of PR is in itself insufficient to induce decidualization in mice or humans. Initiation of HESC differentiation is strictly dependent upon elevated cAMP levels and sustained activation of the protein kinase A pathway, which in turn sensitize the cells to P4 (5, 6). Once decidualized, the endometrium becomes inextricably dependent upon continuous P4 signaling for homeostasis, and in the absence of pregnancy, falling P4 levels trigger a cascade of events that results in apoptosis, proteolytic breakdown of the superficial endometrium, focal bleeding, and menstrual shedding (7).

HESCs also abundantly express the androgen receptor (AR) (8, 9), yet little is known about the function of this nuclear receptor family member in the decidual process. AR

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Abbreviations: AR, Androgen receptor; DHT, dihydrotestosterone; EGFP, enhanced green fluorescent protein; F-actin, filamentous actin; HESC, human endometrial stromal cell; IGFBP-1, insulin-like growth factor-binding protein-1; MLC2, light chain of myosin 2; MPA, medroxyprogesterone acetate; NT, nontargeting; P4, progesterone; PIAS1, protein inhibitor of activated STAT1; PR, P4 receptor; PRL, prolactin; RB, retinoblastoma protein; RTQ, real-time quantitative; siRNA, small interfering RNA; STAT3, signal transducer and activator of transcription 3; SUMO, small ubiquitin-like modifier.

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and PR are phylogenetically closely related and share 54 and 80% sequence homology in their ligand- and DNA-binding domains, respectively (10). AR expression, which is confined to the stroma in cycling endometrium, decreases during the secretory phase, although the receptor remains detectable in the decidua of early pregnancy (11, 12). Serum androgen levels fluctuate throughout the menstrual cycle, with levels peaking around ovulation (13, 14). However, tissue androgen levels and conversion of androstenedione to testosterone are higher in secretory than proliferative endometrium (15). Moreover, a rise in circulating androgen levels in the late luteal phase is associated with a conception cycle and levels continue to rise in early pregnancy (16). Interestingly, both lack and excess of circulating androgens in premature ovarian failure and polycystic ovary syndrome, respectively, are associated with increased risk of early fetal loss and late obstetric complication due to impaired placental function, such as preeclampsia (17–19).

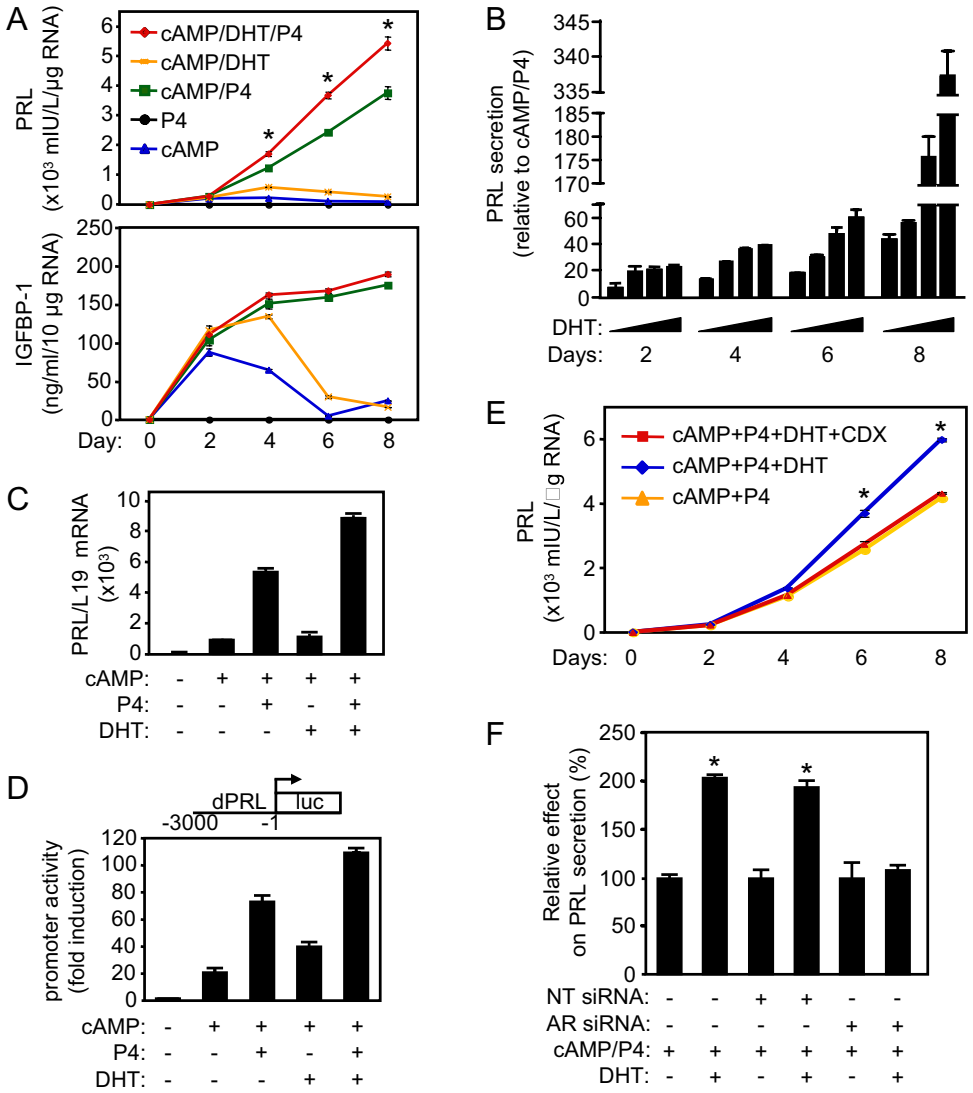

FIG. 1. DHT selectively enhances decidual PRL expression in a time-dependent manner. A, Primary HESCs were cultured in the presence of 8-br-cAMP, P4, and DHT as indicated. The medium was collected and cells harvested every 48 h. The data represent the mean PRL (*upper panel*) and IGFBP-1 (*lower panel*) concentrations (±SD) in the supernatant, with normalization for RNA content at each time point, of triplicate cultures. Significant differences were found in terms of PRL secretion between cultures treated with cAMP/P4 and cAMP/P4/DHT at 4, 6, and 8 d (*, $P < 0.05$). B, Primary HESCs were decidualized with 8-Br-cAMP, P4, and increasing concentrations of DHT (0.001, 0.01, 0.1, and 1 μM). The medium was collected and assayed for PRL concentration. The data are expressed as percentage increase over cAMP+P4 alone (mean ±SD). C, RTQ-PCR analysis was carried out for PRL mRNA levels in cultures treated as in A. The results show mean PRL mRNA levels (±SD) normalized to L19 mRNA of three independent cultures. D, HESCs were treated as in A for 48 h followed by transfection with dPRL-3000/Luc. Subsequently, the cells were maintained in the same culture conditions for 24 h. Luciferase and galactosidase assays were performed, and the results represent the mean (±SD) of triplicate measurements of one representative experiment. E, Primary HESCs were cultured in the presence of 8-br-cAMP, P4, DHT, and bicalutamide (Casodex; CDX) as indicated and PRL secretion determined as described above. PRL secretion was significantly different between cAMP/P4/DHT- and cAMP/P4/DHT/CDX-treated cultures (*, $P < 0.05$). F, HESCs, decidualized with a combination of 8-Br-cAMP, P4, and DHT for 48 h, were either mock-transfected or transfected with NT or AR siRNAs. The treatments were continued for 72 h and the supernatants assayed for PRL. The data represent the relative effect of DHT (percent) on decidual PRL secretion (mean ± SD) of triplicate cultures normalized to total protein content of each well (*, $P < 0.01$).

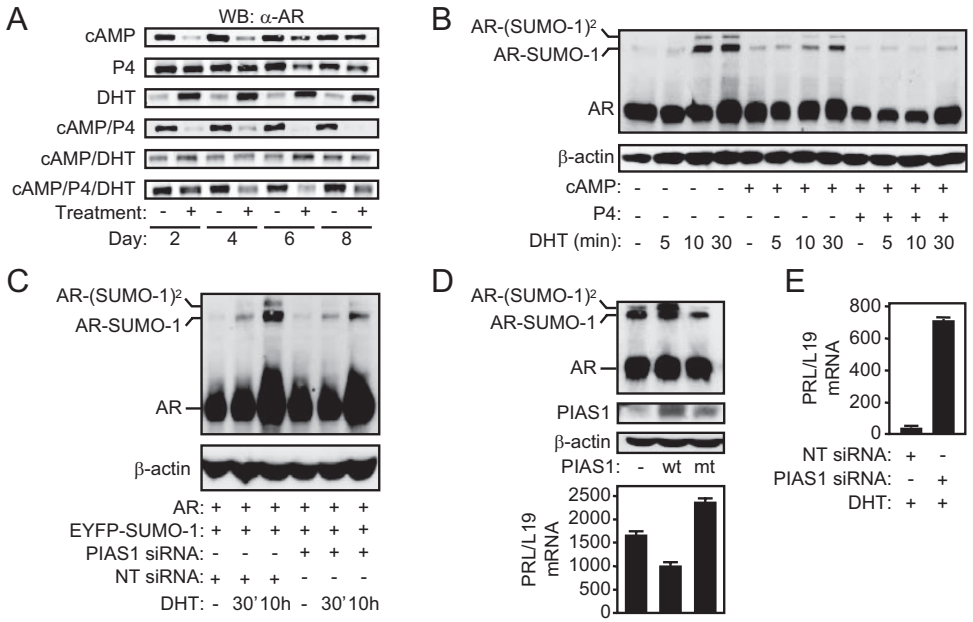

FIG. 2. PIAS1 attenuates AR sumoylation and regulates endogenous androgen responses in differentiating HESCs. A, DHT antagonizes AR down-regulation in decidualizing HESCs. Primary cultures were treated with a combination of 8-Br-cAMP, P4, DHT, and MPA, as indicated. Whole-cell lysates, extracted every 48 h for 8 d, were immunoprobed for AR expression. B, Primary cultures transfected with AR and EGFP-SUMO-1 were treated with vehicle or decidualized with 8-Br-cAMP with or without P4 for 48 h and then pulsed with DHT, as indicated. Total protein lysates were probed for AR expression. Single and double EGFP-SUMO-1-modified AR species are indicated. β-Actin served as a loading control. C, HESCs were transfected with AR, EGFP-Sumo-1, and either NT siRNA or siRNA targeting PIAS1. Cells were left untreated for 2 d and then treated with DHT as indicated. D, Parallel cultures were treated with 8-Br-cAMP and DHT for 48 h and then subsequently transfected with pSG5 or pSG5-PIAS1 (wt or mt) and were analyzed for PRL mRNA expression by RTQ-PCR (*lower panel*) and immunoprobed for AR and PIAS1 (*upper panel*), as indicated. E, Undifferentiated HESCs were transfected with NT or PIAS1 siRNAs and treated 2 d later with vehicle or DHT for 48 h. The results show mean (±SD) PRL transcript levels normalized to L19 mRNA of three independent cultures.

These observations provide compelling but circumstantial evidence that androgens play a role in decidual-trophoblast interactions in pregnancy. We now demonstrate that decidualization of HESCs is associated with increased responsiveness to androgen signaling. Compared with PR, AR controls a much smaller but focused network of genes essential for cytoskeletal organization and cell cycle regulation in decidualizing endometrium.

Materials and Methods

Primary endometrial cell culture

The Local Research and Ethics Committee at Hammersmith Hospitals NHS Trust approved the study, and patient consent was obtained before tissue collection. HESC cultures were established as previously described (2). Cultures were decidualized with 0.5 mM 8-Br-cAMP (Sigma Chemical Co., St. Louis, MO) and medroxyprogesterone acetate (MPA; Sigma), P4 (Sigma), dihydrotestosterone (DHT; Sigma), or bicalutamide (Casodex; AstraZeneca, London, UK), all at 1 μM bar for DHT, which was used at 0.1 μM concentration unless stated otherwise.

Transfections

Primary HESCs were transfected with DNA vectors or small interfering RNA (siRNA) by the calcium phosphate coprecipitation method using the Profection mammalian transfection kit (Promega, Madison, WI), as previously described (2). All transfections were performed in triplicate in 24-well plates and repeated at least three times. The expression plasmids for AR, PR-B, PIAS1, PIAS1(C351S, W372A), and EGFP-SUMO1 have been described (2, 6). The reporter constructs dPRL3000/Luc and PRE2/-32dPRL/Luc were a gift from B. Gellersen (Endokrinologikum Hamburg, Hamburg, Germany) The concentration of reporter constructs and expression vectors was 400 and 100 ng/well, respectively. The control vector pCH110 (50 ng/well), which leads to constitutive β-galactosidase expression, was used to compare transfection efficiency. For gene silencing studies, HESCs were cultured in six-well plates until confluency and transiently transfected with 100 nM of the following siRNA reagents (Dharmacon, Lafayette, CO): si*CONTROL* nontargeting (NT) siRNA Pool, AR si*GENOME* SMARTpool siRNA, PR si*GENOME* SMARTpool siRNA, and PIAS1 si*GENOME* SMARTpool siRNA.

Western blot analysis and prolactin (PRL) and insulin-like growth factor-binding protein-1 (IGFBP-1) assays

Whole-cell lysates and nuclear protein fractions were obtained as described elsewhere (4, 6). Proteins (30 μg) were separated on a 10% SDS-polyacrylamide gel before electrotransfer onto nitrocellulose membrane (Amersham, Little Chalfont, UK). The following primary antibodies were used: monoclonal AR (Biogenix, San Ramon, CA), mouse monoclonal PR (Novocastra Laboratories, Newcastle-Upon-Tyne, UK), mouse monoclonal β-actin (Abcam, Cambridge, UK), rabbit total and phosphorylated (Ser^{473}) AKT (Cell Signaling, Hitchin, UK), rabbit total and phosphorylated ($Thr^{202/204}$) ERK1/2 (Cell Signaling), mouse β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit phosphorylated ($Ser^{807/811}$) pRB (Cell Signaling), rabbit phosphorylated MLC (Ser^{19}) (Cell Signaling), rabbit IL1R1 (Abcam), and rabbit signal transducer and activator of transcription 3 (STAT3) and STAT5b (Upstate Biotechnology, Lake Placid, NY). Primary antibodies were diluted to 1:1000 except β-actin, which was used at 1:100,000. Secondary antibodies were diluted at 1:2000 dilution and protein complexes visualized with a chemoluminescent detection kit (Amersham). PRL in the HESC culture media was measured by microparticle enzyme immunoassay (AxSYM system; Abbott Laboratories, North Chicago, IL). IGFBP-1 levels in culture media were determined using an amplified two-step sandwich-type immunoassay (R&D Systems, Minneapolis, MN).

Microarray and real-time quantitative (RTQ)-PCR

Gene expression profiling was performed on four independent primary cultures, established from proliferative-phase biopsies. Total RNA was isolated from cultured HESCs using Stat-60 (Tel-Test, Friendswood, TX). Genomic DNA was removed by deoxyribonuclease treatment, and the quality of the RNA was evaluated using a Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA). All subsequent steps were carried out at the Finnish DNA-Microarray Centre using the Sentrix Human Illumina 6 V1 Expression BeadChips (Illumina, San Diego, CA), which contains over 47,000 known genes, gene candidates, and splice variants. Three hundred nanograms of each RNA sample, with 260/280 and 28S/18S ratio of greater than 1.8, was used to make double-stranded cDNA and then biotinylated cRNA using the Illumina RNA TotalPrep Amplification Kit (Ambion Inc., Austin, TX). Labeled cRNA was purified and hybridized to the BeadChip at 55 C, for 17 h after the Illumina Whole-Genome Gene Expression Protocol for BeadStation. Hybridized biotinylated cRNA was detected with cyanine3-streptavidin (Amersham). Arrays were scanned with the Illumina BeadArray Reader, which is a confocal-type imaging system with about 0.8-μm resolution and 532-nm laser illumination. The normalization and statistical analyses of the microarrays were performed using the statistical software R package limma (http://www.R-project.org). Genes of coefficient of variation values higher than 0.8 were filtered out from the analysis in preprocessing. The same software was used for single-gene analyses including fold-change calculations. The normalized data were analyzed by pair-wise comparisons to create a list of differentially expressed genes. Because we have shown that HESCs decidualized for 72 h are only modestly sensitive to androgen signaling, differentially expressed genes were defined by a lower boundary of a 99% confidence interval of fold change greater than 1.2 as validated by Student's *t* test ($P < 0.01$). To interpret the biological significance of differentially expressed genes, a gene ontology analysis was conducted using Ingenuity Pathways Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com). RTQ-PCR analysis was performed as previously described (4). All measurements were performed in triplicate. Gene-specific primer pairs were designed using the ABI Primer Express software (supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Fluorescence microscopy, motility, and proliferation assays

Immunofluorescence analysis was performed on cells seeded onto glass coverslips, fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton, and stained with tetramethylrhodamine isothiocyanate-labeled phalloidin (Chemicon, Temecula, CA) and counterstained with 4′,6-diamidino-2-phenylindole. The number of cells with actin stress fibers per 100 cells was determined by an independent assessor, blinded to the treatment, in three independent experiments. Cell motility was assessed by time-lapse microscopy using an inverted microscope with a motorized stage. Images were captured every 15 min over a 48-h period using a Hamamatsu C4742-95 CCD camera, and the distance each cell moved was analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD). Triplicate experiments were performed, and at least 15 cells per field of view, chosen randomly, were analyzed. Proliferation was ascertained using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega).

Results and Discussion

Increased androgen responses in decidualizing HESCs

Secretion of PRL and IGFBP-1 in response to cAMP and P4 signaling is the hallmark of decidual transformation of HESCs (2). To test whether androgens modify this differentiation process, primary HESC cultures were treated with 8-Br-cAMP, P4, and DHT, either alone or in combination over a time course lasting 8 d. Treatment with P4 alone was insufficient to trigger expression of either differentiation marker (Fig. 1A). In contrast, 8-Br-cAMP rapidly increased

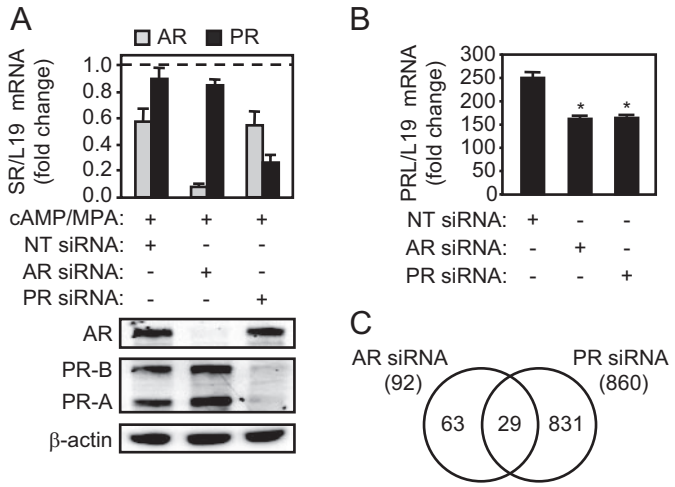

FIG. 3. AR and PR knockdown perturbs the expression of distinct gene sets in decidualizing HESCs. A, Validation of AR and PR silencing. The *upper panel* shows RTQ-PCR analysis of AR and PR transcript levels in cells transfected with NT, AR, or PR siRNAs before treatment with 8-Br-cAMP and MPA for 72 h. AR and PR mRNA levels were normalized to that of L19 mRNA, and the results are the mean (±SEM) of four separate cultures measured in triplicate. The results are fold change relative to transcript levels in undifferentiated cells transfected with NT siRNA (*dotted line*). The *lower panel* shows Western blot analysis of AR and PR expression in protein lysates from parallel cultures. β-Actin served as a loading control. B, RTQ-PCR analysis of PRL transcript levels, normalized to L19 mRNA, in cells first transfected with NT, AR, or PR siRNA followed by differentiation with 8-Br-cAMP and MPA for 72 h. The results are fold induction of PRL mRNA expression relative to the levels in undifferentiated cells transfected with NT siRNA. *, $P < 0.01$. C, Venn diagram showing the number of differentially expressed genes in decidualizing cells upon AR or PR knockdown.

PRL and IGFBP-1 secretion, but expression declined after 4 d of treatment. As expected (2), enhanced and sustained expression of both IGFBP-1 and PRL in long-term cultures required both 8-Br-cAMP and P4 signaling. DHT also modestly enhanced the expression of both marker proteins in 8-Br-cAMP-treated cultures, but in contrast to P4, DHT was insufficient to sustain the decidual response in prolonged culture. Interestingly, DHT markedly enhanced PRL but not IGFBP-1 secretion in cultures treated with 8-Br-cAMP plus P4. This androgen response in decidualizing cultures increased in magnitude over time in a dose-dependent manner (Fig. 1B). RTQ-PCR analysis demonstrated that the changes in PRL transcript levels mirrored those at the protein level (Fig. 1C). In addition, transfection studies with dPRL3000/Luc, a luciferase reporter construct coupled to 3 kb of the decidua-specific PRL promoter region (20), revealed that the pattern of PRL expression in response to cAMP, P4, and DHT corresponded to promoter activation (Fig. 1D).

To determine whether AR mediates the effect of DHT on PRL expression, primary cultures were first treated with 8-Br-cAMP plus P4 in the presence or absence of DHT and the nonsteroidal pure AR antagonist bicalutamide. This antiandrogen entirely negated the ability of DHT to enhance PRL secretion (Fig. 1E). Next, we transfected primary cultures, pretreated for 48 h with 8-Br-cAMP and P4 in the presence or absence of DHT, with either NT siRNA or siRNA targeting AR. Notably, AR depletion was very efficient (see

TABLE 1. Genes down-regulated upon AR knockdown

Gene symbol	Gene name	Fold change
AR	AR (DHT receptor)	−1.81
CUTL1 (37)	Cut-like 1 CCAAT displacement protein (*Drosophila*)	−1.49
WASPIP (38, 39)	Wiskott-Aldrich syndrome protein interacting protein	−1.48
DUSP3	Dual-specificity phosphatase 3 (vaccinia virus phosphatase VH1-related)	−1.46
LOC255065	LOC255065	−1.45
IL1R1 (32, 40)	IL-1 receptor type I	−1.43
PFTK1 (41)	PFTAIRE protein kinase 1	−1.42
PLA2G4F	Phospholipase A2, group IVF	−1.37
FAM135A	Family with sequence similarity 135, member A	−1.35
ALEX2 (42)	Armadillo repeat protein ALEX2	−1.35
SDCCAG1	Serologically defined colon cancer antigen 1	−1.35
GNPDA1	Glucosamine-6-phosphate deaminase 1	−1.33
LPGAT1	Lysophosphatidylglycerol acyltransferase 1	−1.33
WDSUB1	WD repeat, sterile α-motif and U-box domain containing 1	−1.32
CAST (43)	Calpastatin	−1.32
ZBTB1	Zinc finger and BTB domain containing 10	−1.28
LOC286470	LOC286470	−1.27
PLS3 (44)	Plastin 3 (T isoform)	−1.27
ARHGEF7 (45)	ρ-Guanine nucleotide exchange factor (GEF) 7	−1.27
ACTR3 (46)	ARP3 actin-related protein 3 homolog (yeast)	−1.27
AMBRA1 (47)	Autophagy/beclin-1	−1.27
PB1	Polybromo 1	−1.26
FLJ00060	Hypothetical gene FLJ00060	−1.25
KLHDC5	Kelch domain containing 5	−1.25
NR2F2	Nuclear receptor subfamily 2 group F member 2	−1.25
WDR40A	WD repeat domain 40A	−1.23
GDI1 (48)	GDP dissociation inhibitor 1	−1.22
RPC8	RNA polymerase III subunit RPC8	−1.22
C5orf24	Chromosome 5 open reading frame 24	−1.22
WBSCR16	Williams-Beuren syndrome chromosome region 16	−1.22
AHI1	Abelson helper integration site	−1.21
FBXW1B	F-box and WD-40 domain protein 1B	−1.21
POMT2 (49)	Protein-*O*-mannosyltransferase 2	−1.21
SMTN (50)	Smoothelin	−1.21
MADHIP	Mothers against decapentaplegic homolog (*Drosophila*) interacting protein	−1.20
C19orf26	Chromosome 19 open reading frame 26	−1.20
DYRK1A (51, 52)	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	−1.20

Gene symbols in *bold* indicate genes known to be regulated in endometrium. Genes implicated in cytoskeletal organization are referenced.

Fig. 3A) and abolished the ability of DHT to enhance PRL secretion in cells differentiated with 8-Br-cAMP and P4 (Fig. 1F). Together, the results unequivocally demonstrate that androgen actions in decidualizing HESCs are dependent upon AR activation.

Decidualization decreases small ubiquitin-like modifier (SUMO)-1 modification of the activated AR

We postulated that enhanced AR expression could account for the gradual increase in androgen sensitivity upon HESC differentiation. However, treatment of primary cultures with 8-Br-cAMP alone resulted in rapid but transient reduction in AR levels (Fig. 2A). Conversely, P4 had little effect on AR levels in short-term cultures but down-regulated receptor levels after 6–8 d of treatment. Combined 8-Br-cAMP plus P4 treatment resulted in a rapid and sustained decrease in cellular AR levels. DHT strongly increased AR levels in undifferentiated cells (Fig. 2A), as described in other cell systems (21), but only partially antagonized the down-regulation of the receptor in decidualizing cells. Thus, as reported for P4 (2), increased sensitivity to androgens in HESCs is paradoxically associated with decreasing receptor levels.

In the case of P4, increased responsiveness has been linked to global changes in cellular sumoylation upon HESC differentiation (6). More specifically, decidualization is characterized by a gradual decline in the expression of the E3 SUMO ligase protein inhibitor of activated STAT1 (PIAS1), resulting in attenuated ligand-dependent sumoylation of PR, increased transcriptional activity, and enhanced receptor turnover. PIAS1 also serves as an E3 ligase for AR (22). This prompted an analysis of AR sumoylation in undifferentiated and decidualizing HESCs. Untreated cultures and cells first decidualized with 8-Br-cAMP or 8-Br-cAMP plus P4 for 48 h were transfected with expression vectors encoding AR and enhanced green fluorescent protein (EGFP)-tagged SUMO-1 and pulsed 24 h later with DHT. Immunoblotting of cell lysates with an anti-AR antibody demonstrated the presence of two slower migrating forms of AR in undifferentiated cells, first apparent after 10 min DHT stimulation, which represent SUMO-1 modification of the two known acceptor sites (K386 and K520) in AR (Fig. 2B) (22). Compared with undifferentiated HESCs, DHT-dependent sumoylation of AR was attenuated in cells treated with 8-br-cAMP and much more so in cultures decidualized with 8-Br-cAMP plus P4. Next, we confirmed in COS-1 cells that increasing expression

of PIAS1 enhances ligand-dependent AR sumoylation (supplemental Fig. 1) and, conversely, that PIAS1 knockdown reduces AR SUMO-1 modification in HESCs treated with DHT (Fig. 2C). To explore whether PIAS1 regulates endogenous androgen responses in HESCs, we first overexpressed wild-type PIAS1 or a PIAS1 mutant (C351S, W372A) devoid of E3-ligase activity in cells decidualized with 8-Br-cAMP and DHT for 48 h. Parallel cultures were harvested after 24 h for protein and mRNA analyses. As shown in Fig. 2D, wild-type but not mutant PIAS1 enhanced SUMO-1 modification of AR, which corresponded to a 50% decrease in PRL mRNA expression. Notably, expression of the E3-deficient mutant antagonized endogenous PIAS1, resulting in decreased AR sumoylation and higher PRL mRNA levels. Furthermore, PIAS1 knockdown in undifferentiated HESCs was sufficient to induce PRL expression in response to DHT without the need of additional decidualizing stimuli (Fig. 2E). Together, the results demonstrate that down-regulation of PIAS1 upon decidualization sensitizes HESCs not only to P4 (6) but also to androgen signaling.

Identification of decidual AR and PR target genes

MPA, a 17-OH P4 derivative with known androgenic actions (23), is widely used in combination with 8-Br-cAMP to differentiate HESCs *in vitro* (2, 20). We confirmed that MPA, like DHT but not P4, enhances cellular AR levels in HESCs, induces its nuclear accumulation, and transactivates the receptor in a reporter assay (supplemental Fig. 2). We exploited the progestogenic and androgenic properties of MPA to search for specific AR- and PR-dependent genes in decidualizing HESCs. Four separate primary cultures were first transfected with either NT siRNA oligos or a siRNA pool targeting AR or PR and then treated with 8-Br-cAMP plus MPA for 72 h. Parallel cultures were harvested for mRNA and protein analysis. As shown in Fig. 3A, the siRNA knockdown approach for AR and PR was effective and selective at both the mRNA and protein level. Furthermore, knockdown of either receptor was equally efficient in attenuating PRL mRNA expression in differentiating HESCs (Fig. 3B). Total RNA was then processed for genome-wide expression profiling, and the data were interrogated using parametric statistical testing. Figure 3C represents the Venn diagram of regulated genes identified by the following pair-wise comparisons: AR siRNA-transfected cells (AR) *vs.* NT siRNA-transfected cells and PR siRNA-transfected cells (PR) *vs.* NT siRNA-transfected cells. AR knockdown affected the expression of a relatively small pool of genes. Of the 92 transcripts deregulated upon AR depletion, the expression of 42.4 and 57.6% of transcripts were up- and down-regulated, respectively (Tables 1 and 2). In contrast, PR knockdown perturbed the expression of 860 genes, 55.6% of which were up-regulated and 44.4% down-regulated. Tables 3 and 4 list the 50 most induced and repressed PR-dependent genes, respectively. We identified only 29 genes under control of both nuclear receptors in decidualizing cells, although 10 were regulated in an opposing manner (Table 5). Thus, the data confirm the major role of PR in regulating decidual gene expression and define, for the first time, a smaller but distinct set of genes under AR control.

TABLE 2. Genes up-regulated upon AR knockdown

Gene symbol	Gene name	Fold change
DIPA	Hepatitis δ-antigen-interacting protein A	1.45
CADPS2	Ca^{2+}-dependent activator protein for secretion 2	1.41
UGP2	UDP-glucose pyrophosphorylase 2	1.39
MCM4	MCM4 minichromosome maintenance deficient 4 (*S. cerevisiae*)	1.37
OIP5	Opa-interacting protein 5	1.33
CDT1	DNA replication factor	1.33
POLR1D	Polymerase (RNA) I polypeptide D 16 kDa	1.30
CCDC99	Coiled-coil domain containing 99	1.26
FDXR	Ferredoxin reductase	1.26
TMEM160	Transmembrane protein 160	1.25
TBC1D13	TBC1 domain family member 13	1.25
C3orf26	Chromosome 3 open reading frame 26	1.25
NDFIP2	Nedd4 family interacting protein 2	1.25
ANKRD36	Ankyrin repeat domain 36	1.25
WDR51A	WD repeat domain 51A	1.24
IQCC	IQ motif containing C	1.23
MEIS2	Meis1 myeloid ecotropic viral integration site 1 homolog 2 (mouse)	1.22
LOC121642	Similar to prostate cancer antigen-1	1.22
ACAS2	Acetyl-coenzyme A synthetase 2 (AMP forming)-like	1.22
RGS10	Regulator of G-protein signaling 10	1.21
CHTF18	CTF18 chromosome transmission fidelity factor 18 homolog (*S. cerevisiae*)	1.21
PDE7B	Phosphodiesterase 7B	1.20
RPL10A	Ribosomal protein L10a	1.20
HBLD1	HESB like domain containing 1	1.20
ECE2	Endothelin converting enzyme 2	1.20
PRO0386	Hypothetical protein PRO0386	1.20

Gene symbols in *bold* indicate genes known to be regulated in endometrium.

TABLE 3. Top 50 down-regulated genes upon PR knockdown

Gene symbol	Gene name	Fold change
APCDD1	Adenomatosis polyposis coli down-regulated 1	−5.37
CNR1	Cannabinoid receptor 1	−4.21
LOC347348	Similar to heat-shock 27-kDa protein (HSP27)	−3.34
CHST7	Carbohydrate (*N*-acetylglucosamine 6-*O*) sulfotransferase 7	−3.19
HSPB1	Heat-shock 27-kDa protein 1	−3.19
RASD1	RAS dexamethasone-induced	−3.12
FKBP5	FK506 binding protein 5	−3.10
IGF1	IGF-I (somatomedin C)	−3.04
RORB	RAR-related orphan receptor B	−3.01
C13orf33	Chromosome 13 open reading frame 33	−3.00
ACPL2	Acid phosphatase-like 2	−2.71
SORBS1	Sorbin and SH3 domain containing 1	−2.59
PCDH19	Protocadherin 19	−2.59
HSD11B1	Hydroxysteroid (11-β) dehydrogenase 1	−2.56
AOX1	Aldehyde oxidase 1	−2.51
PDLIM1	PDZ and LIM domain 1 (elfin)	−2.51
RASL10B	RAS-like family 10 member B	−2.5
BTBD3	BTB (POZ) domain containing 3	−2.46
MDM1	Nuclear protein double minute 1	−2.46
ZNF145	Zinc finger protein 145 (Kruppel-like expressed in promyelocytic leukemia)	−2.41
ADAMTS1	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif 1	−2.41
IMPA2	Inositol(myo)-1(or 4)-monophosphatase 2	−2.38
NRXN3	Neurexin 3	−2.32
HSPB6	Heat-shock protein, α-crystallin-related, B6	−2.32
METTL7A	Methyltransferase like 7A	−2.28
OSR2	Odd-skipped-related 2A protein	−2.27
SIPA1L2	Signal-induced proliferation-associated 1 like 2	−2.25
DSIPI	δ-Sleep inducing peptide immunoreactor	−2.24
PRPS2	Phosphoribosyl pyrophosphate synthetase 2	−2.21
GATA6	GATA binding protein 6	−2.20
PPAP2B	Phosphatidic acid phosphatase type 2B	−2.20
PIK3R1	Phosphoinositide-3-kinase regulatory subunit polypeptide 1 (p85 α)	−2.16
SERPINE1	Serine (or cysteine) proteinase inhibitor clade E	−2.15
XYLT1	Xylotransferase 1	−2.14
ATAD2	ATPase family, AAA domain containing 2	−2.13
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	−2.13
ABLIM3	Actin-binding LIM protein family member 3	−2.12
CARD9	Caspase recruitment domain family member 9	−2.12
LARGE	Like-glycosyltransferas	−2.09
NPR1	Natriuretic peptide receptor A/guanylate cyclase A	−2.09
PGR	Progesterone receptor	−2.08
DKK1	Dickkopf homolog 1 (*Xenopus laevis*)	−2.05
RAB40A	RAB40A member RAS oncogene family	−2.03
LMCD1	LIM and cysteine-rich domains 1	−2.02
RACGAP1	Rac GTPase activating protein 1	−2.00
SLC27A3	Solute carrier family 27 (fatty acid transporter) member 3	−1.96
FLJ11539	Hypothetical protein FLJ11539	−1.95
SLC7A8	Solute carrier family 7 (cationic amino acid transporter y+ system) member 8	−1.95
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	−1.94
TNFRSF1B	TNF receptor superfamily member 1B	−1.92

Gene symbols in *bold* indicate genes known to be regulated in endometrium.

Because all cultures were decidualized before array analysis, we cross-referenced our genes lists with the Endometrium Database Resource (http://endometrium.bcm.tmc.edu/edr/) and annotated the tables to indicate genes already reported to be regulated upon endometrial differentiation. RTQ-PCR validation of genes under the putative control of AR (*IL1R1*, *DUSP3*, and *OIP5*), PR (*MMP10*, *TWIST1*, and *RASD1*), or both AR and PR (*KCNK3*, *PCDH7*, and *WNT4*) yielded no false-positive results (Fig. 4). Interestingly, not all identified target genes were dependent upon ligand activation of the receptor. For instance, *DUSP3* (dual-specificity phosphatase 3) mRNA levels remained unchanged upon HESC differentiation, yet transfection of AR siRNA resulted in a 3-fold decrease in the transcript levels of this negative regulator of the MAPK pathway. Similarly, *MMP10* (matrix metalloproteinase 10) expression was strongly repressed in differentiating HESCs, and PR knockdown not only reversed this repression but also elicited a 4-fold increase in *MMP10* mRNA levels when compared with undifferentiated cells, suggesting that even the unliganded PR plays a role in repressing *MMP* expression in human endometrium.

TABLE 4. Top 50 up-regulated genes upon PR knockdown

Gene symbol	Gene name	Fold change
CXCR4	Chemokine (C-X-C motif) receptor 4	6.69
MMP10	Matrix metalloproteinase 10 (stromelysin 2)	5.29
STC1	Stanniocalcin 1	5.20
KIAA1199	KIAA1199 protein	5.01
FJX1	Four jointed box 1 (Drosophila)	4.81
TNFRSF11B	TNF receptor superfamily member 11b (osteoprotegerin)	3.99
ABCG1	ATP-binding cassette sub-family G (WHITE) member 1	3.76
MMP11	Matrix metalloproteinase 11 (stromelysin 3)	3.56
IER3	Immediate-early response 3	3.35
SLC16A6	Solute carrier family 16 (monocarboxylic acid transporters) member 6	3.14
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15 inhibits CDK4)	2.86
GDF15	Growth differentiation factor 15	2.76
IL13RA2	IL-13 receptor α2	2.75
ANGPTL2	Angiopoietin-like 2	2.74
THBS2	Thrombospondin 2	2.72
NR4A2	Nuclear receptor subfamily 4 group A member 2	2.63
C10orf10	Chromosome 10 open reading frame 10	2.63
SOX4	SRY (sex determining region Y)-box 4	2.56
FAM43A	Family with sequence similarity 43, member A	2.53
TEK	TEK tyrosine kinase endothelial	2.50
DPYSL4	Dihydropyrimidinase-like 4	2.48
NNAT	Neuronatin	2.45
MEX3A	Mex-3 homolog A	2.45
PDE4B	Phosphodiesterase 4B cAMP-specific	2.41
FOXP1	Forkhead box P1	2.32
AMSH-LP	Associated molecule with the SH3 domain of STAM (AMSH) like protein	2.32
FRMD4	FERM domain containing 4	2.29
TWIST1	Twist homolog 1)	2.25
ARNT2	Aryl-hydrocarbon receptor nuclear translocator 2	2.24
CNIH3	Cornichon homolg 3	2.23
PPFIBP2	PTPRF interacting protein binding protein 2 (liprin β 2)	2.22
LOH11CR2A	Loss of heterozygosity11 chromosomal region 2	2.22
EHZF	Early hematopoietic zinc finger	2.19
DIO2	Deiodinase iodothyronine type II	2.19
ABCA6	ATP-binding cassette sub-family A (ABC1) member 6	2.18
CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	2.18
TNFRSF21	TNF receptor member 21	2.16
FLJ22536	Hypothetical locus LOC401237	2.16
FHOD3	Formin homology 2 domain containing 3	2.15
WNT2	Wingless-type MMTV integration site family member 2	2.15
F13A1	Coagulation factor XIII A1 polypeptide	2.14
KIAA1370	Hypothetical protein LOC56204	2.14
CSRP2	Cysteine- and glycine-rich protein 2	2.07
CHAC1	ChaC, cation transport regulator homolog 1	2.06
CREB5	cAMP responsive element binding protein 5	2.06
PBEF1	Pre-B-cell colony enhancing factor 1	2.04
FGF9	Fibroblast growth factor 9	2.04
MGC13057	Hypothetical protein MGC13057	2.04
FAP	Fibroblast activation protein α	2.02
LOC221091	Hypothetical protein LOC221091	2.01

Gene symbols in *bold* indicate genes known to be regulated in endometrium.

PR is indispensable for activation of secondary signaling pathways upon decidualization

Microarray analyses have been extensively used to examine endometrial responses to P4 in humans and various animal models (24–26). Our gene profiling complements these studies and confirms that PR controls the expression of a network of at least 860 genes in decidualizing HESCs. Ingenuity Pathway Analysis clustered PR-dependent genes into 28 different functional molecular and cellular categories (supplemental Fig. 3), and many can be functionally linked to known P4 actions in the endometrium. For instance, P4 critically ensures tissue integrity of the decidualizing endometrium before menstruation and during pregnancy (3, 7), and not unexpectedly, several genes repressed in a PR-dependent manner encode for matrix metalloproteinases (*MMP2*, *-8*, *-10*, *-11*, and *-27*), death receptors of the tumor necrosis factor receptor superfamily (*TNFRSF10B*, *-10D*, *-11B*, *-19*, and *-21*), apoptosis mediators (*e.g. MCL1*, *NR4A2*, *BCL2L10*, *TRAF4*, *TP53INP*, *MOAP1*, *SEMA3F*, *DAD1*, *CIAPN1*, *DEDD*, and *MAP3K5*), and oxidative stress defenses and DNA repair (*e.g. TXNRD1*, *HNOX1*, *PPP1R15A*, and *XRCC5*).

Although insufficient to trigger HESC differentiation,

TABLE 5. Genes regulated by AR and PR

Gene symbol	Gene name	AR siRNA fold change	PR siRNA fold change
INHBA	Inhibin β A	−1.56	2.38
KCNK3	Potassium channel subfamily K member 3	−1.55	−1.36
FADS2	Fatty acid desaturase 2	−1.45	−1.55
LMOD1 (53)	Leiomodin 1 (smooth muscle)	−1.43	−1.67
WNT4	Wingless-type MMTV integration site family member 4	−1.39	−1.60
ELOVL4	Elongation of very long chain fatty acids (FEN1/Elo2 SUR4/Elo3 yeast)-like 4	−1.36	1.40
GPR125 (54)	G protein-coupled receptor 125	−1.30	1.37
KIAA1377	KIAA1377 protein	−1.27	−1.28
LRRK1 (55)	Leucine-rich repeat kinase 1	−1.24	−1.35
LRCH2	Leucine-rich repeats and calponin homology (CH) domain containing 2	−1.23	1.49
CTSO (56)	Cathepsin O	−1.21	−1.23
CKAP4 (57)	Cytoskeleton-associated protein 4	−1.21	−1.22
TEAD3 (58, 59)	TEA domain family member 3	−1.20	−1.26
LOC401627	Similar to hypothetical protein FLJ33610	−1.20	−1.21
TCEAL7	Transcription elongation factor A (SII)-like 7	−1.20	1.59
MRAS (60)	Muscle RAS oncogene homolog	−1.20	1.34
MCM2	MCM2 minichromosome maintenance deficient 2 mitotin (*S. cerevisiae*)	1.39	−1.25
PCDH7	BH-protocadherin (brain-heart)	1.38	1.74
PCDH7	BH-protocadherin (brain-heart)	1.28	1.63
HNRPH2	Heterogeneous nuclear ribonucleoprotein H2 (H')	1.27	1.23
WEE1	WEE1 homolog (*S. pombe*)	1.26	1.32
IMPDH2	IMP (inosine monophosphate) dehydrogenase 2	1.25	−1.24
ACSL1	Acyl-CoA synthetase long-chain family member 1	1.23	−1.43
DPP4	Dipeptidylpeptidase 4 (CD26 adenosine deaminase complexing protein 2)	1.23	1.51
XTP3TPA	XTP3-transactivated protein A	1.23	1.21
XAB1	XPA binding protein 1	1.22	1.43
PAK1	PAK1 interacting protein 1	1.22	1.35
BM88	BM88 antigen	1.20	1.28
NAP1L1	Nucleosome assembly protein 1-like 1	1.20	−1.29

Gene symbols in *bold* indicate genes known to be regulated in endometrium. Genes implicated in cytoskeletal organization are referenced.

P4 is essential for maintaining the decidual phenotype both *in vivo* and *in vitro*. Compelling evidence has emerged to indicate that sustained expression of the decidual phenotype is also dependent on autocrine or paracrine signals, resulting in activation of various secondary signaling pathways (5, 27, 28). Strikingly, a significant number of PR-dependent genes encode for ligands, membrane-bound receptors, and intermediates in various signal transduction pathways (supplemental Fig. 4). This prompted us to examine the expression and/or activation status of critical signal intermediates in the STAT, MAPK (ERK1/2), PI3K, TGFβ/SMAD, and WNT/β-catenin pathways in undifferentiated HESCs and decidualized cells transfected with AR or PR siRNAs. In agreement with the gene profile, PR knockdown selectively abolished the induction of STAT3 and STAT5b upon HESC differentiation (Fig. 5). Total ERK1/2, AKT, SMAD2, and β-catenin levels remained unchanged upon differentiation of HESCs. However, decidualization was accompanied by a down-regulation in activated (phosphorylated) AKT levels and a reciprocal increase in ERK1/2 phosphorylation, yet the activation status of either pathway was unaffected by AR or PR depletion. In contrast, PR knockdown eliminated nuclear accumulation of activated SMAD2 and β-catenin in differentiating HESCs. The data imply that a substantial proportion of PR-dependent decidual genes are regulated indirectly, via autocrine or paracrine activation of the WNT/β-catenin, TGFβ/SMAD, and STAT pathways.

AR regulates cytoskeletal organization and cell cycle inhibition

Ingenuity pathway analysis complemented by manual mining of available literature implicated 40% (21 of 53) of genes down-regulated upon AR silencing in the regulation of cell morphology, cytoskeletal organization, and cell motility (Tables 1 and 5). This prompted us to examine more closely the differentiation-associated changes in the actin cytoskeleton by phalloidin staining of filamentous actin (F-actin). As shown in Fig. 6A, decidualization is characterized by a dramatic increase in F-actin polymerization and stress fiber formation. However, the proportion of cells that express elongated stress fibers was reduced by approximately 50% upon AR knockdown, whereas PR silencing had no apparent effect (Fig. 6A and supplemental Fig. 5). Time-lapse microscopy demonstrated that decidualization is also associated with a dramatic decrease in basal cell motility, which was partially reversed upon AR knockdown (Fig. 6B). Actin-myosin interactions are essential for cell motility and promoted by phosphorylation of the regulatory light chain of myosin 2 (MLC2) (29). In agreement with the motility studies, AR knockdown was sufficient to reverse loss of MLC2 phosphorylation upon decidualization of HESCs (Fig. 6C).

In addition to cell motility, the actin cytoskeleton is involved in many other biological functions, including endo- and exocytosis, cytokinesis, and signal transduction (30, 31),

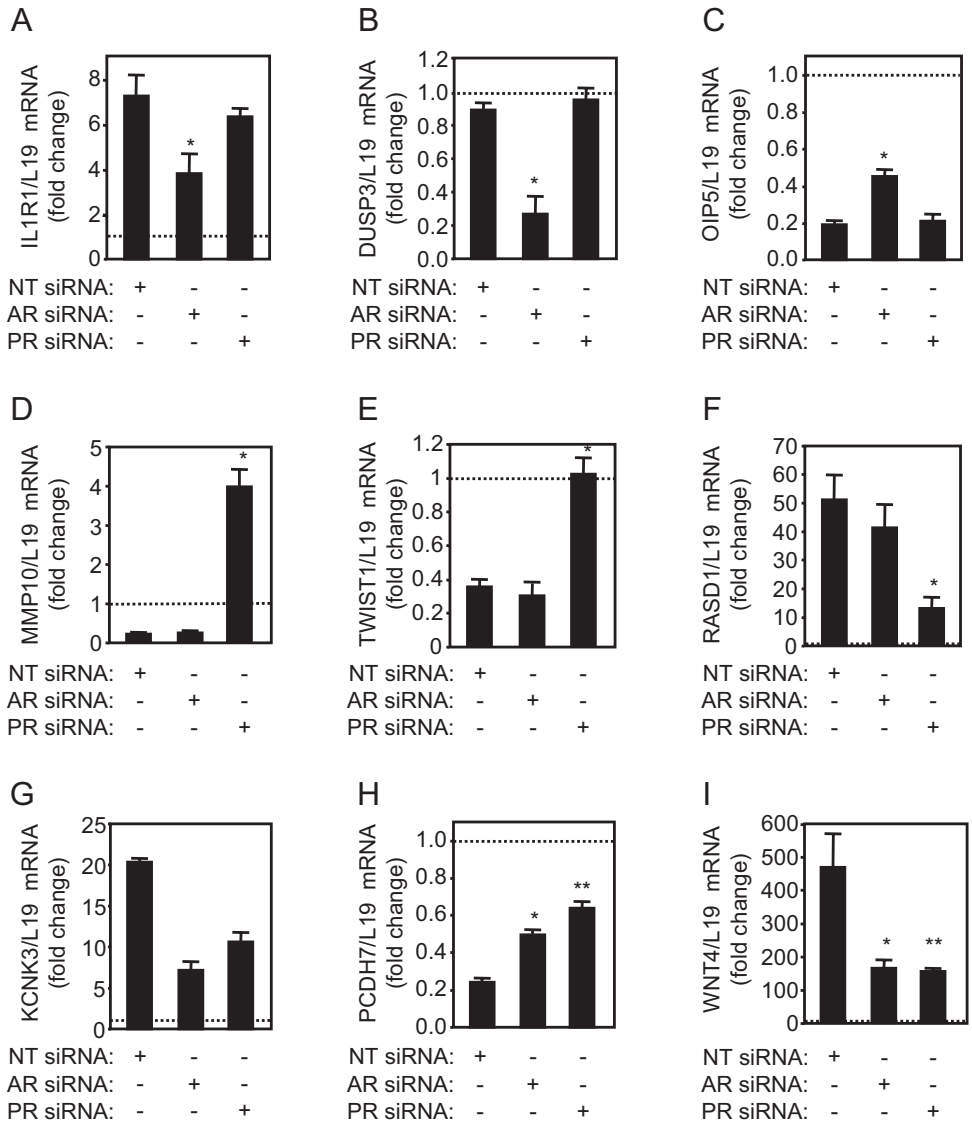

FIG. 4. Validation of putative AR- and PR-dependent genes. For microarray validation, three separate cultures were first transfected with NT, AR, or PR siRNA and then treated with 8-Br-cAMP and MPA for 72 h, and mRNA levels of the indicated putative target genes were measured in triplicate for each sample by RTQ-PCR. The data normalized to L19 are expressed as fold change (±SEM) relative to expression levels in undifferentiated HESCs transfected with NT siRNA (*dotted lines*). *, $P < 0.05$; **, $P < 0.001$.

underscoring the importance of AR in regulating decidual cell function. Importantly, induction of the IL-1 receptor (IL1R1) in decidualizing cells is under AR control (Fig. 4). Embryonic signals, and in particular IL-1β, have been shown to activate focal adhesion kinase (FAK) and to further promote cytoskeletal reorganization in decidual cells (32). Together, these observations suggest AR plays a major role in coordinating decidual-trophoblast interactions during early pregnancy. This conjecture is further supported by the observation that inactivation of decidual RhoA, a Rho GTPase family member essential for cytoskeletal organization, blocks outgrowth but not attachment of blastocysts in a coculture model (33).

In silico analysis further revealed that several genes upregulated in decidualizing cells upon AR depletion, and thus normally repressed in an AR-dependent manner, are involved in various aspects of cell cycle regulation (*e.g. NAP1L1*, *WEE1*, *BM88*, *XTP3TA*, and *IMPDH2*) including DNA replication licensing (*e.g. CDT1*, *MCM4*, and *MCM2*) and chromatid separation (*e.g. CHTF18*, *DIPA*) (Tables 2 and 5). This expression profile points toward a role for AR in safeguarding the genetic stability of the endometrium during rapid cyclic remodeling. Functionally, AR knockdown enhanced proliferation of HESCs decidualized with 8-Br-cAMP and MPA (Fig. 6D). In contrast, proliferation was modestly but consistently reduced upon PR knockdown. Inactivation of retinoblastoma protein (RB) by hyperphosphorylation enables the expression of E2F-target genes essential for coordinating entry into S phase of the cell cycle (34). As shown in Fig. 6E, differentiation of HESCs is strongly associated with loss of RB phosphorylation but much less so upon AR knockdown.

In summary, we have shown that HESCs become increasingly responsive to androgen signaling upon differentiation. This increased sensitivity to sex steroids, whether androgens or P4, is directly linked to global changes in cellular sumoylation and, more specifically, to differentiation-dependent down-regulation of PIAS1, the E3 SUMO-1 ligase of AR and PR. In comparison to PR, AR governs the expression of a limited decidual gene pool,

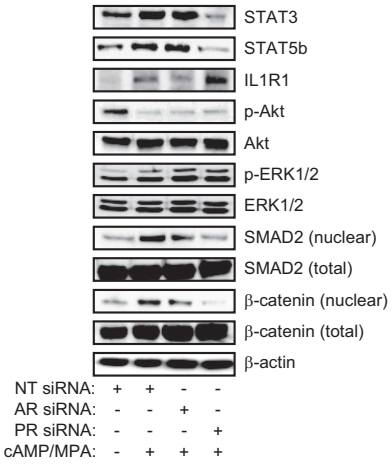

FIG. 5. PR regulates STAT, TGFβ/SMAD, and WNT/β-catenin signaling in decidualizing cells. Whole-cell lysates or nuclear protein fractions from HESCs, transfected first with NT, AR, or PR siRNA and then treated with 8-Br-cAMP and MPA for 72 h, were immunoprobed for various signal intermediates, as indicated. β-Actin served as a loading control.

responsible for cytoskeletal organization and inhibition of cell motility and proliferation. These cell functions under AR control may be critical for coordinated trophoblast invasion and placental development. This notion is supported by the observations in female AR-deficient mice, demonstrating that uterine responses to exogenous gonadotropins are impaired before developing premature ovarian failure (35). Furthermore, the earliest reproductive defect in these mice is a dramatic reduction in the number of pups per litter, and pregnancy is further characterized by placentamegaly, which suggests a compensatory response to defective uterine remodeling (35, 36). However, additional experiments are required to exclude the possibility that these early uterine defects in AR-deficient mice are a consequence of impaired ovarian steroidogenesis.

The identification of human AR signature genes could be exploited to assess the decidual responses before pregnancy, especially in patients with relative androgen deficiency, including older women and patients with premature ovarian failure receiving fertility treatment with donor oocytes. Such translational studies may provide the *in vivo* rational for the targeted use of selective AR modulators for the prevention of associated pregnancy complications.

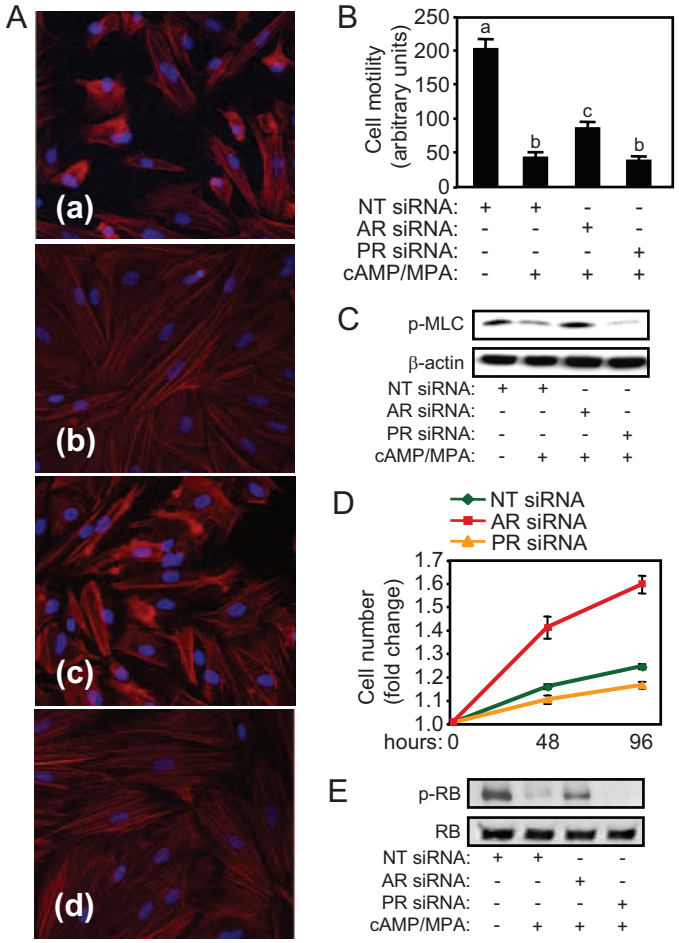

FIG. 6. AR controls cytoskeletal organization, cell motility, and proliferation in differentiating HESCs. A, Phalloidin staining of F-actin in undifferentiated HESCs transfected with NT siRNA (a) and cells decidualized with cAMP and MPA for 72 h after transfection with NT siRNA (b), AR siRNA (c), or PR siRNA (d). B, Motility of HESCs transfected and treated as in A was analyzed by time-lapse microscopy, quantified, and expressed in arbitrary units. The results are the mean (±SD) of triplicate analyses. *Different letters above the error bars* indicate that those groups are significantly different from each other at $P < 0.01$. C, Protein lysates obtained from parallel cultures were immunoprobed for phosphorylated MLC2. β-Actin served as a loading control. D, Primary cultures were first transfected in six-well plates with NT, AR, or PR siRNAs, replated in 96-well plates, and treated with 8-Br-cAMP and MPA, and cell viability was measured at the indicated time points. The results show the relative fold change in cell number, and the data are the mean (±SD) of triplicate measurements. E, Protein lysates from HESCs transfected with NT, AR, or PR siRNA, then treated with 8-Br-cAMP and MPA for 72 h, were subjected to Western blot analysis for total and phosphorylated RB (p-RB) expression.

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