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DOWNREGULATION OF THE HISTONE METHYLTRANSFERASE EZH2 CONTRIBUTES TO THE EPIGENETIC PROGRAMMING OF DECIDUALIZING HUMAN ENDOMETRIAL STROMAL CELLS

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Abstract

Differentiation of human endometrial stromal cells (HESCs) into decidual cells represents a highly coordinated process essential for embryo implantation. We show that decidualizing HESCs downregulate the histone methyltransferase enhancer of Zeste homologe 2 (EZH2), resulting in declining levels of trimethylated lysine 27 of histone 3 (H3K27me3) at the proximal promoters of key decidual marker genes, *PRL* and *IGFBP1*. Loss of H3K27me3 was associated with a reciprocal enrichment in acetylation of the same lysine residue, indicating active remodeling from repressive to transcriptionally permissive chromatin. Chromatin immunoprecipitation coupled with DNA microarray demonstrated that decidualization triggers genome-wide changes in H3K27me3 that only partly overlap those observed upon EZH2 knockdown in undifferentiated HESCs. However, gene ontology analyses revealed that gain and loss of the repressive H3K27me3 mark upon differentiation as well as EZH2 knockdown occurred at loci enriched for genes functionally implicated in cell proliferation and responses to stimulus, respectively. In agreement, EZH2 knockdown in undifferentiated HESCS was sufficient to augment the induction of decidual marker genes in response to cyclic AMP and progesterone signaling. Thus, loss of EZH2-dependent methyltransferase activity in the endometrium is integral to the process of chromatin remodeling that enables acquisition of a decidual phenotype in response to differentiation cues.

Differentiation of human endometrial stromal cells (HESCs) into decidual cells represents a highly coordinated process essential for embryo implantation. Here we show that decidualization during the mid-secretory phase of the cycle coincides with a profound downregulation of enhancer of Zeste homolog 2 (EZH2), the enzymatic subunit of the repressive Polycomb complex 2 that trimethylates lysine 27 of histone 3 (H3K27me3). EZH2 was also profoundly downregulated upon differentiation of primary HESCs, which coincided with a gradual loss of the H3K27me3 mark at the proximal promoters of key decidual marker genes, *PRL* and *IGFBP1*. Loss of H3K27me3 at these loci was further associated with a reciprocal enrichment in acetylation of the same lysine residue (H3K27ac), indicating active remodeling from repressive to transcriptionally permissive chromatin. EZH2 knockdown in undifferentiated HESCS reduced the abundance of H3K27me3 at the proximal *PRL* and *IGFBP1* promoters and augmented the induction of these marker genes in response to cyclic AMP and progesterone signaling. Chromatin immunoprecipitation coupled with DNA microarray demonstrated that decidualization triggers genome-wide changes in H3K27 methylation that are largely distinct from those observed upon EZH2 knockdown in undifferentiated HESCs. However, gene ontology analyses revealed that gain and loss of the repressive H3K27 mark under both experimental conditions occurred at loci functionally enriched for genes implicated in cell proliferation and responses to stimulus, respectively. Thus, loss of EZH2-dependent methyltransferase activity in the endometrium is integral to the process of chromatin remodeling that enables acquisition of a decidual phenotype in response to differentiation cues.

Introduction

The postovulatory rise in progesterone levels drives the differentiation of the endometrium in preparation of pregnancy, a highly coordinated and sequential process characterized by secretory transformation of the glandular epithelium, influx of specialized immune cells, decidualization of the stroma, and vascular remodeling (1-3). Although progesterone signaling is indispensible, the sequential nature of this differentiation process is thought to reflect the actions of locally produced cytokines and growth factors. For example, decidualization, which denotes the differentiation of endometrial stromal cells into epitheloid decidual cells, is a classical progesterone-dependent process, although this process is initiated *in vivo* only approximately 9 days after the ovulatory surge in circulating progesterone levels (4-6). Similarly, purified human endometrial stromal cells (HESCs) in culture are also largely refractory to progesterone signaling, despite abundantly expressing the nuclear progesterone receptors PR-A and –B (4,7,8). However, progesterone does acquire control over the expression of decidual markers genes, such as *PRL* and *IGFBP-1*, upon simultaneous activation of the cAMP pathway (4,9-11). Endometrial cAMP levels also increase *in vivo* during the luteal phase, probably reflecting the induction of local factors that activate adenylate cyclase in stromal cells, such as relaxin, corticotropin-releasing hormone, and prostaglandin E2 (5).

Multiple mechanisms have been shown to underpin the convergence of the cAMP and progesterone signal pathways in HESCs. For example, cAMP inhibits ligand-dependent sumoylation of PR (11,12), a posttranslational modification that limits the transactivation capacity of this nuclear receptor (13). Increased cAMP levels also induce the expression of several transcription coregulators of PR, including FOXO1, STAT5, and C/EBPβ, and disrupt the binding of the receptor to

specific corepressors, such as NCoR and SMRT (14-18). While all these observations indicate that PR activity in HESCs is tightly controlled it does not explain well the highly temporal regulation of the decidual process during the cycle. One, as yet untested, possibility is that decidual transformation of the endometrial stroma in the cycle is dependent on coordinated chromatin modifications that create permissive and repressive transcriptional environments enabling large gene networks to respond to differentiation signals.

Numerous DNA and histone modifying enzymes govern the accessibility of the transcriptional machinery to chromatin, thus determining if a gene is silenced, activated, or poised to respond to a stimulus (19-21). One of the most widely studied histone modifiers is enhancer of Zeste homolog 2 (EZH2), which along with the embryonic ectoderm development (EED) and the zinc finger protein suppressor of zeste 12 homologue (SUZ12), makes up the Polycomb-repressive complex 2 (PRC2). Within this repressive complex EZH2 serves as the active enzyme that catalyzes the trimethylation of H3 on Lys 27 (H3K27me3) leading to gene silencing (22-24). Importantly, aberrant expression of EZH2 occurs in a variety of hormone-dependent malignancies, including endometrial, breast and prostate cancers (25-29). Furthermore, ovarian hormones reportedly regulate EZH2 expression and activity in a variety of cell types (30). These observations prompted us to examine the expression of EZH2 in human endometrium and to determine if cycle-dependent changes in EZH2 methylation activity plays a role in differentiation of HESCs into specialized decidual cells.

Materials and Methods

Primary cell culture This study was approved by the Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee (1997/5065). Endometrial samples were obtained from pre-menopausal consented women without uterine pathology. Written informed consent was obtained from all participating subjects prior tissue collection. HESCs were isolated and established cultures were maintained as described previously (15). For *in vitro* decidualization, cultures were treated with 0.5mM 8-Br-cAMP (Sigma, St Louis, Mo, USA) and 1μM medroxyprogesteroneacetate (MPA) (Sigma).

Transient transfection of primary cultures

Primary HESCS were transfected by calcium phosphate coprecipitation using the ProFection Mammalian Transfection kit (Promega, Madison, Wi, USA) according to manufacturer's instructions. For EZH2 gene-silencing HESCS were transiently transfected with the following small interfering RNA (siRNA) siCONTROL nontargeting (NT) siRNA pool (Dharmacon, Lafayette, Co, USA) and EZH2 siGENOME SMARTpool siRNA (Dharmacon). For EZH2 overexpression the *EZH2* in pCMV-SPORT6 (Open Biosystems, Huntsville, Al, USA) vector was used.

Western blot analysis

Whole cell protein extracts were obtained by direct lysis in Laemmli buffer. Proteins were resolved by SDS-PAGE, transferred to a PVDF membrane and probed with antibodies raised against EZH2 (NCL-L-EZH2; Leica Biosystems, Newcastle upon Tyne, UK), EZH1 (ab13665; Abcam, Cambridge, UK), H3K27me3 (07-449;

Millipore, Billerica, Ma, USA), H3K27ac (ab4729; Abcam), β-actin (ab-8226; Abcam). The proteins were visualized by incubation with HRP-conjugated secondary antibodies (Roche, Wellyn Garden City, UK) and the chemiluminescence detected using the ECL+ kit (GE Healthcare, Indianapolis, In, USA).

Real-time quantitative PCR (RT-qPCR)

RNA was extracted using STAT-60 (AMS Biotech, Abingdon, UK) and following manufacturer's instructions. cDNA was synthesized using the SuperScript First-Strand Synthesis for RT-PCR (Invitrogen, Carlsbad, CA, USA) with oligo-dT primers (Invitrogen) after having treated the extracted RNA with amplification grade DNase I (Invitrogen). Quantitative PCR (qPCR) was carried out on BioRad Opticon Monitor 3 Real-Time PCR System. PCR reactions were set up using SYBR Green JumpStart Taq (Sigma), 0.20 μM of each primer, 0.5 μl of template in a 15 μl reaction. The following program was run on the thermocycler: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C° for 15 seconds, 60°C for 1 minute and 72°C for 30 seconds. Input variance was normalized against the expression of the *L19* gene, which encodes for a non-regulated universally expressed ribosomal protein.

Confocal microscopy

HESCS were cultured on 4-well chamber slides and decidualized for 2, 4 and 8 days. The cells were fixed with 4% paraformaldehyde (Thermoscientfic, Waltham, MA, USA). Subsequently they were permeabilized with 0.5% Triton-X and blocked with 7.5% normal goat serum and 3% BSA in PBS. The slides were hybridized with the primary antibody and then with the secondary one conjugated with Alexa Fluor 594. The chambers were mounted in Vectashield with DAPI. Alexa Fluor 594 and DAPI

were visualized under a Leica SP5 confocal microscope with a 63× oil-immersion objective.

Immunohistochemistry

Paraffin-embedded, formalin-fixed endometrial tissue sections were placed on 1% w/v polylysine coated slides. Immunostaining was performed using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA), according to manufacturer's instructions. The sections were stained with EZH2 antibody (NCL-L-EZH2; Leica).

Chromatin immunoprecipitation (ChIP)

HESCS were cultured in 10 cm culture dishes, fixed with 1% formaldehyde and incubated for 10 minutes at 37°C. After having stopped the fixation with 125 mM glycine the nuclei were isolated by incubating at 4°C for 10 minutes in 1 ml Swelling buffer (25 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.1% Nonidet). The cells were then scraped and homogenized with a dounce homogenizer. The samples were centrifuged for 3 minutes at 16000g at 4°C and the nuclei, collected in a pellet, resuspended in 500 µl SDS lysis buffer (1% SDS, 1% Triton-X100, 0.5% deoxycholate, 10 mM EDTA, 500 mM Tris-HCl pH 8.1) and sonicated for 30 minutes (with 30 second cycles) at 4°C on high power in a Diagenode Bioruptor sonicator. The resulting suspension was centrifuged for 15 minutes at 16000 g at 4°C and the supernatant diluted ten times in IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl) and then precleared at 4 °C for 3 hours with Protein A Dynabeads (Invitrogen). The chromatin was then complexed overnight at 4°C with the antibody bound to Protein A Dynabeads and washed with the following buffers: Low Salt Buffer (0.1% SDS, 1% Triton-X100, 2 mM EDTA,

20 mM Tris-HCl pH 8.1, 150 mM NaCl), High Salt Buffer (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl Buffer (250 mM LiCl, 1% Nonidet, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), TE buffer (10 mM Tris-HCl pH8, 1 mM EDTA) before eluting the chromatin with 250 µl Elution buffer (1% SDS, 100 mM $NaHCO_3$) and incubated at room temperature for 15 minutes. 200 mM NaCl was added to reverse crosslink the proteins and the DNA. After an overnight incubation at 65°C 10 mM EDTA, 40 mM Tris-HCl pH 8 and 40 µg/ml Protease K (Sigma) was added and the sample incubated for a further hour at 55°C prior to proceeding with the DNA purification using QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands). All the buffers were supplemented with protease inhibitors, 1 mM phenylmethanesulfonylfluoride (PMSF) and 10mM sodium butyrate. The following antibodies were used in the ChIP experiments: H3K27me3 (07-449; Millipore), H3K27ac (ab4729; Abcam) and as negative control the rabbit polyclonal anti-mouse IgG (M7023, Sigma) was used.

ChIP-chip array

Chromatin was immunoprecipitated with an H3K27me3 specific antibody. ChIP DNA was amplified using the WGA2 Kit (Sigma), following manufacturer's instructions. The ChIP-chip assays were performed in triplicate, each representing a primary culture from an individual patient. A total of 12 primary cultures were assayed. Labeling of ChIP DNA and input, hybridization to NimbleGen Human ChIP-chip 3x720K RefSeq promoter arrays (Roche), and scanning were performed by NimbleGen in their service laboratory. Peak detection and statistical analysis was carried out using Partek® Genomic Suite ™ (Partek, St. Louis, MO, USA).

Results

Cycle-dependent expression of EZH2 in human endometrium

EZH2 expression during the menstrual cycle was examined in 5 proliferative and 5 secretory endometrial biopsies. Western blot analysis of whole tissue lysates showed a marked decrease in the expression of this methyltransferase during the secretory phase of the cycle (Fig. 1A & B). To further explore the dynamics of this down-regulation, we focused on the expression of EZH2 during this phase. RT-qPCR analysis on timed endometrial biopsies revealed a 3-fold decrease in EZH2 transcript levels with the onset of the mid-secretory phase. This reduction in EZH2 mRNA level was then maintained for the remainder of the cycle (Fig. 1C). Tissue sections obtained between day 14 and 27 were subjected to immunohistochemistry. As shown in Fig. 1C, EZH2 immunoreactivity was prominent in the epithelial glandular compartment during the early secretory phase. It was also abundantly expressed in stromal cells, although the staining was less homogenous. As the cycle progressed, a marked loss of EZH2 expression was apparent in epithelial cells, resulting in virtual lack of signal in this cellular compartment by the end of the cycle. A similar trend was apparent in the stroma, although individual cells strongly expressing EZH2 were still present during the late-secretory phase. Taken together, these data suggest that the progression of the menstrual cycle results in a gradual but marked loss of EZH2 expression in differentiating human endometrium.

Loss of EZH2 expression in decidualizing HESCS

Elevated intracellular cAMP levels initiate differentiation of HESCS while progesterone is essential for the maintenance and enhancement of the decidual phenotype (5). As EZH2 expression is downregulated in the stromal compartment of

differentiating endometrium we examined if this would be the case upon decidualization of primary HESCs *in vitro*. Primary cultures were stimulated with 8-Br-cAMP and the progestin MPA for various time-points (Fig. 2A). Total mRNA and protein were extracted from parallel cultures to examine the expression of EZH2. Unexpectedly, transcript levels were already 80% lower in cells decidualized for 2 days and this level of repression was maintained throughout the entire time-course (Fig. 2A, top panel). The decline in EZH2 mRNA expression was paralleled at protein level, although the reduction was more gradual. Upon 8 days of differentiation, expression of this methyltransferase was below the level of detection on Western blot analysis (Fig. 2A, bottom panel). The loss of EZH2 upon decidualization of HESCS was further confirmed by confocal microscopy (Fig. 2B).

To provide insights into the mechanism of EZH2 repression, primary cultures were treated with 8-Br-cAMP, MPA, alone or a combination, for 48 hours. EZH2 transcripts decreased modestly upon treatment with 8-Br-cAMP, whereas MPA reduced the expression level by approximately 50%. However, a combination 8-Br-cAMP and MPA yielded an additive effect, resulting in approximately 80% reduction in EZH2 mRNA levels (Fig 2C, top panel). Again, the regulation of EZH2 transcripts was recapitulated at protein level (Fig 2C, bottom panel). Thus, both the cAMP and progesterone signal transduction pathways play a role in EZH2 silencing in differentiating HESCS, with progesterone being the dominant signal.

Interplay between acetylation and trimethylation of H3K27

EZH2 catalyzes trimethylation of H3K27, a histone tail modification associated with repressive chromatin and gene silencing (31). To examine if loss of EZH2 impacts on global cellular H3K27me3 levels, confocal microscopy as well as Western blot

analysis were carried out on undifferentiated HESCS and cultures treated with 8-Br-cAMP and MPA for various time-points. Unexpectedly, differentiation of HESCs for as long as 8 days was not associated with a discernible change in overall methylation of H3K27 (Fig. 3A & B). This suggested that other enzymes with histone H3 methylase activity are likely to be present in decidualizing cells. As shown in Fig 3A (right panel), EZH1, a functional homolog of EZH2 (32), is indeed expressed in HESCs and, if anything, the abundance of this methyltransferase modestly increases upon treatment with 8-Br-cAMP and MPA.

Next, we examined if loss of EZH2 impacts on the methylation status of H3K27 at the transcriptional start site (TSS) of highly induced decidua-specific genes. *PRL* and *IGFBP-1* are the most widely studied decidual marker genes and their transcriptional regulation has been extensively studied (14,33-36). Notably, PRL in the endometrium is transcribed from an alternative promoter upstream of a noncoding exon, located approximately 6 kb upstream of the pituitary-specific TSS (33). ChIP analysis was carried out using a H3K27me3-specific antibody followed by amplification of a 98 bp fragment encompassing the -332 to -270bp regulatory region of the decidual-specific *PRL* TSS, which contains several response elements required for cAMP- and progesterone-dependent regulation (12,15,36,37). Time-course analysis revealed that HESC differentiation is associated with a gradual but dramatic loss of this mark at this locus (Fig. 3B). Loss of H3K27me3 was even more rapid and pronounced at the proximal *IGFBP1* promoter (-263 to -33bp relatively to the TSS). Thus, the marked decline of H3K27me3 at specific loci in the absence of a global change in levels suggests that decidualization is associated with a dynamic, albeit specific, redistribution of the mark.

Acetlyation of H3K27 (H3K27ac) antagonizes PRC2-dependent gene

silencing. Furthermore, acetylation and trimethylation of H3K27 are mutually exclusive as both modifications compete for binding to the same lysine residue (38). To determine if complementarity between these marks exists in HESCS, we first analyzed global levels of H3K27ac in undifferentiated and decidualizing cells. Confocal microscopy revealed an increase in H3K27ac upon decidualization in HESCS, apparent after 2 days but even more pronounced after 4 and 8 days of differentiation (Fig. 4A). This was further confirmed by Western blot analysis, demonstrating a gradual increase in the abundance of this mark in decidualizing cultures (Fig. 4B). To verify if loss of H3K27me3 is accompanied by a gain in acetylation at specific loci, ChIP with a H3K27ac-specific antibody followed by qPCR amplification of the proximal decidual *PRL* and *IGFBP1* promoters was carried out. As shown in Fig. 4C, differentiation of HESCS is indeed associated with rapid and marked increase in H3K27ac signal upstream of the TSS of both genes. This was especially true for the *IGFBP1* promoter, where the abundance of this modification increased by several multitudes over time.

EZH2 down-regulation is permissive for decidualization

Next, we explored the functional consequences of EZH2 downregulation on the expression of decidual marker genes. First, we used siRNA to silence EZH2 expression in primary cultures, which were then left untreated or decidualized with 8-Br-cAMP and MPA for 2 days. The knockdown was highly effective and reduced EZH2 expression below the level of detection on Western blot analysis (Fig. 5A). Interestingly, EZH2 silencing was sufficient to decrease H3K27 trimethylation on the decidual *PRL* and *IGFBP1* promoters by 60% and 90%, respectively (Fig. 5B). Notably, EZH2 knockdown enhanced acetylation of the same residue in H3 at the

IGFBP1 but only marginally on *PRL* promoter (Fig. 5C). Furthermore, knockdown of this methyltransferase in undifferentiated cells enhanced the subsequent induction of both PRL and IGFBP-1 transcripts in response to 8-Br-cAMP and MPA treatment (Fig. 5D). There was also a consistent but very discrete increase in basal expression levels of these marker genes upon EZH2 knockdown. To confirm that EZH2 inhibits decidualization, primary HESC cultures were transfected with an expression vector encoding EZH2 and then differentiated with 8-Br-cAMP and MPA for 2 days. Overexpression was confirmed by Western blot analysis (Fig. 5E). RT-qPCR analysis of parallel cultures demonstrated that exogenous EZH2 expression reduces the induction of IGFBP-1 mRNA and, even more pronounced, of PRL transcripts in differentiating HESCs (Fig 5F). Together, the data demonstrate that loss of EZH2 alone is insufficient to induce a decidual phenotype yet determines the cellular responsiveness to differentiation stimuli.

Genome-wide redistribution of H3K27me3 upon decidualization and in response to EZH2 knockdown

Our results indicated that loss of EZH2 expression in decidualizing cells results in loss of H3K27me3 signal at specific loci yet overall levels of this repressive histone modification seem to be maintained. To determine if decidualization is associated with genome-wide redistribution of H3K27 methylation, we carried out ChIP-chip arrays on undifferentiated HESCs and cells treated with 8-Br-cAMP and MPA for 8 days, a time-point at which EZH2 expression is virtually undetectable (Fig. 2A). Chromatin from three independent, paired undifferentiated and decidualizing cultures, was immunoprecipitated with the H3K27me3 antibody, labeled, and hybridized to a Roche NimbleGen Human ChIP-chip 3x720K RefSeq promoter array. We identified a

significant change ($P < 0.05$) in H3K27me3 signal at 3008 genomic regions. Of these, there was a reduction in methylation in 75% and enrichment in 25% upon differentiation of HESCs into decidual cells. Gene ontology (GO) analysis revealed that gain of this repressive modification was strongly enriched at promoters of genes involved in transcriptional regulation (enrichment score: 7.5; $P < 0.001$) whereas loss of the mark was prominent at genes implicated in response to stimulus (enrichment score: 12.5; $P < 0.001$) (Fig. 6A). As anticipated, one genomic region characterized by reduced levels of trimethylated H3K27 in differentiating HESCs was the *IGFBP1* promoter (Fig. 6B). The decidua-specific *PRL* promoter, however, was not represented on the array.

To determine if this genome-wide redistribution of H3K27me3 upon decidualization of HESCs is caused by the loss of EZH2, we repeated the ChIP-chip arrays but this time on chromatin immunoprecipitated from three independent undifferentiated primary cultures first transfected with non-targeting or EZH2 siRNA. Silencing EZH2 expression was sufficient to significantly alter the abundance of H3K27me3 at 2029 distinct genomic regions and a comparable number of loci displayed a reduction or enrichment in histone modification (53% and 47%, respectively). Furthermore, cross-referencing of the two data sets revealed altered H3K27me3 at 567 genomic regions in both decidualizing HESCs and upon EZH2 knockdown in undifferentiated cultures (Fig. 6C). However, the direction of change was discordant in 229 regions (40%). In other words, EZH2 knockdown in undifferentiated cells only recapitulated the change in H3K27 methylation at 338 of 3008 (11%) genomic regions altered upon decidualization. These 388 highly EZH2-responsive regions were also subjected to GO analysis where loss and gain of the H3K27me3 repressive mark was found to occur prominently in the proximity of genes

involved in response to stimuli (enrichment score: 28; $P < 0.05$), and cellular growth (enrichment score: 6.8 ; $P < 0.05$), respectively (Fig. 6D).

Discussion

Like other ligand-activated nuclear receptors, progesterone triggers a conformational change in PR, which leads to dissociation of chaperone proteins, receptor dimerization, and binding to specific DNA recognition sequences in the promoters of target genes (39). However, interaction of the activated PR with DNA is in itself insufficient to alter gene expression as nuclear receptors do not possess chromatin modifying activities necessary to enable or prevent recruitment of the basal transcriptional machinery (40-43). Remodeling of local chromatin depends on recruitment of coregulators, broadly divided into coactivators and corepressors, to the DNA-bound receptor (40,41). Based on their mechanisms of action, nuclear receptor coactivators can be categorized into three major function complexes: (i) the SWI/SNF complex, which remodel the local chromatin structure through adenosine triphosphate-dependent histone acetylation; (ii) the SRC complex, which contain acetyltransferases (e.g. CBP, p300, and the p300/CBP-associated factor) and methyltransferases (e.g. CARM1 and PRMT1); and (iii) mediator complex, involved in the activation of RNA polymerase II and initiation of transcription. Although the mechanism of agonist-bound PR-dependent gene repression is still unclear, it is widely assumed to involve interaction with NCoR, SMRT or possibly RIP140, corepressors capable of recruiting DNA- and histone-methyltransferases complexes (44,45).

In this model, nuclear receptors like PR are viewed as pioneer factors responsible for initiating the process of chromatin remodelling near the TSSs of target genes. However, this paradigm is being profoundly challenged by novel techniques that allow genome-wide mapping of binding of nuclear receptors to DNA (19,46,47). Contrary to expectations, a majority of nuclear binding events do not occur proximal

to TSSs of target genes but at large distances from promoters (47-49). Moreover, rather than the activated receptor inducing a permissive chromatin environment that enables transcription, most activated receptors will bind at preexisting sites that are constitutively accessible. For example, the binding pattern of the activated glucocorticoid receptor to DNA, and subsequent gene regulation, was recently shown to be highly cell-specific and comprehensively predetermined by basal differences between cell types in chromatin structure (46). When extrapolated to the cycling endometrium, these observations strongly suggest that acquisition of responsiveness to differentiation signals must be preceded by, or at least occur in concert with, genome-wide remodelling of the chromatin.

This study provides evidence that loss of EZH2 activity in HESCs contributes to the chromatin changes necessary for expression of a decidual phenotype in response to differentiation cues. While EZH2 transcript levels fell rapidly in response to cAMP and progestin treatment, protein levels declined more gradually. The kinetics of this response correlated well with the decline in H3K27me3 at the *PRL* and *IGFBP1* promoters and, inversely, with the level of transcriptional activation of these marker genes (4,12). Furthermore, a parallel gain in the competing activation mark H3K27ac was observed at both promoters upon decidualization of HESCs, although the amplitude of this response was more pronounced at the TSS of *IGFBP1*. Interestingly, a recent study reported that different modifications are predictive of the expression levels of genes that are either rich or poor in the CpG content of their promoters. In particular H3K27ac is strongly associated with activation of high but not low CpG content promoters (50). In keeping with this model, acetylation of H3K27 upon HESC differentiation was not only more pronounced at the *IGFBP1* promoter, which has 68 CpGs, but EZH2 knockdown was sufficient to enrich the

mark. In contrast, the decidua-specific *PRL* promoter is poor in CpG content and enrichment in H3K27ac was strictly dependent on cAMP and MPA stimulation. In a previous study, we demonstrated that CBP/p300, coactivators that catalyze acetylation of H3K27, are indispensible for *PRL* expression in differentiating HESCs (51), suggesting that recruitment of these histone modifiers to decidual promoters requires binding of specific transcriptional complexes induced by cAMP and MPA signaling. In other words, while loss of EZH2-dependent methyltransferase activity contributes to creating a transcriptionally permissive chromatin environment, these changes alone are insufficient to trigger the expression of decidual marker genes in undifferentiated HESCs, or even upon stimulation with only MPA (data not shown).

Downregulation of EZH2 expression in decidualizing HESCs was not paralleled by a decline in global cellular H3K27me3 levels as determined by Western blot analysis or confocal imaging. However, our ChIP-chip array study suggested a net reduction in chromatin-bound methylated H3K27 after 8 days of differentiation as 3-times more genomic regions were reduced than enriched in this modification. Nevertheless, residual H3 methylase activity remained apparent in differentiating HESCs, even with EZH2 expression below the level of detection. This residual activity is likely accounted for by the continuous expression of EZH1. Like EZH2, EZH1 integrates in PCR2, although there is evidence that these complexes differ in their repressive roles. EZH1 has relative weak intrinsic histone methyltransferase activity and is thought to elicit its role by compacting chromatin through interaction with nucleosomes (52). Interestingly, EZH2 expression is widely associated with cellular proliferation, whereas EZH1 is reportedly more abundant in non-proliferative cells (52). This general pattern of expression fits well with our observation that EZH2 but not EZH1 is lost upon differentiation of HESCs.

In addition to DNA methylation, over 60 different residues within histone tails have been identified as targets for various posttranslational modifications, including methylation, acetylation, ubiquitination, phosphorylation and sumoyaltion (21,53). Mining of existing gene expression revealed that decidualization of HESCs is associated with altered expression of members of several classes of chromatin modifying enzymes, including DNA methyltransferases (e.g. *DNMT3a* and *DNMT3b*), DNA binding proteins (e.g. *UFRH1*) and histone modifying enzymes (e.g. *SUV420H1*). Similarly, the expression of DNMT1 and MDB2, a methyl-CpG binding protein, in human endometrium has been shown to be cycle-dependent (54). Because of the interdependency of various chromatin modifications (50,55), it was anticipated that EZH2 knockdown in undifferentiated HESCs would at best only partially recapitulate the changes in H3K27me3 observed upon decidualization. This was indeed the case. Only 11% of the genomic regions altered in H3K27me in response to prolonged cAMP and MPA stimulation were modified in a similar manner upon EZH2 knockdown in untreated cells. These loci could be viewed as highly-dependent on EZH2 for remodeling. Interestingly, GO analysis revealed that those regions that gain the transcriptionally repressive H3K27me mark upon decidualization of HESCs are enriched for genes associated with growth. Even more strikingly, the same analysis of regions that lost the mark, thus acquiring a transcriptionally permissive chromatin environment, revealed a preponderance of genes functionally associated with responsiveness to stimuli. Thus the result of the ChIP-chip arrays further supports the notion that downregulation of EZH2 is a key event that renders endometrial cells responsive to differentiation and other environmental cues.

Taken together our data show that cAMP and progesterone signaling reshapes the chromatin landscape of HESCs, which in turn enables regulation of large gene

networks that underpin the expression of a decidual phenotype. Extrapolated to the *in vivo* situation, our findings suggest that cycle-dependent changes in chromatin structure is a major determinant in the cellular responsiveness to differentiation signals and the subsequent acquisition of a decidual phenotype. In light of the crucial role of EZH2 in this process, it seems likely that perturbations in this or other chromatin remodeling enzymes underpin reproductive failure.

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References

1. Gellersen, B., Brosens, I.A. and Brosens, J.J. (2007) Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Seminars in reproductive medicine*, **25**, 445-453.
2. Wynn, R.M. (1974) Ultrastructural development of the human decidua. *American journal of obstetrics and gynecology*, **118**, 652-670.
3. Brosens, J.J. and Gellersen, B. (2006) Death or survival--progesterone-dependent cell fate decisions in the human endometrial stroma. *Journal of molecular endocrinology*, **36**, 389-398.
4. Brosens, J.J., Hayashi, N. and White, J.O. (1999) Progesterone receptor regulates decidual prolactin expression in differentiating human endometrial stromal cells. *Endocrinology*, **140**, 4809-4820.
5. Gellersen, B. and Brosens, J. (2003) Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair. *The Journal of endocrinology*, **178**, 357-372.
6. de Ziegler, D., Fanchin, R., de Moustier, B. and Bulletti, C. (1998) The hormonal control of endometrial receptivity: estrogen (E2) and progesterone. *Journal of reproductive immunology*, **39**, 149-166.
7. Mote, P.A., Balleine, R.L., McGowan, E.M. and Clarke, C.L. (1999) Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle. *The Journal of clinical endocrinology and metabolism*, **84**, 2963-2971.
8. Li, X. and O'Malley, B.W. (2003) Unfolding the action of progesterone receptors. *The Journal of biological chemistry*, **278**, 39261-39264.

9. Brosens, I. (1999) Management of ovarian endometriomas and pregnancy? *Fertility and sterility*, **71**, 1166-1167.

10. Brar, A.K., Frank, G.R., Kessler, C.A., Cedars, M.I. and Handwerger, S. (1997) Progesterone-dependent decidualization of the human endometrium is mediated by cAMP. *Endocrine*, **6**, 301-307.

11. Jones, M.C., Fusi, L., Higham, J.H., Abdel-Hafiz, H., Horwitz, K.B., Lam, E.W. and Brosens, J.J. (2006) Regulation of the SUMO pathway sensitizes differentiating human endometrial stromal cells to progesterone. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 16272-16277.

12. Cloke, B., Huhtinen, K., Fusi, L., Kajihara, T., Yliheikkila, M., Ho, K.K., Teklenburg, G., Lavery, S., Jones, M.C., Trew, G. *et al.* (2008) The androgen and progesterone receptors regulate distinct gene networks and cellular functions in decidualizing endometrium. *Endocrinology*, **149**, 4462-4474.

13. Abdel-Hafiz, H., Takimoto, G.S., Tung, L. and Horwitz, K.B. (2002) The inhibitory function in human progesterone receptor N termini binds SUMO-1 protein to regulate autoinhibition and transrepression. *The Journal of biological chemistry*, **277**, 33950-33956.

14. Christian, M., Zhang, X., Schneider-Merck, T., Unterman, T.G., Gellersen, B., White, J.O. and Brosens, J.J. (2002) Cyclic AMP-induced forkhead transcription factor, FKHR, cooperates with CCAAT/enhancer-binding protein beta in differentiating human endometrial stromal cells. *The Journal of biological chemistry*, **277**, 20825-20832.

15. Christian, M., Pohnke, Y., Kempf, R., Gellersen, B. and Brosens, J.J. (2002) Functional association of PR and CCAAT/enhancer-binding protein beta

isoforms: promoter-dependent cooperation between PR-B and liver-enriched inhibitory protein, or liver-enriched activatory protein and PR-A in human endometrial stromal cells. *Molecular endocrinology (Baltimore, Md*, **16**, 141-154.

16. Pohnke, Y., Kempf, R. and Gellersen, B. (1999) CCAAT/enhancer-binding proteins are mediators in the protein kinase A-dependent activation of the decidual prolactin promoter. *The Journal of biological chemistry*, **274**, 24808-24818.

17. Mak, I.Y., Brosens, J.J., Christian, M., Hills, F.A., Chamley, L., Regan, L. and White, J.O. (2002) Regulated expression of signal transducer and activator of transcription, Stat5, and its enhancement of PRL expression in human endometrial stromal cells in vitro. *The Journal of clinical endocrinology and metabolism*, **87**, 2581-2588.

18. Wagner, B.L., Norris, J.D., Knotts, T.A., Weigel, N.L. and McDonnell, D.P. (1998) The nuclear corepressors NCoR and SMRT are key regulators of both ligand- and 8-bromo-cyclic AMP-dependent transcriptional activity of the human progesterone receptor. *Molecular and cellular biology*, **18**, 1369-1378.

19. Wiench, M., Miranda, T.B. and Hager, G.L. Control of Nuclear Receptor Function by Local Chromatin Structure. *The FEBS journal*.

20. Sharov, A.A., Nishiyama, A., Piao, Y., Correa-Cerro, L.S., Amano, T., Thomas, M., Mehta, S. and Ko, M.S. Responsiveness of genes to manipulation of transcription factors in ES cells is associated with histone modifications and tissue specificity. *BMC genomics*, **12**, 102.

21. Kouzarides, T. (2007) Chromatin modifications and their function. *Cell*, **128**, 693-705.

22. Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S. and Zhang, Y. (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science (New York, N.Y*, **298**, 1039-1043.

23. Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A. and Pirrotta, V. (2002) Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell*, **111**, 185-196.

24. Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P. and Reinberg, D. (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes & development*, **16**, 2893-2905.

25. Karanikolas, B.D., Figueiredo, M.L. and Wu, L. Comprehensive evaluation of the role of EZH2 in the growth, invasion, and aggression of a panel of prostate cancer cell lines. *The Prostate*, **70**, 675-688.

26. Gonzalez, M.E., Li, X., Toy, K., DuPrie, M., Ventura, A.C., Banerjee, M., Ljungman, M., Merajver, S.D. and Kleer, C.G. (2009) Downregulation of EZH2 decreases growth of estrogen receptor-negative invasive breast carcinoma and requires BRCA1. *Oncogene*, **28**, 843-853.

27. Wei, Y., Xia, W., Zhang, Z., Liu, J., Wang, H., Adsay, N.V., Albarracin, C., Yu, D., Abbruzzese, J.L., Mills, G.B. *et al.* (2008) Loss of trimethylation at lysine 27 of histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. *Molecular carcinogenesis*, **47**, 701-706.

28. Berezovska, O.P., Glinskii, A.B., Yang, Z., Li, X.M., Hoffman, R.M. and Glinsky, G.V. (2006) Essential role for activation of the Polycomb group

(PcG) protein chromatin silencing pathway in metastatic prostate cancer. *Cell cycle (Georgetown, Tex*, **5**, 1886-1901.

29. Kleer, C.G., Cao, Q., Varambally, S., Shen, R., Ota, I., Tomlins, S.A., Ghosh, D., Sewalt, R.G., Otte, A.P., Hayes, D.F. *et al.* (2003) EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 11606-11611.

30. Bredfeldt, T.G., Greathouse, K.L., Safe, S.H., Hung, M.C., Bedford, M.T. and Walker, C.L. Xenoestrogen-induced regulation of EZH2 and histone methylation via estrogen receptor signaling to PI3K/AKT. *Molecular endocrinology (Baltimore, Md*, **24**, 993-1006.

31. Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vanderwinden, J.M. *et al.* (2006) The Polycomb group protein EZH2 directly controls DNA methylation. *Nature*, **439**, 871-874.

32. Ezhkova, E., Pasolli, H.A., Parker, J.S., Stokes, N., Su, I.H., Hannon, G., Tarakhovsky, A. and Fuchs, E. (2009) Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. *Cell*, **136**, 1122-1135.

33. Gellersen, B., Kempf, R., Telgmann, R. and DiMattia, G.E. (1994) Nonpituitary human prolactin gene transcription is independent of Pit-1 and differentially controlled in lymphocytes and in endometrial stroma. *Molecular endocrinology (Baltimore, Md*, **8**, 356-373.

34. Lynch, V.J., Brayer, K., Gellersen, B. and Wagner, G.P. (2009) HoxA-11 and FOXO1A cooperate to regulate decidual prolactin expression: towards

inferring the core transcriptional regulators of decidual genes. *PloS one*, **4**, e6845.

35. Gao, J., Mazella, J. and Tseng, L. (2002) Hox proteins activate the IGFBP-1 promoter and suppress the function of hPR in human endometrial cells. *DNA and cell biology*, **21**, 819-825.

36. Al-Sabbagh, M., Fusi, L., Higham, J., Lee, Y., Lei, K., Hanyaloglu, A.C., Lam, E.W., Christian, M. and Brosens, J.J. NADPH oxidase-derived reactive oxygen species mediate decidualization of human endometrial stromal cells in response to cyclic AMP signaling. *Endocrinology*, **152**, 730-740.

37. Telgmann, R., Maronde, E., Tasken, K. and Gellersen, B. (1997) Activated protein kinase A is required for differentiation-dependent transcription of the decidual prolactin gene in human endometrial stromal cells. *Endocrinology*, **138**, 929-937.

38. Tie, F., Banerjee, R., Stratton, C.A., Prasad-Sinha, J., Stepanik, V., Zlobin, A., Diaz, M.O., Scacheri, P.C. and Harte, P.J. (2009) CBP-mediated acetylation of histone H3 lysine 27 antagonizes Drosophila Polycomb silencing. *Development (Cambridge, England)*, **136**, 3131-3141.

39. Maruyama, T. and Yoshimura, Y. (2008) Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium. *Endocrine journal*, **55**, 795-810.

40. Han, S.J., Lonard, D.M. and O'Malley, B.W. (2009) Multi-modulation of nuclear receptor coactivators through posttranslational modifications. *Trends in endocrinology and metabolism: TEM*, **20**, 8-15.

41. Lonard, D.M. and O'Malley, B.W. (2006) The expanding cosmos of nuclear receptor coactivators. *Cell*, **125**, 411-414.

42. Thakur, M.K. and Paramanik, V. (2009) Role of steroid hormone coregulators in health and disease. *Hormone research*, **71**, 194-200.

43. Onate, S.A., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science (New York, N.Y*, **270**, 1354-1357.

44. Li, J., Wang, J., Wang, J., Nawaz, Z., Liu, J.M., Qin, J. and Wong, J. (2000) Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. *The EMBO journal*, **19**, 4342-4350.

45. Kiskinis, E., Hallberg, M., Christian, M., Olofsson, M., Dilworth, S.M., White, R. and Parker, M.G. (2007) RIP140 directs histone and DNA methylation to silence Ucp1 expression in white adipocytes. *The EMBO journal*, **26**, 4831-4840.

46. John, S., Sabo, P.J., Thurman, R.E., Sung, M.H., Biddie, S.C., Johnson, T.A., Hager, G.L. and Stamatoyannopoulos, J.A. Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. *Nature genetics*, **43**, 264-268.

47. Wiench, M. and Hager, G.L. Expanding horizons for nuclear receptors. *EMBO reports*, **11**, 569-571.

48. Hakim, O., Sung, M.H. and Hager, G.L. 3D shortcuts to gene regulation. *Current opinion in cell biology*, **22**, 305-313.

49. Carroll, J.S., Liu, X.S., Brodsky, A.S., Li, W., Meyer, C.A., Szary, A.J., Eeckhoute, J., Shao, W., Hestermann, E.V., Geistlinger, T.R. *et al.* (2005) Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell*, **122**, 33-43.

50. Karlic, R., Chung, H.R., Lasserre, J., Vlahovicek, K. and Vingron, M. Histone modification levels are predictive for gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 2926-2931.

51. Christian, M., Marangos, P., Mak, I., McVey, J., Barker, F., White, J. and Brosens, J.J. (2001) Interferon-gamma modulates prolactin and tissue factor expression in differentiating human endometrial stromal cells. *Endocrinology*, **142**, 3142-3151.

52. Margueron, R., Li, G., Sarma, K., Blais, A., Zavadil, J., Woodcock, C.L., Dynlacht, B.D. and Reinberg, D. (2008) Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. *Molecular cell*, **32**, 503-518.

53. Li, B., Gogol, M., Carey, M., Pattenden, S.G., Seidel, C. and Workman, J.L. (2007) Infrequently transcribed long genes depend on the Set2/Rpd3S pathway for accurate transcription. *Genes & development*, **21**, 1422-1430.

54. van Kaam, K.J., Delvoux, B., Romano, A., D'Hooghe, T., Dunselman, G.A. and Groothuis, P.G. Deoxyribonucleic acid methyltransferases and methyl-CpG-binding domain proteins in human endometrium and endometriosis. *Fertility and sterility*, **95**, 1421-1427.

55. Lee, J.S., Smith, E. and Shilatifard, A. The language of histone crosstalk. *Cell*, **142**, 682-685.

Figure Legends

Figure 1. Cycle-dependent expression of EZH2 in human endometrium. A. Protein lysates from proliferative (PE) and secretory (SE) endometrium were subjected to Western blot analysis and immunoprobed for EZH2. β-actin served as a loading control. Panel on the left shows densitometric analysis of Western blots using Image J. **B.** *EZH2* transcript levels in timed endometrial biopsies obtained during the early- (ES, n=5), mid- (MS, n=5) and late- (LS, n=5) secretory phase of the cycle were determined by RT-qPCR. The data, normalized to *L19* mRNA, are expressed in arbitrary units (a.u.; mean ± SEM). **C.** Endometrial tissue sections obtained at different phases of the cycle were immunostained for EZH2 (brown). The data shown in this figure are representative of 3 or more independent experiments.

Figure 2. EZH2 is down-regulated upon decidualization A. confluent HESC cultures were either untreated (day 0) or decidualized with 8-Br-cAMP and MPA for the indicated time-points. *EZH2* expression at mRNA and protein level was determined in parallel cultures by RT-qPCR (upper panel) and Western blot analysis (lower panel). The RT-qPCR data show the fold change (± SEM of triplicate measurments) in EZH2 transcript levels upon treatment relatively to expression in untreated cells. **B.** EZH2 expression in endometrial stromal cells cultured in the presence or absence of 8-Br-cAMP and MPA for 2, 4 and 8 days. The intensity of EZH2 (red channel) and DAPI nuclear staining (blue channel) were captured by confocal microscopy. **C.** confluent HESC cultures either untreated or decidualized with 8-Br-cAMP (cAMP), MPA, or a combination for 2 days. *EZH2* expression at

mRNA and protein level was determined in parallel cultures by RT-qPCR (upper panel) and Western blot analysis (lower panel). The data show the fold change (± SEM of triplicate measurments) in EZH2 transcript levels upon treatment relatively to expression in untreated cells.

Figure 3. H3K27 methylation in undifferentiated and decidualizing HESCs. A. Primary HESCs treated with 8-Br-cAMP and MPA for the indicated timepoints were fixed in formaldehyde, stained, and subjected to confocal microscopy. The upper panel represents H3K27me3 staining (red channel), whereas the lower panel shows the corresponding nuclear DAPI staining (blue channel). **B.** Total protein lysates of HESCS treated as above were subjected to Western blot analysis and immunoprobed for EZH1 and H3K27me. β-actin was used as a loading control. **C.** Chromatin extracted from HESC first decidualized for 2, 4 or 8 days was immunoprecipitated with antibodies against H3K27me3 and IgG. The chromatin, normalized to the input, was analyzed by qPCR (ChIP-qPCR) with primers specific for the promoter regions of *PRL* (left panel) and *IGFBP1* (right panel). Data are expressed as the fold change (± SEM of triplicate measurments) relatively to the abundance of chromatin-bound H3K27me3 in untreated cells. The data shown in this figure are representative of 3 independent experiments.

Figure 4. Dynamic changes in H3K27 acetylation upon decidualization of HESCs. A. Endometrial stromal cells were cultured in the presence or absence of 8-Br-cAMP and MPA for the indicated time-points. The intensity of H3K27ac (green channel) and the corresponding DAPI nuclear staining (blue channel) were captured by confocal microscopy. **B.** Whole cell protein lysates of HESCS treated as above

were subjected to Western blot analysis using an H3K27ac-specific antibody. β-actin served as a loading control. **C.** ChIP was carried out on HESCs decidualized for the indicated time-points using antibodies against H3K27ac and IgG. qPCR was caried out on the ChIPed DNA with primers specific for the proximal promoter regions of *PRL* (left panel) and *IGFBP1* (right panel) and normalized to the input. Data are expressed as the fold change (± SEM of triplicate measurements) relatively to the abundance of chromatin-bound H3K27ac in untreated cells. The data shown in this figure are representative of 3 independent experiments.

Figure 5. EZH2 downregulation is permissive for the induction of decidual marker genes. A. Total cell lysates from primary HESC cultures first transfected with either non-targeting (NT) or EZH2 siRNA and then treated with cAMP and MPA for 2 days were subjected to Western blot analysis and probed with EZH1-, EZH2-, and H3K27me3-specific antibodies. β-actin served as a loading control. **B.** ChIP with H3K27me3 and IgG antibodies was performed on cultures treated as above. The percipitated DNA fragments were analyzed by qPCR and normalized to the input. The primers used were specific for the proximal promoter regions of *IGFBP1* (left panel) and *PRL* (right panel). Data are expressed as the fold change (± SEM of triplicate measurments) relatively to the abundance of chromatin-bound H3K27me3 in untreated cells. **C.** ChIP was carried out with H3K27ac-specific antibody under the experimental conditions described above. **D.** PRL and IGFBP-1 transcript levels were measured by RT-qPCR in HESCs first transfected with either non-targeting (NT) or EZH2 siRNA and then treated with cAMP and MPA for 2 days. The data show fold-change (± SEM of triplicate measurments) in expression relative to untreated cells transfected with NT siRNA. **E.** Protein lysates extracted from cells

first transfected with an empty control vector (pCMV) or a plasmid encoding EZH2 (pCMV-EZH2) vector and then treated with decidualization stimuli for 2 days were subjected to Western blot analysis and immunoprobed for EZH2. β-actin was used as a loading control. **F.** Total RNA from parallel cultures was examined for the expression of IGFBP-1 (upper panel) and PRL (lower panel) transcripts by RT-qPCR. The data (mean ± SEM) are presented as fold-induction and the results are representative of 3 repeat experiments.

Figure 6. Genome-wide redistribution of H3K27me3 in decidualizing HESCs and upon EZH2 knockdown in undifferentiated HESCs. A. Venn diagram indicating the number of genomic loci significantly ($P < 0.05$) altered in H3K27 methylation as determined by ChIP-chip analysis of HESCs either decidualized with 8-Br-cAMP and MPA for 8 days (left) or upon EZH2 knockdown (right). **B**. Example of altered H3K27me3 peaks in the genomic region encompasssing the *IGFBP1* promoter. The blue line represents the peaks in the undifferentiated cells whereas the red line indicate the corresponding relative level of H3K27me3 in paired cultures decidualized with 8-Br-cAMP and MPA for 8 days. **C**. GO analysis of genes in the proximity of genomic loci characterized by significant loss (X panel) or gain (X panel) in H3K27me3 upon decidualization of HESCs. **D**. GO enrichment analysis of promoter regions altered in H3K27 methylation in response to differentiation as well as siRNA-mediated EZH2 knockdown in undifferentiated cultures.