The Poly(C)-Binding Protein-1 Regulates Expression of the Androgen Receptor

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The androgen receptor (AR) is a ligand-dependent transcription factor, expressed in male and female reproductive organs, and essential for normal reproduction in both sexes. The levels of AR are tightly controlled in androgen-responsive cells in which it plays a central role in the regulation of target gene expression. The AR is abundantly expressed in human endometrial stromal cells (HESCs), but levels decline markedly after differentiation into decidual cells in vivo and in primary cultures. Decidualization profoundly down-regulated AR protein levels with no discernible effect on either AR mRNA or protein stability, suggesting that loss of the receptor was a consequence of translational inhibition. Here we show that HESCs express three RNA-binding proteins, Hu antigen R and the poly(C)-binding proteins PCBP1 and PCBP2, that reportedly target the 3’-untranslated region of AR transcripts. Only PCBP1 expression was enhanced in secretory endometrium in vivo and in decidualizing HESCs. Furthermore, knockdown of PCBP1 in decidualizing cells was sufficient to restore AR protein levels, indicating that loss of the AR protein is primarily the consequence of a translational block. PCBP1 also blocked AR translation in a cell-free system, although this did not require binding to the 3’-untranslated region of the receptor mRNA. Furthermore, knockdown of PCBP1 in the prostate cancer LNCaP cell line also increased AR protein. Therefore, PCBP1 plays a major role in the dynamic expression of AR in both male and female androgen-responsive cells.

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Hormonal responses in reproductive tissues are mediated by members of the ligand-dependent nuclear receptor superfamily that include estrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR). Although androgens are best known for their role in male reproduction, the AR is present in both male and female reproductive organs and is essential for normal reproductive function in both sexes (1, 2). In the absence of ligand, the AR is predominantly cytoplasmic in a large dynamic heterocomplex composed of heat shock proteins. Ligand binding induces a conformational change in the AR protein, and it is translocated to the nucleus in which it binds the promoters of androgen-regulated genes. The processes affected by AR include cell proliferation and differentiation, which are especially well defined in prostate cancer (3, 4).

In women, the AR is essential in the ovary for normal folliculogenesis, and its absence leads to premature ovarian failure (1). The receptor is also abundantly expressed in the human endometrium, a tissue that is exquisitely sensitive to the actions of many steroid hormones. In response to fluctuating levels of ovarian estrogens and progesterone, the endometrium undergoes waves of proliferation, differentiation, menstrual shedding, and regeneration. These hormonal responses are regulated by coordinated activation of the ER and PR, respectively. The

Abbreviations: AR, Androgen receptor; B-Br-cAMP, 8-bromoadenosine-cAMP; ER, estrogen receptor; HESC, human endometrial stromal cell; hnRNP, heteronuclear ribonucleoprotein; HuR, Hu antigen R; miRNA, microRNA; NT, nontargeting; P4, progesterone; PCBP, poly(C)-binding protein; PR, progesterone receptor; RBP, RNA-binding protein; RNP, ribonucleoprotein; RTQ-PCR, real-time quantitative PCR; siRNA, small interfering RNA; UTR, untranslated region.
pattern of AR expression in the endometrial stromal compartment resembles that of PR, with the highest levels occurring during the proliferative phase, followed by declining receptor levels throughout the secretory phase of the cycle (5–7). Circulating androgen levels also fluctuate during the menstrual cycle with levels peaking around the time of ovulation, although the tissue concentration of androgens is higher in secretory than proliferative endometrium, reflecting expression of 17β-hydroxysteroid dehydrogenase and increased local conversion of androstenedione to testosterone (8–10). Yet in contrast to ER and PR, the role and regulation of AR in the endometrium remains poorly understood. Recently we have shown that human endometrial stromal cells (HESCs) become increasingly responsive to androgens on differentiation into decidual cells, despite a relative decline in AR levels (11). Moreover, knockdown experiments demonstrated a nonredundant function for the activated AR in cytoskeletal organization and cell cycle regulation in decidualizing cells (11).

Regulation of steroid receptor expression in the endometrium has largely been studied in terms of signaling pathways and transcription factors that control activation of the encoding genes. However, the flow of information from the genome to the proteome remains tightly controlled beyond transcription by several additional regulatory systems, including the microRNA (miRNA) machinery and ribonucleoprotein (RNP) enzymes (12, 13). In recent years tremendous progress has been made in characterizing how miRNAs, which are genomically transcribed noncoding about 20–30 nucleotide RNA molecules, regulate gene expression by controlling the stability and/or translation of mRNAs. Two major enzymes, DICER and DROSHA, are responsible for the generation of mature miRNAs and gene silencing (14). To date, in excess of 700 miRNAs have been identified in mammalian cells, many of which display tissue-specific expression patterns. RNAs, whether protein coding or not, exist in cells in RNP complexes that are typically composed of numerous RNA-binding proteins (RBPs). RNP complexes critically govern gene expression by controlling mRNA splicing (the spliceosome) and protein synthesis (the ribosome) (15, 16).

RBPs exert several other essential functions, including modulation of mRNA stability through binding to cis-regulatory elements residing in the mRNA 3′-untranslated region (UTR) of target transcripts. This is aptly illustrated by the cross talk between heteronuclear ribonucleoprotein (hnRNP) D and ER. The activated ER regulates the expression of hnRNP D, which in turn stabilizes ER mRNA, thereby providing a feed-forward signal (17, 18). In the context of AR, three RBPs, Hu antigen R (HuR) and the poly(C)-binding protein (PCBP)-1 and PCBP2, have been shown to bind the 3′-UTR of AR mRNA (19), although the functional consequences of these interactions on AR expression have not yet been studied. HuR is a member of the embryonic lethal abnormal vision/Hu family of RBPs, capable of modulating gene expression by regulating mRNA stability, nuclear export, and translation (20–22). PCBP1 and PCBP2, two highly homologous members of the hnRNP K homology domain family of RBPs (23), have been shown to promote translation of certain transcripts, such as α-globulin and erythropoietin mRNAs (24), but to translationally silence others, including 15-lipoxygenase and CCAAT/enhancer-binding protein-α mRNAs (25, 26). Interestingly, PCBP1 has also been implicated in transcriptional regulation and, more recently, in mRNA splicing (27, 28).

This study examined the posttranscriptional mechanisms that regulate AR expression in decidualizing HESCs and prostate cancer LNCaP cells. We show that translation of AR transcripts is inhibited on differentiation of HESCs in response to cAMP and progesterone (P4) signaling, through a mechanism that does not involve the miRNA machinery. Whereas HuR and PCBP2 are expressed in HESCs and capable of regulating AR expression, it is the induction of PCBP1 in response to cAMP and P4 signaling that limits translation of AR transcripts in decidualizing cells. Knockdown of PCBP1 in LNCaP cells also led to an increase in AR protein, indicating a central role for this RBP in controlling AR expression in androgen responsive cells.

Materials and Methods

Primary endometrial cell culture and timed tissue sampling

The local Research and Ethics Committee at Hammersmith Hospitals National Health Service Trust approved the study, and patient consent was obtained before tissue collection. Endometrial biopsies were obtained by curettage from women aged 18–40 yr at the time of diagnostic laparoscopy, diagnostic hysteroscopy, or laparoscopic sterilization. All women had regular menstrual cycles and received no hormonal treatment in the 3 months before biopsy. HESC cultures were established as previously described (29). Cultures were decidualized with 0.5 mm 8-bromoadenosine-cAMP (8-Br-cAMP; Sigma, St. Louis, MO) and 1 μM P4 (Sigma). Actinomycin D, cycloheximide, and MG132 were purchased from Sigma and used at concentrations of 5 μg/ml, 10 μg/ml, and 20 μM, respectively.

Timed endometrial biopsies were taken from fertile women with regular menstrual cycles who had not used hormonal preparations in the preceding 3 months. The samples were classed as early to midproliferative when taken within 10 d of the last menstrual period. Secretory samples were obtained from women between 5 and 9 d after the LH surge. For the purposes of RNA analysis, the biopsies were obtained at room temperature in approximately 5× volume of RNAlater (Ambion, Austin, TX) and stored as per the manufacturer’s instructions until RNA extraction.
Plasmids and transfections
Primary HESCs were transected 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 (29). The expression plasmid consisting of only the human AR coding sequence without any 3’ UTR, cloned into pSG5, has previously been described (30). The expression vector pcDNA3.1-HuR was a gift from Joan Steitz (Howard Hughes Medical Institute, Yale University, New Haven, CT) (21), whereas both pSG5-PCBP1 and pSG5-PCBP2 were gifts from Matthias Hentze (European Molecular Biology Laboratory, Heidelberg, Germany) (31). The 634-bp 3’UTR of the AR mRNA (genomic coordinates chrX: 66726674–66727308) was cloned downstream of the Luc2P (destabilized luciferase reporter) gene to generate the expression vector pcDNA3.1/Luc-AR3UTR. A control expression vector pcDNA3.1/Luc containing only the Luc2P gene encoding sequence in pcDNA3.1/DV5-His-TOPO (Invitrogen, Carlsbad, CA) was also generated.

For gene-silencing studies, HESCs were cultured in six-well plates until confluency and transiently transfected with 80 nM of siCONTROL nontargeting (NT) siRNA pool, DICER siGENOME SMARTpool siRNA, or PCBP1 siGENOME SMARTpool siRNA (Dharmacon, Lafayette, CO). LNCaP cells were kindly provided by Dr. C. Bevan (Dharmacon, Lafayette, CO) and transfected with siRNA using Dharmafect 4 reagent following the manufacturer’s instructions.

In vitro translation
The Retic lysate IVT kit (Ambion) in vitro translation kit was used to synthesize AR or luciferase protein in the presence or absence of PCBP1 following the manufacturer’s protocol. Briefly, a master mix of nuclease-free water, 20 μl of translation mix (-Met), 50 μM methionine, rabbit reticulocyte lysate, and in vitro-transcribed AR or luciferase mRNA, prepared from pSG5-AR, pcDNA3.1/Luc-AR3UTR, or pcDNA3.1/Luc-AR3UTR+UC with Megascript T7 kit (Ambion) was made up on ice. Five-microliter aliquots of this master mix were then added to tubes on ice. Each aliquot was then spiked with 1 μl of in vitro-translated PCBP1 or 1 μl of an unprogrammed in vitro translation reaction. The aliquots were then incubated at 30 C to allow in vitro translation to proceed. AR translation reactions were stopped at the indicated time points by adding 6 μl of 2× Laemmli buffer. Samples were then stored at −20 C until required for SDS-PAGE. Luciferase translation reactions were stopped after 1.5 h incubation by placing the tubes on ice. Luciferase assay was carried out for each IVT reaction as follows: 2 μl of IVT was mixed with 8 μl of PBS in a white 96-well plate and then 100 μl of luciferase assay reagent (Promega) was added just before reading luminescence on a Victor II plate reader (PerkinElmer, Boston, MA). Each luciferase assay was carried out in triplicate.

Western blot analysis and immunohistochemistry
Whole-cell lysates were obtained as described elsewhere (30, 32), and 30 μg of protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel before electrotransfer onto nitrocellulose membrane (Amersham, Little Chalfont, UK). The following primary antibodies were used: mouse monoclonal AR (Biogenex, San Ramon, CA), mouse monoclonal β-actin (Abcam, Cambridge, UK), mouse monoclonal DICER (Abcam), mouse monoclonal HuR (Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal PCBP1 (Santa Cruz), and goat polyclonal PCBP2 (Santa Cruz). 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 chemiluminescent detection kit (GE Healthcare, Indianapolis, IN). Densitometric analysis was performed with ImageJ software (http://rsb.info.nih.gov/ij/). For the determination of AR protein decay, the relative abundance of AR protein was calculated for each time point and plotted as a function of time. Subsequently the slope (k) was derived from a linear equation ln C = ln C0 − kt, and where C is the relative level of AR protein in HESCs. A tissue microarray of normal, proliferative, and secretory endometrium was constructed as described previously (33) and stained with monoclonal antibody to AR (Biogenex) at a dilution of 1:30 after antigen retrieval according to the manufacturer’s data sheet. Histological and immunohistochemical assessments were performed by two independent pathologists.

Real-time quantitative PCR (RTQ-PCR)
RTQ-PCR analysis was performed as previously described (32). Briefly, RNA was extracted from cultured HESCs or timed biopsies using the RNA STAT-60 reagent (Tel-Test, Friendswood, TX) as per the manufacturer’s instructions. The RNA concentrations were determined spectrophotometrically. Genomic DNA was then removed by deoxyribonuclease I treatment (Invitrogen). Equal amounts of total RNA were reversed transcribed using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen), and the resulting first-strand complementary DNA was diluted and used as a template in the RTQ-PCR analysis. Detection of relevant mRNA was performed with SYBR Green master mix (Applied Biosystems, Foster City, CA). All reactions were carried out on an ABI PRISM 7700 sequence detection system (Applied Biosystems) for 40 cycles (95 °C for 15 sec, 60 °C for 1 min) after 10-min incubations at 95 °C. Data analysis was carried out using the relative standard curves method. L19 represents a nonregulated gene and its expression served as an internal control and was used to normalize for variances in input cDNA. All measurements were performed in triplicate. Amounts of AR mRNA molecules were calculated for each time point and plotted as a function of time. Subsequently the half-life was calculated using \( t_{1/2} = \frac{0.693}{k} \), where \( k \) is the slope derived from a linear equation \( \ln C = \ln C_0 - kt \), and where \( C \) is the relative level of AR mRNA in HESCs (34).

The following gene-specific primer pairs were designed using the ABI Primer Express software: L19 sense (5’-GCCAGCGCGCCGCAA-3’) and L19 antisense (5’-GCGGAAGGGTACACGCAAT-3’) and AR sense (5’-GCCACCTACCCAGCCTCTG-3’) and AR antisense (5’-CCCCATTTCCGCTTCTAACACA-3’) and DICER sense (5’-TTGGTAAAGTGAACCCGAAA-3’) and DICER antisense (5’-ACTGCTGACCTCCCCTCGTGAAC-3’) and HuR sense (5’-GAAGTACGGAGTACGCGTTT-3’) and HuR antisense (5’-TTTAACACGAACTCTGACCTGAAA-3’) and PCBP1 sense (5’-CCTGAGGGACCACTCCTAGA-3’) and PCBP1 antisense (5’-TTCGACCTGGCCAGTACGA-3’) and PCBP2 sense (5’-CGCAATTCCCTCTGAGATGTGAC-3’).

Statistical analysis
All experiments were performed on three or more separate primary cultures. Statistical analysis was performed using Student’s t test after normalization of the data with the level of significance defined as \( P < 0.05 \).
Results

Regulation of AR expression in differentiating HESCs in vivo and in vitro

AR expression in cycling endometrium was examined by immunostaining of a tissue microarray, consisting of 15 core biopsies taken throughout the menstrual cycle. In agreement with previous studies (7) AR expression in the endometrium was confined to the stromal cell compartment. During the proliferative phase, in excess of 90% of stromal cells displayed strong nuclear staining. AR immu-

FIG. 1. AR expression is down-regulated upon endometrial differentiation. A, Immunohistochemistry of timed endometrial biopsies showed that AR expression is confined to endometrial stromal cells. The relative proportion of AR-positive cells (dark gray/black) decreased markedly from proliferative (P) to early-secretory (ES) and late-secretory (LS) phase of the cycle. Negatively stained cells are light gray. B, RNA from 10 proliferative (P) and 10 secretory (S) biopsies were subjected RTQ-PCR analysis. AR mRNA levels, normalized to L19, are expressed in arbitrary units (a.u.). Medians are indicated as horizontal bars, and the data show a significant reduction in AR transcript levels in secretory when compared with proliferative samples (P < 0.01). C, HESCs were cultured in the absence or presence of 8-Br-cAMP and P4 (cAMP/P4), as indicated. Treatment media were replenished every 2 d and protein and RNA harvested after 8 d. The top panel represents AR mRNA (±SEM) expression, normalized to L19. The bottom panel is a Western blot of AR protein expression with β-actin serving as a loading control. D, HESCs cultured in the absence or presence of cAMP/P4 for 8 d were treated with MG132 for up to 3 h, as indicated. A Western blot was performed for AR protein expression with β-actin serving as a loading control. E, HESCs were cultured in the absence or presence of cAMP/P4 for 2 d and extracted protein was subjected to Western analysis (left panel) or treated with cycloheximide for a total of 12 h, and protein was extracted at the time points indicated. A Western blot was performed for AR protein expression. A linear regression plot of relative AR protein levels (±SEM), as determined by densitometry, are plotted on an ln (loge) scale as a function of time. The decay of AR protein was similar in both untreated or decidualized cells (k = −0.1007 and k = −0.1245, respectively).
noreactivity was confined to about 50% of cells in early secretory endometrium and was almost undetectable in decidualizing stromal cells during the late secretory phase of the cycle (Fig. 1A). Next, we examined whether AR regulation at protein level was accompanied by similar changes at mRNA level. RTQ-PCR analysis of timed biopsies demonstrated that AR transcript levels in midsecretory endometrium were on average 3-fold lower when compared with midproliferative samples (Fig. 1B, P < 0.01). Thus, at first glance, the down-regulation of AR expression in differentiating HESCs is entirely accounted for by inhibition in transcriptional expression.

HESCs differentiate in culture in response to elevated intracellular cAMP levels, although maintenance and augmentation of the decidual phenotype is strictly dependent on continuous P4 signaling (35). We previously reported that a combination of 8-Br-cAMP and P4 induces a rapid and sustained down-regulation in AR protein levels in primary HESCs (11). To examine whether these effects on AR expression also reflect transcriptional regulation, primary HESCs were cultured in the presence of a decidualizing stimulus of 8-Br-cAMP in combination with P4. RNA was extracted after 8 d of treatment and subjected to RTQ-PCR analysis. The treatment induced only a very minor reduction in AR mRNA levels (Fig. 1C, upper panel). However, as anticipated (11), Western blot analysis of parallel cultures showed a marked reduction in AR protein on 8-Br-cAMP and P4 treatment (Fig. 1C, lower panel). AR protein levels were unaffected by MG132 treatment (Fig. 1D), indicating that the down-regulation in decidual cells did not reflect enhanced proteasomal degradation of the receptor.

AR protein turnover in HESCs was investigated after the addition of the protein synthesis inhibitor cycloheximide to cells cultured for 2 d in the absence or presence of 8-Br-cAMP and P4. As expected, treatment with a decidualizing stimulus resulted in down-regulation of AR protein (Fig. 1E, left panel). By increasing the exposure time, AR protein was detectable on Western blot analysis in decidualizing cells. The rate of AR protein degradation was comparable between undifferentiated (k = −0.1007) and decidualizing (k = −0.1245) HESCs (Fig. 1E, center and right panel). Hence, loss of AR expression in HESCs decidualized with 8-Br-cAMP and P4 is not attributable to either transcriptional inhibition or increased protein turnover, suggesting that the mechanism of regulation may lie at the level of translation of receptor transcripts.

**DICER knockdown does not affect AR expression**

To the best of our knowledge, miRNAs that target AR have not yet been described. However, miRNAs play an important role in endometrial gene expression and studies in mice have shown that DICER, a major enzyme responsible for the generation of mature miRNAs and gene silencing, is indispensable for female reproduction (36). As shown in Fig. 2, DICER is expressed in decidualized HESCs and transfection of targeting siRNA results in efficient knockdown at the protein (Fig. 2A) and mRNA levels (Fig. 2B). Depletion of DICER did not affect AR mRNA expression levels (Fig. 2B) or reverse the inhibition of AR protein on treatment of primary cultures with 8-Br-cAMP and P4 (Fig. 2C).

**Role of HuR in endometrial AR expression**

In the absence of evidence implicating the miRNA machinery, we next focused on the potential role of the RBPs HuR, PCBP1, and PCBP2 in regulating AR expression in differentiating HESCs. These RBPs are trans-acting proteins that reportedly bind directly to cis elements in the 3’-UTR of AR transcripts (19). However, the role of these RBPs in regulating AR expression has as yet not been stud-
HuR regulates AR expression. A, HESCs were transfected with a control plasmid (pcDNA3.1) or an expression vector encoding HuR. After 48 h, parallel protein and RNA samples were assessed for AR expression. The RTQ-PCR data represent mean AR mRNA levels (±SEM) levels, normalized to L19. β-Actin served as a loading control for Western blot analysis. B, Primary cultures were transfected as for A and remained untreated for 48, after which the cells were treated with 5 µg/ml actinomycin D. RNA was extracted at the indicated time points and subjected to RTQ-PCR analysis. The linear regression plots of AR mRNA levels (±SEM) are plotted on an ln (log,.) scale as a function of time. The half-life of AR mRNA in cells transfected with pcDNA3.1 was 9.65 h, whereas this was increased to 38.79 h in cells transfected with HuR. C, HuR transcript levels were compared between proliferative (P) and secretory (S) biopsies. Individual measurements are presented and the horizontal bars represent the median (P < 0.05). D and E, Total RNA and protein were extracted from parallel HESC cultures treated with or without 8-Br-cAMP and P4 for the indicated time points. Mean AR mRNA (±SEM) levels were normalized to L19, whereas β-actin served as a loading control for the Western analysis. F, HESCs, decidualized or not for 48 h with 8-Br-cAMP and P4, were treated with 5 µg/ml actinomycin D for the indicated time points. After actinomycin D treatment for 8 h, the half-life was comparable between undifferentiated (16.15 h) and differentiated cells (12.89 h) (P > 0.05). *P < 0.01; **P < 0.001.

Figure 3B shows that HuR overexpression indeed stabilizes AR mRNA, increasing the half-life from approximately 9.7 to 38.8 h. Next, we examined whether HuR expression is regulated on endometrial differentiation in vivo or in vitro. Analysis of the timed biopsy samples revealed a significant down-regulation of HuR transcript levels on transformation of proliferative endometrium to the secretory phenotype (Fig. 3C; P < 0.05). Somewhat surprisingly, HuR mRNA levels increased marginally on differentiation of primary HESCs with 8-Br-cAMP and P4 (Fig. 3D), although this was not mimicked at protein level (Fig. 3E). Moreover, actinomycin D treatment of undifferentiated HESCs or cells first stimulated with 8-Br-cAMP and P4 demonstrated that decidualization only very modestly enhances AR mRNA turnover, an effect that was not statistically significant (Fig. 3F). Together, these results suggest that HuR is unlikely to be a major regulator of AR expression in differentiating HESCs.

Role of PCBP1 and PCBP2 in endometrial AR expression

In view of the above findings, we next explored the role of PCBP2 in regulating AR expression in HESCs. In contrast to HuR, overexpression of PCBP2 in HESCs had no effect on AR mRNA levels but modestly reduced AR protein expression (Fig. 4A), suggesting that this RBP interferes with the translation of AR transcripts. However, PCBP2 mRNA levels were comparable in proliferative and secretory endometrial biopsy samples (Fig. 4B). Moreover, PCBP2 protein levels remained unaltered on decidualization of primary HESC cultures (Fig. 4C), despite a modest increase in the transcripts that encode this RBP (Fig. 4D).

To complete our survey, the role of PCBP1 was investigated. Like PCBP2, overexpressed PCBP1 had no effect on the abundance of AR transcripts but significantly reduced AR protein levels, and the magnitude of this response was consistently greater when compared with PCBP2 (Fig. 5A). Furthermore, unlike HuR and PCBP2, PCBP1 transcript levels significantly increased on secre-
PCBP1 Inhibits AR Expression

Fig. 4. Expression and function of PCBP2. A, HESCs were transfected with a control plasmid (pSG5) or a vector encoding PCBP2. After 48 h, parallel cultures were harvested and RNA and protein subjected to RTQ-PCR and Western blot analysis, respectively. AR mRNA levels are expressed as mean ± SEM, normalized to L19, whereas β-actin served as a loading control for the Western analysis. B, PCBP2 mRNA expression was determined in 10 proliferative (P) and 10 secretory (S) endometrial biopsies control for the Western analysis. B, PCBP2 mRNA expression was determined in 10 proliferative (P) and 10 secretory (S) endometrial biopsies by RTQ-PCR. The bars represent median expression levels, which did not differ between the two phases of the cycle (P > 0.05). C and D, Parallel endometrial cultures, maintained in 2% dextran-coated charcoal-stripped serum or treated with or without 8-Br-cAMP and P4, were harvested for RNA and protein analyses at the indicated time points. The data represent mean PCBP2 mRNA (±SEM) levels normalized to L19, whereas β-actin served as a loading control for the Western analysis. *, P < 0.001.

Discussion

We demonstrated that decidualization of HESCs, in vitro as well as in vitro, is associated with a decline in AR levels, confirming previous microarray and immunohistochemical studies (6, 7, 37, 38). The observation that P4 is capable of reducing AR levels in HESCs has also been reported (39), and conversely, treatment of women with antiprogestins has been shown to enhance endometrial AR expression (6, 40). The androgen dihydrotestosterone is reported to decrease AR mRNA levels but increases AR protein in HESCs and a variety of other cell types (41–43). Indeed, endometrial AR expression is known to increase in female to male transsexuals treated with long-terms androgens as well as postmenopausal women treated with testosterone as part of hormone replacement therapy (44, 45). It has been shown that conformational changes in AR on ligand binding promote nuclear compartmentalization of the receptor and reduces its degradation (46, 47). Inhibition of AR mRNA expression on androgen exposure also involves several, and often cell-specific, mechanisms. For example, dihydrotestosterone reduces AR gene transcription but also stabilizes AR transcripts in prostate cancer (LNCaP) cells, resulting in a net reduction in total AR mRNA levels. Yet in the breast cancer cell line MDA453, dihydrotestosterone destabilizes AR mRNA without affecting AR gene expression (42).

Although AR transcript levels are lower in secretory compared with proliferative endometrium, our data indi-
FIG. 5. PCBP1 inhibits AR protein expression. A, Parallel HESC cultures transiently transfected with the pSGS control plasmid or a PCBP1 expression vector were harvested 48 h later for RNA and protein analyses. The relative abundance of AR transcripts (mean ± SEM) was normalized to L19, whereas β-actin served as a loading control for the Western analysis. B, RTQ-PCR analysis showed significantly higher PCBP1 mRNA levels in secretory (S) when compared with proliferative (P) endometrium (P < 0.05). C and D, PCBP1 expression in parallel cultures, untreated or decidualized with 8-Br-cAMP and P4 for the indicated time points, was determined by RTQ-PCR and Western blot analyses. E, Primary cultures were decidualized with 8-Br-cAMP and P4 for 4 d and then transfected with either NT or PCBP1 siRNA. After another 4 d of treatment, PCBP1 mRNA and protein levels were determined in parallel cultures by RTQ-PCR and Western blot analyses, respectively. F, Luciferase activity from protein generated by a 1.5-h in vitro translation reaction, in the presence of in vitro-translated PCBP1 (+) or an unprogrammed in vitro translation reaction (−), with RNA prepared transcribed from pcDNA3.1/Luc-AR3'UTR or pcDNA3.1/Luc. G, Western blot for AR generated by in vitro translation for the indicated time points, in the presence of in vitro-translated PCBP1 (+) or an unprogrammed in vitro translation reaction (−), from RNA transcribed from pSGS-AR. H, Western blot for PCBP1, AR, and β-actin in LNCaP cells transfected with either NT or PCBP1 siRNA. *, P < 0.05; **, P < 0.0001.

cate that additional posttranscriptional pathways play a major role in inhibiting AR expression, especially on decidualization of the stroma during the late luteal phase of the cycle. Treatment of primary cultures with 8-Br-cAMP

and P4 resulted in a dramatic loss of AR protein, whereas transcript levels were only marginally down-regulated. Decidualization of HESCs had little or no effect on AR protein or mRNA turnover, indicating that the dramatic reduction in receptor levels could be due to translational inhibition. Knockdown of DICER, a ribonuclease III-like enzyme essential for processing of premiRNAs (14), had no effect on either AR mRNA or protein levels, which prompted us to focus on the role of RBPs known to bind to a highly conserved uridine-cytosine-rich motif within the 3' UTR of the AR mRNA (19).

We have shown not only that HuR, PCBP2, and PCBP1 are expressed in HESCs but also that they are capable of modulating AR expression. As mentioned, HuR is a ubiquitously expressed RBP that stabilizes target mRNAs and, depending on environmental signals, promotes or represses translation (20–22). Overexpression of HuR in HESCs resulted in a 5-fold increase in AR mRNA levels, although the increase in AR protein was much more modest. HuR expression was not regulated on decidualization, rendering it unlikely that it accounts for the decrease in AR mRNA translation upon cAMP and P4 signaling. Yet these observations do not necessarily negate an important role for HuR in decidual cell function, especially under conditions of oxidative stress. Indeed, HuR has been shown to translocate from the nucleus to the cytoplasm in response to stress signals in which it then serves to stabilize and translate specific mRNA targets, many of which encode for proteins involved in apoptotic and stress responses (48, 49). Whereas the role of HuR in endometrial stress responses warrants further investigation, the observation that it can modulate AR expression may be of particular relevance to endocrine-dependent cancers, including prostate cancer, which are often characterized by either overexpression or increased cytoplasmic localization of HuR (50, 51).
The mammalian PCBP family consists of five members (PCBP1-4 and hnrNP K). PCBP1 and PCBP2 not only are the best-characterized family members but also share the highest level of amino acid sequence homology (89%) (23). Upon overexpression, both RBPs reduce AR protein but not mRNA levels in HESCs, although this response was consistently more pronounced with PCBP1. Moreover, expression of PCBP1 but not PCBP2 increases during the secretory phase of the cycle as well as in decidualizing primary HESC cultures. PCBP2 has perhaps been most extensively characterized for its ability to stimulate internal ribosomal entry site-mediated translation (52), although recent evidence suggest that, like HuR, it also plays an important role in cellular defenses against oxidative and inflammatory stress (53, 54). PCBP1, on the other hand, has emerged as a key coordinator of the transcriptional, splicing, and translational events in response to mitogenic stimuli (28, 55). This notion is based on the observation that PCBP1 is phosphorylated by the p21-activated kinase, which in turn results in increased nuclear retention of this RBP, stimulation of eukaryotic translation initiation factor 4E expression, and enhanced PCBP1-dependent splicing events (28). Whether this function of PCBP1 is maintained in differentiating HESCs is unclear because the decidual process is associated with a marked decrease in p21-activated kinase mRNA levels. Instead, we propose that PCBP1 serves to limit AR protein levels in decidualizing human endometrium, a notion supported by several strands of evidence. First, forced expression of PCBP1 in proliferating cells, when levels are low, reduces AR protein but not mRNA levels. Second, decidualizing cells express more PCBP1 and siRNA directed silencing of this RBP antagonizes AR protein down-regulation, again without affecting mRNA levels. Although PCBP1 was identified as RBP capable of binding the 3′-UTR of AR (19), we found that this interaction is not essential for inhibition of AR mRNA translation, at least not in vitro. Furthermore, we found PCBP1-dependent regulation of AR levels is not restricted to HESCs. PCBP1 is expressed in prostate cancer LNCaP cells, and AR protein is elevated when PCBP1 is depleted. Therefore, PCBP1 is a potential new target for modulating androgen action in prostate cancer.

In summary, this study revealed a hitherto unexplored level of control that regulates AR expression in androgen-responsive tissues including the cycling human endometrium and prostate cancer cells. The dramatic loss of AR protein in the endometrial stromal compartment during the late secretory phase of the cycle is the consequence of the imposition of a translational block, mediated at least in part by PCBP1, concomitantly with a decline in AR mRNA expression. The existence of multiple and complex levels of regulation emphasizes that coordinated expression of steroid receptors is critical for endometrial function. Many common reproductive disorders, such as pelvic endometriosis, are increasingly linked to aberrant steroid hormone signaling in the endometrium. Whether perturbations in the posttranscriptional pathways that control endometrial gene expression underpin these endometrial disorders and reproductive failure warrants further investigation.

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