Analysis of chromatin accessibility in decidualizing human endometrial stromal cells

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ABSTRACT: Spontaneous decidualization of the endometrium in response to progesterone signaling is confined to menstruating species, including humans and other higher primates. During this process, endometrial stromal cells (EnSCs) differentiate into specialized decidual cells that control embryo implantation. We subjected undifferentiated and decidualizing human EnSCs to an assay for transposable accessible chromatin with sequencing (ATAC-seq) to map the underlying chromatin changes. A total of 185,084 open DNA loci were mapped accurately in EnSCs. Altered chromatin accessibility upon decidualization was strongly associated with differential gene expression. Analysis of 1533 opening and closing chromatin regions revealed over-representation of DNA binding motifs for known decidual transcription factors (TFs) and identified putative new regulators. ATAC-seq footprint analysis provided evidence of TF binding at specific motifs. One of the largest footprints involved the most enriched motif—basic leucine zipper—as part of a triple motif that also comprised the estrogen receptor and Pax domain binding sites. Without exception, triple motifs were located within Alu elements, which suggests a role for this primate-specific transposable element (TE) in the evolution of decidual genes. Although other TEs were generally under-represented in open chromatin of undifferentiated EnSCs, several classes contributed to the regulatory DNA landscape that underpins decidual gene expression.—Vrljicak, P., Lucas, E. S., Lansdowne, L., Lucciola, R., Muter, J., Dyer, N. P., Brosens, J. J., Ott, S. Analysis of chromatin accessibility in decidualizing human endometrial stromal cells. FASEB J. 32, 2467–2477 (2018). www.fasebj.org

KEY WORDS: endometrium · decidualization · gene regulation · open chromatin · transposable elements

INTRODUCTION

Decidualization of the endometrium occurs in all mammals in which the implanting embryo breaches the luminal endometrial epithelium and the trophoblast invades the maternal tissues (1). This process—defined by the transformation of endometrial stromal cells (EnSCs) into epithelioid decidual cells—occurs in concert with the secretory transformation of uterine glands, vascular remodeling, and influx of specialist immune cells, especially uterine NK cells (2). In pregnancy, decidual cells form a protective and nutritive matrix around the early conceptus that enables controlled trophoblast invasion and confers maternal immune tolerance of the antigenically distinct fetus (3–5).

In most mammals, decidualization is triggered by the implanting embryo; however, in a few species—that is, higher primates, 4 species of bats, the elephant shrew, and the common (Cairo) spiny mouse—decidualization is spontaneous, meaning that it is initiated during the mid-luteal phase of each cycle, independently of an implanting embryo (6, 7). Sustained progesterone signaling is essential to maintain the decidual phenotype of differentiated EnSCs (8). In the absence of pregnancy, falling ovarian progesterone production triggers a cascade of inflammatory events in the decidualizing endometrium that, upon recruitment and activation of leukocytes, becomes irreversible and leads to partial tissue destruction, bleeding, and menstrual shedding (9). Recent studies have shown that

ABBREVIATIONS: ATAC, assay for transposable accessible chromatin; bZIP, basic leucine zipper; ChIP-seq, chromatin immunoprecipitation followed by sequencing; DCC, dextran-coated charcoal; EnSC, human endometrial stromal cell; ESR, estrogen receptor; FOSL2, Fos-like antigen; FOXO1, forkhead box O1; GEO, Gene Expression Omnibus; MPA, medroxypregesterone acetate; PGR, progesterone receptor; seq, sequencing; TE, transposable element; TF, transcription factor; TSS, transcription start site

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the inextricable coupling of cyclic decidualization to menstruation and tissue repair bequeaths the uterus with additional reproductive traits, including the ability to reject invasive, but developmentally impaired embryos and to adapt to reproductive failure (10, 11).

Primary human EnSCs readily decidualize in culture in response to cAMP and progestin signaling in a manner that recapitulates closely the in vivo situation (12, 13). This model system has been used extensively to characterize the signaling pathways, downstream transcription factors (TFs), histone and DNA modifications, and gene networks that regulate this differentiation process [reviewed in detail in Gellersen et al. (2)]. Furthermore, DNase-seq has been used to map open chromatin regions, indicative of regulatory DNA, in decidualized EnSCs (14), but the dynamic changes in the chromatin landscape that underpin the transition of undifferentiated EnSCs to specialized decidual cells have not yet been characterized.

In this study, we used an assay for transposase accessible chromatin with sequencing (ATAC-seq) to profile the chromatin landscapes of primary human EnSCs in their undifferentiated state and upon decidualization. ATAC-seq utilizes the highly active transposase, Tn5, to interrogate the accessibility of the genome and map open chromatin regions. These putative cis-regulatory DNA regions can be additionally explored for footprints of TF binding (15). Footprints are small regions of tens of base pairs that are relatively resistant to transposase activity inside an otherwise highly accessible locus. We have generated a comprehensive map of chromatin regions that change dynamically upon decidualization, determined the TF binding motifs that are most enriched upon the opening or closing of chromatin, and interrogated footprints that are indicative of TF binding to cis-regulatory sequences in decidualizing EnSCs. Analysis of a conspicuous footprint that is indicative of the binding of a large protein complex led to the discovery of an abundant triple motif that consists of estrogen receptor (ESR), basic leucine zipper (bZIP), and Pax domain binding sequences in Alu elements, the most abundant transposable elements (TEs) in the human genome. We explored whether specific triple motifs could have been co-opted to regulate decidual gene expression in menstruating primates and examined the involvement of other TEs in the cis-regulatory landscape of differentiating human EnSCs.

MATERIALS AND METHODS

Ethical approval and sample collection

The study was approved by the National Health Service (NHS) National Research Ethics-Hammersmith and Queen Charlotte’s and Chelsea Research Ethics Committee (1997/3065). Endometrial biopsies were obtained from women who attended the Implantation Clinic, a dedicated research clinic at University Hospitals Coventry and Warwickshire National Health Service Trust. Time-midsecretory-phase biopsies were obtained using a Wallach Endocell endometrial sampler with written informed consent in accordance with the Declaration of Helsinki. ATAC-seq analysis was performed on undifferentiated and decidualizing EnSC cultures that were established from 1 nulliparous patient awaiting in vitro fertilization treatment and 2 recurrent miscarriage patients. Biopsies were obtained between 5 and 10 d after LH surge. None of the patients received hormonal therapy for at least 2 cycles before the biopsy cycle.

Primary culture

Endometrial biopsies were collected in DMEM-F12 media that was supplemented with 10% dextran-coated charcoal (DCC)-striped FBS and processed for primary EnSC culture as previously described (16). For decidualization studies, confluent monolayers of human EnSCs were incubated overnight at 37°C with 5% CO2 in phenol red–free DMEM/F-12 that contained 2% DCC with antibiotic/antimycotic and l-glutamine (2% medium). To induce differentiation, cells were treated in 2% DCC with 0.5 mM 8-bromoadenosine-cAMP (Sigma-Aldrich, Poole, United Kingdom) and 1 μM medroxyprogesterone acetate (MPA; Sigma-Aldrich) for the indicated time points. Control cultures were incubated in media that was supplemented with 2% DCC. Primary cultures were subjected to ATAC-seq at passage 2.

ATAC-seq libraries

ATAC-seq was performed as previously described (15, 17), with some modifications. In brief, EnSCs (n = 3) were grown in 35-mm diameter tissue culture dishes. Confluent monolayers were washed with cold Dulbecco’s PBS, then lysed using ice-cold EZ lysis buffer (Sigma-Aldrich). Cells were scraped, then transferred to chilled prelabeled nuclease-free 1.5-ml microcentrifuge tubes. Samples were vortexed, left on ice for 5 min, then pelleted in a fixed-angle refrigerated benchtop centrifuge. The supernatant was carefully removed, and the pellet was washed once in EZ lysis buffer. The nuclear pellet was resuspended in the transposase reaction mix that contained 25 μl Tagment DNA buffer, 5 μl Tagment DNA enzyme, and 20 μl nuclease-free water (Nextera DNA Sample Preparation Kit; Illumina, Cambridge, United Kingdom). The transposition reaction was carried out for either 45 or 90 min at 37°C. Samples were purified using a Zymo DNA Clean and Concentrator-5 Purification Kit according to manufacturer instructions. In brief, DNA-binding buffer was added to the 50-μl sample that was then mixed and transferred to the column. Samples were centrifuged at 17,000 × g for 30 s at room temperature. Flow-through was discarded, 200 μl DNA wash buffer added, and columns were centrifuged as described above. The wash and centrifugation steps were repeated twice. After removing the residual liquid, 23 μl prewarmed elution buffer was added to the columns. Samples were incubated for 2 min at room temperature, then centrifuged for 2 min to elute DNA. After purification, a 20 μl sample was added to a 0.2-ml PCR tube that contained the following reagents (Nextera DNA Sample Preparation Kit and Nextera Index Kit; Illumina): 5 μl index 1, 5 μl index 2, 15 μl Master mix, and 5 μl Primer Cocktail. Amplification was performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the following PCR conditions: 72°C for 3 min, 98°C for 30 s, then 15 cycles of 98°C for 10 s, 63°C for 30 s, and 72°C for 1 min. Libraries were purified using AMPure XP beads according to the Illumina Nextera Kit protocol. Amplified libraries were quantified using Qubit HS DNA Assay on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Paisley, United Kingdom) according to the manufacturer’s instructions. Library sizes were assessed on an Agilent Technologies 2100 Bioanalyzer using the High Sensitivity DNA chip (Agilent Technologies, Santa Clara, CA, USA).

Bioinformatic analysis

ATAC-seq libraries were sequenced with 100-bp paired ends on a HiSeq 2500 (Illumina) and mapped to the hg19 human genome.
assembly using bowtie2-2.2.6 (18) and samttools-1.2.0 (19). Peaks were called on a merged mapped file using MACS-2.1.0 (20), which led to the identification of 202,169 peaks. Of these peaks, 185,087 were deemed high confidence \((q < 1 \times 10^{-4})\) and were used for additional analysis. To determine differential chromatin opening, we used HTSeq-0.6.1 (21) to assign reads to peaks, and DESeq2 (22) was used to identify differential peaks. Peaks were then ranked according to their \(P\) value, taking into account the direction of the fold change, such that lower-rank values represent peaks that open upon decidualization, whereas high-rank values represent closing regions of chromatin. Data have been submitted to Gene Expression Omnibus (GEO; accession number: GSE104720).

**Comparison of peak data with other data sets**

ATAC-seq data were compared with other available chromatin binding and accessibility data sets using BEDTools (23). Chromatin immunoprecipitation followed by sequencing (ChiP-seq) data sets from decidualizing stromal cells were obtained from the GEO for the following TFs: progesterone receptor (PGR; GSM1703567), Fos-like antigen (FOSL2; GSM1703658), and forkhead box O1 (FOXO1; GSM1703607). Formaldehyde-assisted chromosome repositioning (FACeSeq) (24) and ENCODE DNaseI hypersensitivity data were used to associate open chromatin regions in EnSCs overlapped with DNaseI hypersensitivity data for decidualized endometrium and DNaseI hypersensitivity data for decidualized stromal cells were obtained from GEO accession numbers GSM1011119 and GSM61793, respectively, whereas the Encyclopedia of DNA Elements (ENCODE; Stanford University, Stanford, CA, USA) DNase hypersensitivity data for 125 human cell and tissue types were obtained from the University of California, Santa Cruz (UCSC; Santa Cruz, CA, USA) genome browser track (24–26).

**Differential expression of proximal genes**

ENCODE DNase hypersensitivity data were used to associate open chromatin regions and gene promoters with high confidence (25). A distal open chromatin region was deemed to be associated with a gene promoter if it was shown to physically interact and the distance was no more than 10 kb. Gene expression profiles of undifferentiated and decidualizing EnSCs were obtained by performing RNA-seq analysis on 3 independent samples (GEO accession number: GSE104721).

**Motif discovery and identification**

Differentially opening chromatin regions were interrogated for enriched short-sequence motifs using HOMER v.4.8 (27). Background sets were generated from random genomic sequences of selected size near the transcription start site (TSS) of genes \((\pm 50\,\text{kb})\), matching the GC/CpG distribution of the input. To additionally investigate the context of informative motifs, 50-bp sequences that surround enriched motifs were searched with Multiple Em for Motif Elicitation (MEME) and compared with motif databases using TomTom (28). Sequence conservation at each nucleotide position was calculated using WebLogo v.3.2.9 (29). Find Individual Motif Occurrences (FIMO) was used to identify instances of the motif in the Human Genome (30). These locations were intersected with the RepeatMasker database retrieved via the UCSC Table Browser. Average ATAC-seq profiles and footprinting analysis were performed with Wellington (31).

**Conservation of Alu elements in primate genomes**

To examine Alu conservation, Alu elements in the human genome were mapped to other primate species using blastn (32). We examined for conservation a total of 7120 Alu elements of >280 bp in length and overlapping gene-linked ATAC-seq peaks. Nucleotide sequences of 100 bp in length that spanned the 5' and 3' ends and surrounding sequences were mapped to other primate genomes. Alu loci for which orthologous loci in other primates that could not be established with confidence were excluded from the analysis. For remaining loci, the presence or absence of specific Alu elements at the orthologous loci was computed (see examples in Supplemental Fig. 7).

**RESULTS**

**Altered chromatin accessibility upon decidualization**

To map dynamic chromatin changes upon decidualization, nuclei that were isolated from EnSC cultures, either untreated or decidualized with 8-bromoadenosine-cAMP and MPA for 4 d, were processed for ATAC-seq. This analysis accurately mapped the open chromatin regions in both cellular states. For example, expression of prolactin (PRL), a major decidual marker gene, is initiated in the endometrium at an alternative start site located at an additional noncoding exon, exon 1a, ~6-kb upstream of the pituitary-specific TSS (33). As shown in Fig. 1A (upper panel), ATAC-seq analysis revealed selective chromatin opening immediately upstream of the nonpituitary PRL TSS in response to treatment with 8-bromoadenosine-cAMP and MPA, which confirmed that cells responded to deciduogenic signals. Another cardinal decidural marker gene is IGFBP1 (IGF binding protein-1), and, again, chromatin opening across the proximal promoter region was apparent in differentiating EnSCs (Fig. 1A, lower panel). Additional regions of chromatin opening at the IGFBP1 locus were mapped upstream of the TSS and downstream of the termination site.

The density of mapped ATAC-seq peaks as a function of genomic position provides a quantitative measure of chromatin accessibility. A total of 185,084 regions of accessible chromatin were mapped in EnSCs and ranked by the extent of opening or closing upon decidualization (Supplemental Table 1). The basis of a stringent criterion \((\text{Bonferroni adjusted } P < 0.05)\), 1225 and 278 loci opened or closed upon decidualization, respectively. The majority of these loci \((55\%)\) fell into gene introns, and 27% were found within 10 kb upstream of TSSs. Next, we examined the overlap in accessible chromatin regions that were identified by ATAC-seq and existing chromatin profiling data obtained either by DNase-seq analysis of decidualized EnSCs or by formaldehyde-assisted isolation of regulatory elements with sequencing analysis of whole endometrial tissue (14). More than 80% of open chromatin regions that were identified in this study were not mapped previously in either cultured decidual cells or whole endometrium (Supplemental Fig. 1); however, >95% of the open chromatin regions in EnSCs overlapped with DNaseI hypersensitive sites that were identified by DNase-seq analysis of 125 human cell and tissue types in the ENCODE project (25), which indicates that our analysis captured bona fide regulatory DNA regions.

The gain or loss of ATAC-seq peaks upon decidualization indicates altered TF binding that drives differential
though not fully predictive, association with increased or 

designated opening motifs 1

enrichment analysis on the 1255 opening and 278 closing 

motifs in closing motifs 1

expression, we performed 

to gain insight into TFs that govern decidual gene ex-

Motif discovery and footprint analysis

To gain insight into TFs that govern decidual gene ex-

expression, we performed de novo short-sequence motif en-

richment analysis on the 1255 opening and 278 closing 

chromatin regions. This annotation revealed 17 signif-

icantly over-represented motifs in opening peaks (des-

ignated opening motifs 1–17) and 7 over-represented 

motifs in closing motifs 1–7 (Fig. 2A, and Supplemental 

Fig. 2, and Supplemental Table 2). Of note, these motifs 

were associated strongly with the opening or closing 

of chromatin, not only in the subset of peaks used for motif 

discovery, but over the full set of peaks (Fig. 2B and 

Supplemental Fig. 3).

Binding of TFs to open chromatin should result in 

an ATAC-seq footprint. To examine the occupancy of 

informative binding sites, we calculated the average 

ATAC-seq signal centered on each motif (Fig. 2C and 

Supplemental Fig. 4). At the center of these plots, con-

served motif sequences are reflected by spikes that re-

sult from the sequence-specific cutting bias of the Tn5 

transposase. TF occupancy of a motif protects the DNA 

from transposase activity, which causes relative enrich-

ment of reads across the binding site. This gives rise to 

the conspicuous pattern of excess positive and negative 

strand reads at 5’ and 3’ flanking regions of the motif,

respectively. Footprint analysis indicated TF binding to 
cis-regulatory elements in all enriched motifs, except for 
opening motif 10 and closing motif 2. Next, we matched 
the motifs against known TF binding databases and 
examined the gene expression of the putative binding 
TFs in decidualizing EnSCs (Supplemental Fig. 5 and 
Supplemental Table 3). CCAAT/enhancer binding 
protein β and δ, FOSL2 (or FRA2), FOXO1, PGR, and 
signal transducer and activator of transcription 3 and 
5 are essential TFs for decidualization (34–37), whereas 
TEA domain transcription factor 1 has been shown to 
repress decidualization (38). Of note, our top motifs 
in enriched ATAC-seq peaks correlated to high-
affinity binding sites for key decidual TFs (Fig. 2A). 
Furthermore, the most enriched motif in closing regions 
binds TEA domain transcription factor 1. We also com-
pared ATAC-seq regions with existing ChIP-seq binding 
data for FOXO1 (GSM1703607), PGR (GSM1703567), 
and FOSL2 (GSM1703568) in differentiating EnSCs 
(39). FOXO1 and PGR binding was enriched in regions 
of opening chromatin (P < 1 × 10^{-4} and P < 1 × 10^{-15}, 
respectively; Fisher’s exact test for 1000 opening and 
closing peaks), which is consistent with their role as 
core decidual TFs (Supplemental Fig. 6). Of interest, 
the presence of both PGR and FOSL2 binding sites 
within a chromatin region correlated with chromatin 
opening upon decidualization, whereas the presence of 
FOSL2 in the absence of PGR binding correlated with 
chromatin closure.

Next, we mined our RNA-seq data to identify novel 
decidual TFs that may bind other motifs in opening 
chromatin. This exercise yielded several candidate TFs 
that were not yet implicated in the promotion of decidu-
alization, including RAR-related orphan receptor A, aryl 
hydrocarbon receptor nuclear translocator like, and Meis 
homeobox 1. Conversely, down-regulation of runt-related 
transcription factor 1 and 2, SRY-box 12, transcription 
factor 3, and ETS Proto-Oncogene 1 upon decidualization
Figure 2. Gain and loss of TF binding motifs in dynamic chromatin regions. A) Top 5 most enriched TF binding motifs in opening and closing chromatin regions (see Supplemental Fig. 2 for full lists). The frequency (%) of opening or closing peaks featuring a given motif is shown relative to randomly selected genomic regions (±50 kb from TSSs, matching size, and GC/CpG content). On the basis of expression data and motif specificity, the most plausible TF for each motif is shown. B) The frequency of the 10 motifs was also determined across 185,084 open chromatin peaks in EnSCs. The y-axis indicates the frequency of each motif across bins of 10,000 ATAC-seq peaks that are ranked on the x-axis from the most opening (1) to the most closing peak (185,084). Nonshaded bars indicate expected frequency on the basis of genomic background frequency. C) Footprint analysis. (continued on next page)
may account for the loss of chromatin accessibility in differentiating EnSCs. Taken together, our ATAC-seq data capture known modulators of decidualization, identify their putative target loci, and suggest novel TFs.

**Triple motif and Alu repeats**

Footprints around bZIP motifs (e.g., opening motif 1) were noticeably wider than around other motifs (Fig. 2C), which suggests binding of multimeric complexes at these sites. Additional short-sequence motif analysis on 50-bp windows around these putative TF binding sites in opening motif 1 resulted in the discovery of an over-represented triple motif that consisted of ESR, bZIP, and Pax domain binding sequences in close proximity (Fig. 3A). This triple motif occurs 47,499 times in open chromatin regions. A genome-wide search revealed that triple motifs overlaps invariably with the 3’ end of Alu repetitive element sequences (Fig. 3B). Dimeric Alu elements are unique to primates and are classified into subfamilies according to their relative ages (40, 41). Triple motifs were found in all subfamilies of Alu elements, including the oldest, Alu J. Whereas they share the same level of sequence conservation with other Alu regions (Fig. 3C), triple motifs were much more dynamically regulated upon decidualization (Fig. 3D). Indeed, the 3’ end of Alu elements overlaps ATAC-seq peaks more frequently than does the 5’ end \( (P = 2.2 \times 10^{-16}, \text{binomial test}; \text{Fig. 3E}). \)

Next, we identified 7120 Alu elements that overlap with open chromatin regions that are associated with genes and examined their conservation in menstruating primates. Menstruation is well documented in Catarrhines (humans, apes, and Old World monkeys), but is absent in Strepsirrhines (e.g., bushbabies/galagos). The prevalence of menstruation in Platyrrhini (New World monkey) is much less clear, with conflicting observations reported for certain species (7, 42). As shown in Fig. 4, the level of conservation of informative Alu was high in Catarrhines, but not in Strepsirrhines. An intermediate level of conservation was observed in Platyrhini, including squirrel monkey (Saimiri) and marmoset (Callithrix), 2 reportedly nonmenstruating primates. To confirm these findings, we examined a set of genes that were differentially expressed upon decidualization that are putatively regulated by Alu elements in opening or closing ATAC-seq peaks (Supplemental Table 4). Of interest, among these genes are several that encode key decidual TFs—for example, CEBPD and WT1 (Wilms tumor 1)—and signal intermediates, such as PROKI (prokineticin 1) and SGKI (serum/glucocorticoid regulated kinase 1) (13, 43, 44). Taken together, these observations suggest that the triple motif in Alu elements contrives to the cis-regulatory landscape that governs the spontaneous decidualization and menstruation in the primate lineage; however, there was no evidence that Alu repeats in dynamic vs. nondynamic chromatin regions in decidualizing EnSCs were evolutionarily more conserved (Fig. 4).

**Contribution of other TEs**

To determine the involvement of other TEs in the open chromatin landscape of human EnSCs, we overlapped ATAC-seq data with repetitive elements that were identified by RepeatMasker. In 5 of 6 of the most abundant repetitive families, the frequency of overlap with ATAC-seq peaks was lower than expected by chance \( (P < 1 \times 10^{-15}, \text{Fisher’s exact test}) \). Only one family—chicken repeat 1 elements—demonstrated a higher frequency than expected \( (P < 1 \times 10^{-15}, \text{Fisher’s exact test; \text{Fig. 5A}}) \). Next, we examined the contribution of TEs to the dynamic chromatin changes in decidualizing cells by examining their frequency in opening peaks. This analysis identified additional exapted TEs, including the Eutherian-specific hAT-Charlie DNA transposon that has been previously shown to confer cAMP and progesterone responsiveness in decidualizing EnSCs (45). Other notable TEs included Line1 and the exogenous retroviral-derived ERVL (Fig. 5B and Supplemental Fig. 8). Of interest, low complexity and simple repeats were more likely to close during decidualization (Supplemental Fig. 8). Finally, chromatin accessibility was predominantly unchanged upon decidualization in regions of Satellite and ERVK sequences. Thus, whereas repetitive elements are generally under-represented in open chromatin in EnSCs, they nevertheless function frequently as part of the regulatory landscape that renders EnSCs responsive to cAMP and progesterin signaling.

**DISCUSSION**

Decidualization of human EnSCs depends on the convergence of the cAMP and progesterone signaling pathways as well as the induction and/or activation of core decidual TFs (2, 12). Here, we present the first characterization of the changes in chromatin accessibility upon differentiation of human EnSCs into decidual cells. Opening ATAC-seq peaks result from the cooperative binding of TFs in place of a canonical nucleosome. Congruently, the most highly enriched cis-regulatory motifs upon decidualization represent high-affinity binding sites for core decidual TFs, including C/EBPs, FOXO1, signal transducers and activators of transcription, PGR, and members of the activator protein-1 family.
Figure 3. Triple motif in Alu elements. A) Short-sequence motif-finding analysis on 50-bp windows across opening motif 1 revealed a triple motif that consisted of ESR1, bZIP and Pax-like domain binding sequences. B) Triple motif is part of the 3’-end of Alu repetitive elements. Every triple motif in the genome overlaps with the right arm of Alu repetitive elements. C) Conservation, measured as entropy, of full-length Alu sequences in the genome was analyzed together with flanking sequences. Higher entropy, denoted as natural units of information (Nats), indicates higher conservation. D) Aggregated ATAC-seq signals across Alu sequences of at least 300 bp in length, together with 50 bp of surrounding sequence. Red and blue indicate positive and negative strand cuts, respectively. ATAC-seq peaks are enriched over the triple motif region, but are depleted in A-rich regions. E) Frequency of overlap of individual positions within the Alu elements and open chromatin regions. The 3’ end of Alu elements more frequently coincides with open chromatin than does the 5’ end.
of transcription factors, most prominently FOSL2 (2, 34). Although multiple TFs can potentially bind a given motif, the mining of published ChIP-seq data confirms increased binding of both FOXO1 and PGR to opening chromatin regions in decidualizing cells (34, 39). Several studies have reported that cooperation between core decidual TFs, which is often mediated via direct protein-protein interaction, modifies the transcription output of individual TFs (46–48). A case in point is FOSL2, which has been identified as a putative PGR coregulator in a ChIP-seq analysis of the PGR, cistrome, in decidualizing EnSCs (34). Our data demonstrate that the presence of both PGR and FOSL2 binding sites within a DNA region correlates with increased chromatin accessibility upon decidualization, whereas sites with only FOSL2 motifs were more likely to close. We identified a total of 17 enriched motifs in opening chromatin, all of which, bar one (opening motif 10), demonstrated evidence of TF binding in ATAC-seq footprints. Cross-referencing of these binding motifs against TFs that are up-regulated at the mRNA level upon decidualization identified several putative novel decidual transcriptional regulators, including RAR-related orphan receptor A, aryl hydrocarbon receptor nuclear translocator like, and Meis homeobox 1. Reduced chromatin accessibility was equally associated with the loss of binding motifs for TFs that are repressed upon decidualization, most notably, runt-related transcription factor 1 and 2, SRY-box 12, transcription factor, and ETS Proto-Oncogene 1. Whereas gene expression was not fully predicted by chromatin changes at specific loci, overall, the changes in chromatin accessibility—leading to either the loss or enrichment of cis-regulatory DNA elements in both promoters and at distal sites—correlated strongly with differential gene expression in decidualizing cells.

There is compelling evidence that the emergence of decidual cells in Eutherian (placental) mammals was driven by large-scale integration of numerous TEs that contributed cis-regulatory elements that were coopted by EnSC-specific TFs to drive decidual gene expression (14). Most families of repetitive elements, including Alu and L1 elements, are under-represented in the open chromatin landscape of EnSCs, which suggests active repression; however, several families of TEs were over-represented in opening chromatin upon decidualization, which supports the notion that they contributed essential functional DNA sequences that enabled the endometrium to accommodate an invasive placenta. Among Eutherian mammals, spontaneous decidualization coupled with menstruation is a rare phenomenon, with higher primates accounting for 93% of all known menstruating species (6, 7). A striking observation in this study was the discovery of a singular triple TF binding motif, embedded in primate-specific dimeric Alu elements, that was not only linked strongly to chromatin opening upon decidualization, but that also was conserved in menstruating but not in non-menstruating primates. The precise nature of the TF complex that binds this triple motif, which consists of contiguous ESR, bZIP, and Pax domain binding sequences, requires additional investigation, although ESR sites in Alu sequences have been shown previously to be bound in vivo (49, 50). As estradiol is not part of our differentiation protocol, our findings potentially point to a role for the unliganded ESR in regulating decidual gene expression, a phenomenon that has already been described for PGR and androgen receptor (12, 51). Triple motifs in Alu elements are abundant throughout the genome, which indicates that the binding of a multimeric transcriptional complex upon decidualization is likely dependent on the local chromatin context. Whereas our observations are intriguing, it is possible that the emergence of Alu triple motif and menstruation in primates may not be functionally linked, but merely reflects the
Although spontaneous decidualization in primates and other species evolved independently, menstruating mammals share a number of reproductive features, including spontaneous ovulation, a deeply invading hemochorial placenta, and few well-developed offspring. The convergence of these reproductive traits suggests an adaptive value for species that possess them (7); however, the reproductive advantage of menstruation has been the subject of intense controversy (7, 52–54), especially in view of the health burden associated with abnormal menstruation and menstruation-associated disorders, such as endometriosis (2). Furthermore, overwhelming evidence indicates that aberrant decidualization causes a spectrum of clinical disorders, including implantation failure and recurrent pregnancy loss (11, 13, 55). By mapping the dynamic changes in the chromatin landscape in differentiating human EnSCs, our work constitutes a step toward the full elucidation of the regulatory networks that drive decidualization, which ultimately may contribute to the development of more

**Figure 5.** Involvement of TEs in dynamic chromatin changes upon decidualization. A) Relative frequency of ATAC-seq peaks overlapping repetitive elements identified by RepeatMasker. Of the six most abundant TE families in ATAC-seq peaks, only one [chicken repeat 1 (CR1)] exceeds the frequency that is expected by chance. B) The frequency of TE families was also determined across 180,000 open chromatin peaks. The y axis indicates the frequency of each family across bins of 10,000 peaks that are ranked on the x axis from the most opening (1) to the most closing peak (185,084).
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AUTHOR CONTRIBUTIONS

J. J. Brosens and S. Ott conceived of the study; E. S. Lucas and R. Luicciola performed ATAC-seq experiments; J. Muter performed RNA-seq experiments; P. Vrljicak and N. P. Dyer analyzed ATAC-seq data; P. Vrljicak analyzed gene expression data; P. Vrljicak and L. Lansdowne analyzed Alu element sequence data; P. Vrljicak, L. Lansdowne, and J. J. Brosens wrote the manuscript; S. Ott supervised P. Vrljicak, L. Lansdowne, and N. P. Dyer; and J. J. Brosens supervised E. S. Lucas and R. Luicciola.

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