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ELF5-enforced transcriptional networks define an epigenetically regulated trophoblast stem cell compartment in the human placenta

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The first definitive cell fate decision in development occurs at the blastocyst stage with establishment of the trophoblast and embryonic cell lineages. In the mouse, lineage commitment is achieved by epigenetic regulation of a critical gatekeeper gene, the transcription factor *Elf5*, that reinforces placental cell fate and is necessary for trophoblast stem (TS) cell self-renewal. In humans, however, the epigenetic lineage boundary seems to be less stringent since human embryonic stem (ES) cells, unlike their murine counterparts, harbour some potential to differentiate into trophoblast derivatives. Here, we show that ELF5 is expressed in the human placenta in villous cytotrophoblast cells but not in post-mitotic syncytiotrophoblast and invasive extravillous cytotrophoblast cells. ELF5 establishes a circuit of mutually interacting transcription factors with CDX2 and EOMES, and the highly proliferative ELF5$^+$/CDX2$^+$ double-positive subset of cytotrophoblast cells demarcates a putative TS cell compartment in the early human placenta. In contrast to placental trophoblast, however, *ELF5* is hypermethylated and largely repressed in human ES cells and derived trophoblast cell lines, as well as in induced pluripotent stem cells and murine epiblast stem cells. Thus, these cells exhibit an embryonic lineage-specific epigenetic signature and do not undergo an epigenetic reprogramming to reflect the trophoblast lineage at key loci such as *ELF5*. Our identification of the trophoblast-specific transcriptional circuit established by ELF5 will be instrumental to derive human TS cell lines that truly reflect early placental trophoblast and that will be most beneficial to gain insights into the aetiology of common pregnancy complications, including intra-uterine growth restriction and pre-eclampsia.

INTRODUCTION

Intrauterine development depends on the proper differentiation and function of trophoblast cells that emerge as a distinct cell lineage in the first differentiation event after fertilization, forming the outer layer of the blastocyst. Trophoblast cells are essential to mediate implantation of the embryo into the uterus, and go on to form major components of the placenta that ensure normal growth and development of the embryo. Recent insights have substantially advanced our understanding of the early differentiation events that lead up to the establishment of the trophoblast and embryonic cell lineages (1). In this context, an important distinction has to be made between the processes implicated in specification of the first cell lineages and those involved in their stable propagation once they have been established (2).

Early cell lineage specification is achieved by a tight interplay between transcription factor cascades, cell position and

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polarization events as well as cellular signalling pathways (3). The unequivocal fixation of cell lineage fate occurs, at least in the mouse, at the late blastocyst stage when individual cells lose their developmental plasticity and become committed to either an embryonic or trophoblastic lineage fate even when displaced from their normal cell–cell context (4–8). Our recent work has established that this restriction of lineage potency is epigenetically controlled through DNA methylation of the transcription factor *Elf5*: *Elf5* is methylated and stably repressed in the embryonic lineage, but hypomethylated and expressed in the trophoblast lineage where it forms a positive feedback loop with the TS cell genes *Cdx2* and *Eomes*. Thus, ELF5 functions as a gatekeeper, downstream of initial lineage determination, to reinforce commitment to the trophoblast lineage or to abort this pathway in cells of the embryonic lineage (9).

The epigenetic restriction of cell lineage fate has important implications for the developmental potency of stem cells that can be derived from the early embryo. In the mouse, pluripotent embryonic stem (mES) cells have the capacity to differentiate into all cell types of the embryo proper, but are largely excluded from the trophoblast compartment unless genetically altered (10–12). Conversely, trophoblast stem (mTS) cells are restricted towards differentiation into all trophoblast cell types of the placenta, but they cannot contribute to embryonic tissues (13). This developmental restriction correlates with extensive methylation of the *Elf5* promoter in mES cells and *Elf5* hypomethylation in mTS cells (9). In contrast to the situation with mES cells, however, human ES (hES) cells have some potential to differentiate into the trophoblast lineage, both spontaneously and when enhanced by treatment with BMP4 (14,15). Trophoblast differentiation from hES cells involves a characteristic morphological change as the cells form flatter, epithelial-like colonies with single and multinucleated, syncytiotrophoblast-like cells. Pluripotency markers such as *OCT4* (*POU5F1*), *SOX2*, *FGF4* and *NANOG* are down-regulated and genes important for extraembryonic development, including *TCFAP2A*, *GCM1*, β-*HCG*, *CD9* and *HLA-G*, are increased, whereas other genes whose homologues are important for mouse trophoblast, such as *CK7*, *HASH2*, *ESRRB* and *MET*, are not elevated (14,15). The predominant cell type that emerges from the hES-to-trophoblast 'transdifferentiation' is the distinct, terminally differentiated syncytiotrophoblast that is positive for HLA-G and secretes β-HCG. However, despite an overall limited proliferation capacity, continuously dividing cytotrophoblast cell lines have been successfully derived from hES cells by repeated rounds of β-HCG selection (15).

The global gene expression profile and behaviour of hES cells has suggested that they represent a slightly advanced developmental stage when compared with their mES counterpart and are more akin to mouse epiblast-derived stem cells (epiSCs) (16,17). Although epiSCs are derived from embryonic (epiblast) tissue of post-implantation conceptuses, they do express genes that are important for trophoblast differentiation such as *Cdx2*, *Hand1* and *Eomes* when cultured in BMP4, and thus may resemble hES cells in this regard. However, the true trophoblastic characteristics of these cells remain to be determined as the genes assessed are also expressed in the post-implantation embryo itself and do not represent unequivocal markers of trophoblast differentiation. This point is further reinforced by the observation that epiSCs are excluded from contributing to trophoblast tissues in chimeras (17). Because of the paucity of genes with a truly trophoblast-restricted expression pattern that could serve as lineage markers, and the inability to test for trophoblast contribution of hES cells *in vivo* for ethical reasons, it is thus not clear to what extent hES cells have overcome the epigenetic lineage restriction and whether the trophoblast-like cells differentiated from them are fully characteristic of placental trophoblast.

Given that both the establishment of a tight epigenetic boundary between the embryonic and trophoblast lineages and the function of *Elf5* are critical for early mouse development (18–21), we sought to investigate the role and regulation of *ELF5* in the human placenta as well as in hES cells and derived trophoblast cell lines. We set out to determine whether or not *ELF5* serves to identify a TS cell compartment in the human placenta, and whether *ELF5* is epigenetically regulated and forms a lineage barrier similar to the situation in the mouse. Our results show that ELF5, EOMES and CDX2 form a mutually interacting core triumvirate of trophoblast transcription factors in the human placenta, and that ELF5, in conjunction with CDX2, may demarcate a TS cell population within the placental villous cytotrophoblast layer. We find that *ELF5* is unmethylated in early placental trophoblast, but almost fully methylated in hES cells, hES-derived trophoblast cells as well as induced pluripotent stem (iPS) cells and mouse epiSCs. These results indicate that the DNA methylation profile of *ELF5* retains its lineage-specific epigenetic signature, and that the trophoblast cells that differentiate from hES cells are not representative of a true placental TS cell population. Our data have important implications for the derivation of human TS cell lines that fully reflect the differentiative potency of trophoblast in the early human placenta. Such hTS cells would be extremely valuable to study gene function and mechanisms of early trophoblast differentiation at a developmental time where human material is not available, but that is critical in the aetiology of important trophoblast-based complications, including intra-uterine growth restriction and pre-eclampsia.

RESULTS

Expression and epigenetic regulation of *ELF5* in the human placenta

To test for *ELF5* expression in the human placenta, we first employed standard RT–PCR on placental villus samples from various gestational stages between 8 weeks of pregnancy and term. Unlike the mouse *Elf5* gene, the human *ELF5* locus harbours two alternative transcription start sites, one that aligns with the orthologous start site of the mouse gene and produces the *ELF5-2b* isoform (also termed ESE-2b, Ensembl ELF5_201), and the other within the first intron of *ELF5-2b* that gives rise to a longer variant *ELF5-2a* (ESE-2a, Ensembl ELF5_202). An additional splice isoform of *ELF5-2b* has recently been annotated that lacks the coding exons 3 and 4 (*ELF5-2b*Δex3/4, Ensembl ELF5_203) and thus the SAM/Pointed domain, a widespread domain in signalling and nuclear proteins, but it retains the *ets* consensus

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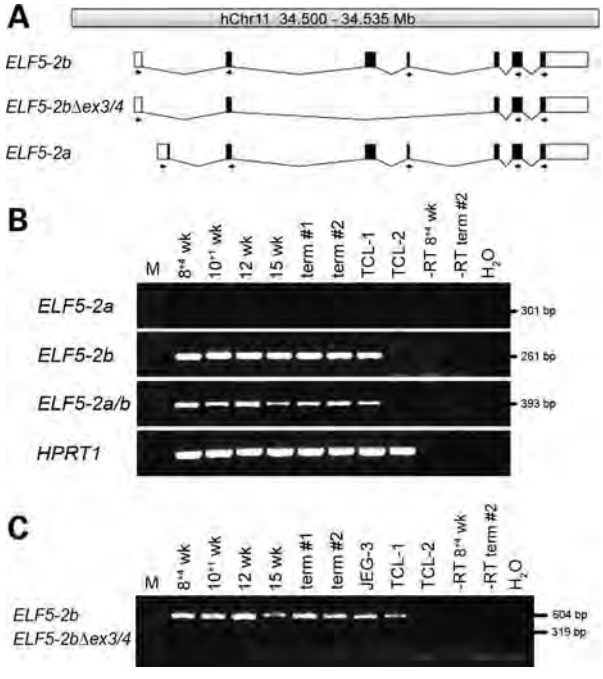

Figure 1. Genomic organization of the human *ELF5* locus and transcript isoform expression in placenta and trophoblast cell lines. (**A**) Diagram of the exon–intron structure of the human *ELF5* locus and annotated splice variants. Position of primers used is indicated. Filled boxes represent open-reading frames and open boxes represent untranslated regions. (**B**) RT–PCR analysis with isoform-specific and common primers reveals that *ELF5-2b* is the expressed splice variant in placenta and the trophoblast-like cell line TCL-1, but that it is absent from the first trimester mesenchymal-like cell line TCL-2. (**C**) RT–PCR with primers spanning exons 3 and 4 demonstrates that the annotated ELF5-2bΔex3/4 variant is not present in placenta and choriocarcinoma and trophoblast-like cell lines JEG-3 and TCL-1.

motif (Fig. 1A). To distinguish which of these isoforms are expressed in the human placenta, we generated common and isoform-specific primer pairs for *ELF5*. *ELF5* mRNA was readily detected throughout gestation in the human placenta with *ELF5-2b* as the major variant (Fig. 1B). *ELF5* was also expressed in the third trimester trophoblast-like cell line TCL-1 and the choriocarcinoma cell line JEG-3, but not in the first trimester mesenchyma-like cell line TCL-2 (Figs 1B and C, 2A, 6B–D). Further, we did not detect the shorter *ELF5-2b*Δex3/4 variant in any of the samples, such that the evolutionarily conserved *ELF5-2b* constitutes the relevant *ELF5* transcript in the human placenta (Fig. 1C).

Next, we investigated in more detail whether placental *ELF5* expression is regulated with gestational age. For this purpose, quantitative RT–PCR (qPCR) analysis was performed on an extended set of placental samples and revealed that *ELF5* was more strongly expressed in the first trimester and was down-regulated by 3–4-fold towards term (Fig. 2A–C). Since the lineage 'gatekeeper' function of *Elf5* is achieved by its tight epigenetic regulation in the mouse, we tested whether the activity state of human *ELF5* is also epigenetically controlled by DNA methylation. Bisulphite sequencing of the *ELF5* promoter between −400 bp and the transcription start site showed very little methylation in the first trimester (11%, most of which derived from one clone), whereas DNA methylation increased to 40–50% in second and third trimester samples (Fig. 2D). These levels of DNA methylation displayed a perfect inverse correlation with *ELF5* gene expression levels, indicating that the transcriptional activity of *ELF5* is epigenetically regulated and repressed by DNA methylation. This epigenetic regulation was also observed in the cell lines analyzed: the *ELF5* promoter region was highly methylated (79.5% in the upstream region) in TCL-2 cells in which *ELF5* is not expressed, but hypomethylated in JEG-3 cells (8% in the upstream region) that are positive for *ELF5* (Fig. 2E). We also tested additional commonly used trophoblast cell lines and found *ELF5* to be hypomethylated and expressed in BeWo and JAR cells, but hypermethylated (and repressed) in HTR-8/SVneo, IST-1 and SWAN-71 cells (Supplementary Material, Fig. S1). The TCL-1 cells that do express *ELF5*, albeit at low levels (Figs 1C and 6B–D), exhibited a particularly interesting, bipartite methylation pattern and prompted us to expand the analyzed region to include 400 bp downstream of the transcription start site. This analysis indicated that the five CpG dinucleotides surrounding the immediate transcriptional start site are critical for *ELF5* activity, and presumably contain transcription factor-binding sites that need to be unmethylated for binding and *ELF5* expression to occur (Fig. 2E).

Expression and localization of key TS cell factors in the human placenta

To correlate the expression pattern of *ELF5* with that of other transcription factors that are critical for trophoblast proliferation and TS cell self-renewal in the mouse, we investigated the expression of *CDX2* and *EOMES* in the same panel of human placental samples. While *EOMES* did not exhibit any obvious transcriptional regulation during pregnancy, an extremely clear-cut pattern was observed for *CDX2* that was expressed in the first trimester but rapidly down-regulated thereafter (Fig. 2A–C). *CDX2* was not detected at all in 15 week and later-stage samples, even when additional PCR cycles were performed (Fig. 2A and not shown). Thus placental *CDX2* expression is restricted to the first trimester and correlates with the developmental period of high *ELF5* expression levels.

Immunofluorescence staining revealed that placental ELF5 is predominantly localized to nuclei of villous cytotrophoblast cells (Fig. 3A and Supplementary Material, Fig. S2). Villous cytotrophoblasts are a proliferative cell population that continuously provides cells to fuse with the overlying post-mitotic syncytiotrophoblast layer, thereby expanding the syncytium. The cell-type-specific expression of ELF5 was confirmed with the pan-trophoblast marker cytokeratin 7 (CK7) and the villous cytotrophoblast cell marker SPINT1 [also known as HAI-1 (22)] that exhibited a perfect co-localization with ELF5 (Fig. 3B and C). Some, albeit considerably weaker, signals for ELF5 were also detected within nuclei of the mesenchymal villous core; in contrast to the prominent ELF5 staining in nuclei of villous cytotrophoblasts, however, the ELF5 distribution in mesenchymal cells appeared to be in a punctate, heterochromatin-associated pattern (not shown). In addition to villous cytotrophoblast, a proliferating cell population is also present at the proximal end of the cytotrophoblast cell columns. Daughter cells migrate distally along the column, and sequentially undergo epithelial–mesenchymal transition to differentiate into invasive extravillous trophoblast

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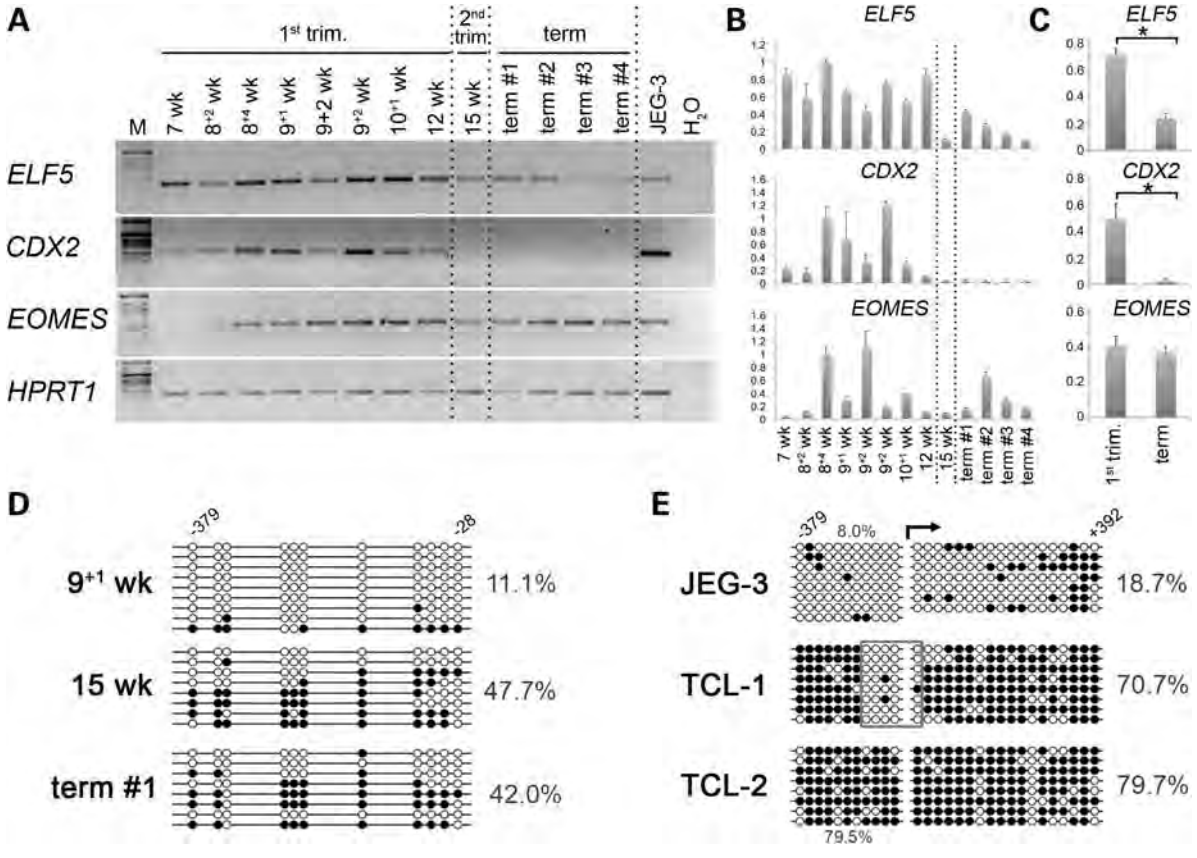

Figure 2. Expression of trophoblast stem cell genes and epigenetic regulation of *ELF5* in placenta throughout gestation. (**A**) RT–PCR analysis of *ELF5*, *CDX2* and *EOMES* (i.e. genes important for trophoblast stem cell self-renewal and proliferation in the mouse) on human placental villous samples ranging from 7 weeks of gestation to term. Four independent term placental samples were investigated. The choriocarcinoma cell line JEG-3 was included as control. Colour-inverted photographs of ethidium bromide stained gels are shown. All three genes are expressed in placenta, but *CDX2* is not detected from the second trimester onwards even when the PCRs are over-cycled. (**B**) Quantitative RT–PCR (qPCR) analysis of *ELF5*, *CDX2* and *EOMES* on the same samples used in (A). *ELF5* is down-regulated in second and third trimesters, whereas no overall regulation with gestational age was observed for *EOMES*. (**C**) Comparison of expression levels between first trimester and term. *ELF5* expression is significantly reduced at term when compared with first trimester, CDX2 is absent from term placentas. (**D**) Bisulphite sequencing analysis of the *ELF5* promoter region. Filled circles indicate methylated cytosine residues. *ELF5* is extremely hypomethylated in the first trimester and acquires higher DNA methylation levels in second and third trimester, correlating with transcriptional down-regulation at these stages. (**E**) DNA methylation analysis of an extended region between −400 bp and +400 bp around the transcriptional start site of *ELF5*. Hypomethylation correlates with *ELF5* expression in JEG-3 cells and, conversely, *ELF5* is hypermethylated and not expressed in TCL-2 cells. The methylation pattern in TCL-1 cells reveals a critical stretch of five CpG residues (grey box) at the immediate transcriptional start site that needs to be unmethylated for *ELF5* to be expressed.

(EVT) cells that penetrate into the underlying uterine stroma, either interstitially or inside maternal spiral arteries (23). ELF5 was detected in the cells at the proximal end of a column, but not further distally; ELF5 was also largely absent from the post-mitotic endovascular and interstitial EVTs within the decidual bed (Fig. 3D and E).

ELF5 expression can be induced by fibroblast growth factor (FGF) signalling in lung epithelial cells and in hypomethylated mES cells causing them to differentiate into trophoblast (9,24). This FGF → ELF5 pathway correlates well with the FGF4 dependence of mTS cells to maintain the stem cell state, and with the expression of the corresponding FGFR2 receptor on the surface of mouse trophoblasts. Notably, FGFR2 has also been detected in cytotrophoblasts of human placental villi (25). We therefore stained first trimester placental villus sections for ELF5 and FGFR2 to detect whether the presence of FGF receptor correlates with expression of ELF5. Indeed, we found that most ELF5-positive villous cytotrophoblasts also stained positive for FGFR2 (Fig. 4A and Supplementary Material, Fig. S3), thus corroborating the link between FGF/FGFR2 and ELF5 within an hTS cell-like compartment in the human placenta.

Because of the strict temporal restriction of *CDX2* expression to the first trimester, we reasoned that the hTS cell-like compartment could be further refined by the presence of this transcription factor. Using dual labelling for ELF5 and CDX2, we found that CDX2 is mostly confined to a subset of ELF5-positive cells within the villous cytotrophoblast layer and at the base of EVT columns (Fig. 4B and Supplementary Material, Fig. S4). Rare examples of villous cytotrophoblasts that expressed only CDX2 and no, or very little, ELF5 were also observed (Supplementary Material, Fig. S4). This pattern resembled that in the mouse where a small $CDX2^+$ population precedes a larger $CDX2^+/ELF5^+$ compartment. Strikingly, however, larger groups of CDX2-positive cells were only observed in 6–8 week placental samples. The number of $CDX2^+$ cells and their expression levels rapidly declined thereafter, and only extremely few, individual

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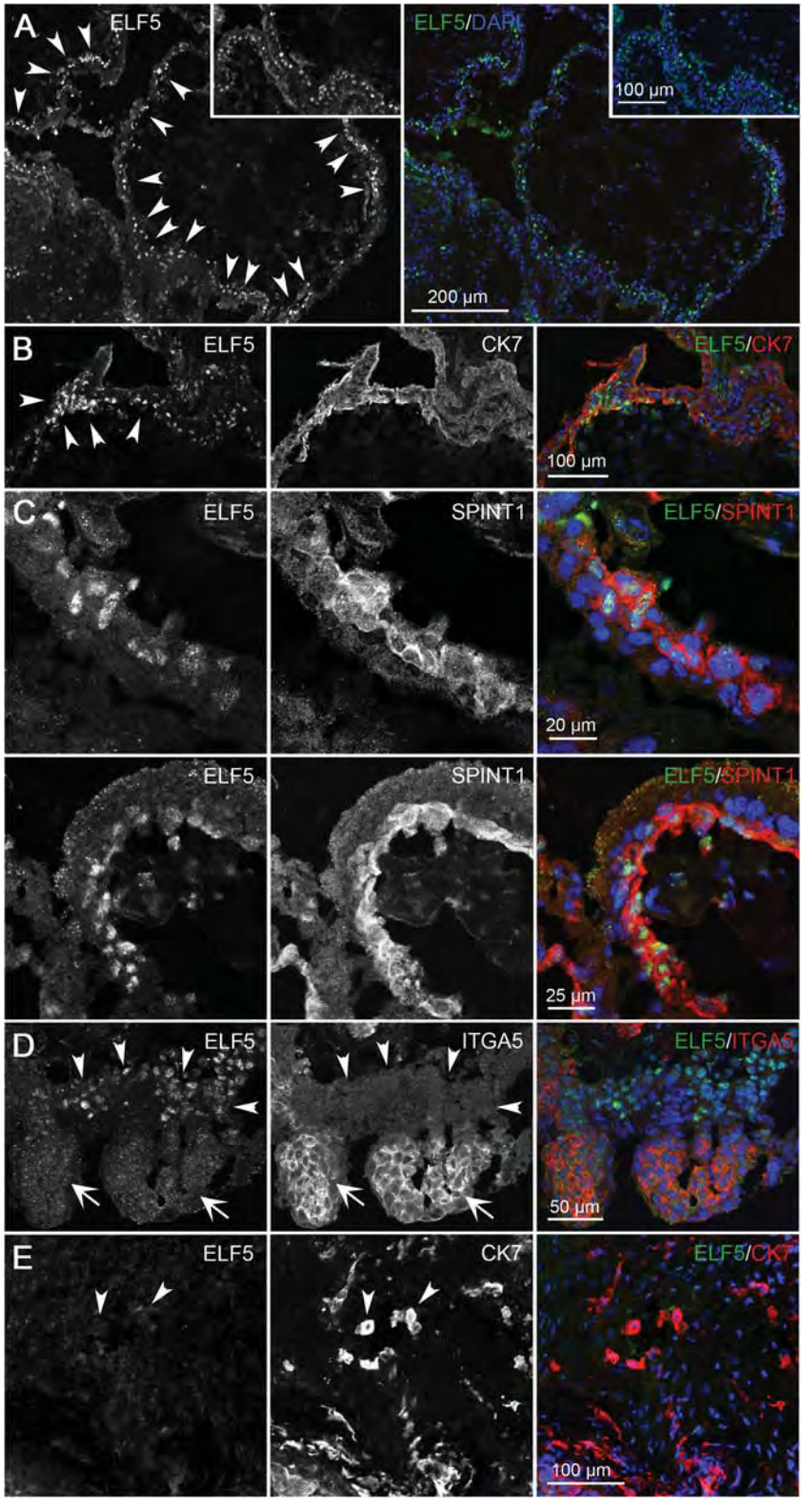

Figure 3. Immunofluorescence localization of ELF5 to cytotrophoblasts in the human placenta. (**A**) Overview of 11 week placental villous cross-section shows ELF5 localization to nuclei of villous cytotrophoblasts, but absence from nuclei of the overlying syncytiotrophoblast layer. Cytotrophoblasts are a proliferative cell population that continuously divide to replenish the overlying syncytium. (**B**) Co-localization with cytokeratin 7 (CK7) confirms the trophoblast identity of ELF5-positive cells. (**C**) Confocal image of a double staining of ELF5 and the villous cytotrophoblast marker SPINT1 (also known as HAI-1) shows that every ELF5-positive nucleus resides within the cytotrophoblast layer. Top row 6 week, bottom row 11 week placenta. (**D**) Confocal image analysis of an 11 week villous section stained for ELF5 and the extravillous cytotrophoblast (EVT) marker integrin alpha-5 (ITGA5). ELF5 is detected only in nuclei at the proliferative base, but not further distal along the EVT column where cells adopt an invasive phenotype and lose proliferative potential. (**E**) ELF5 is also absent from post-mitotic interstitial and endovascular EVTs within the decidual bed.

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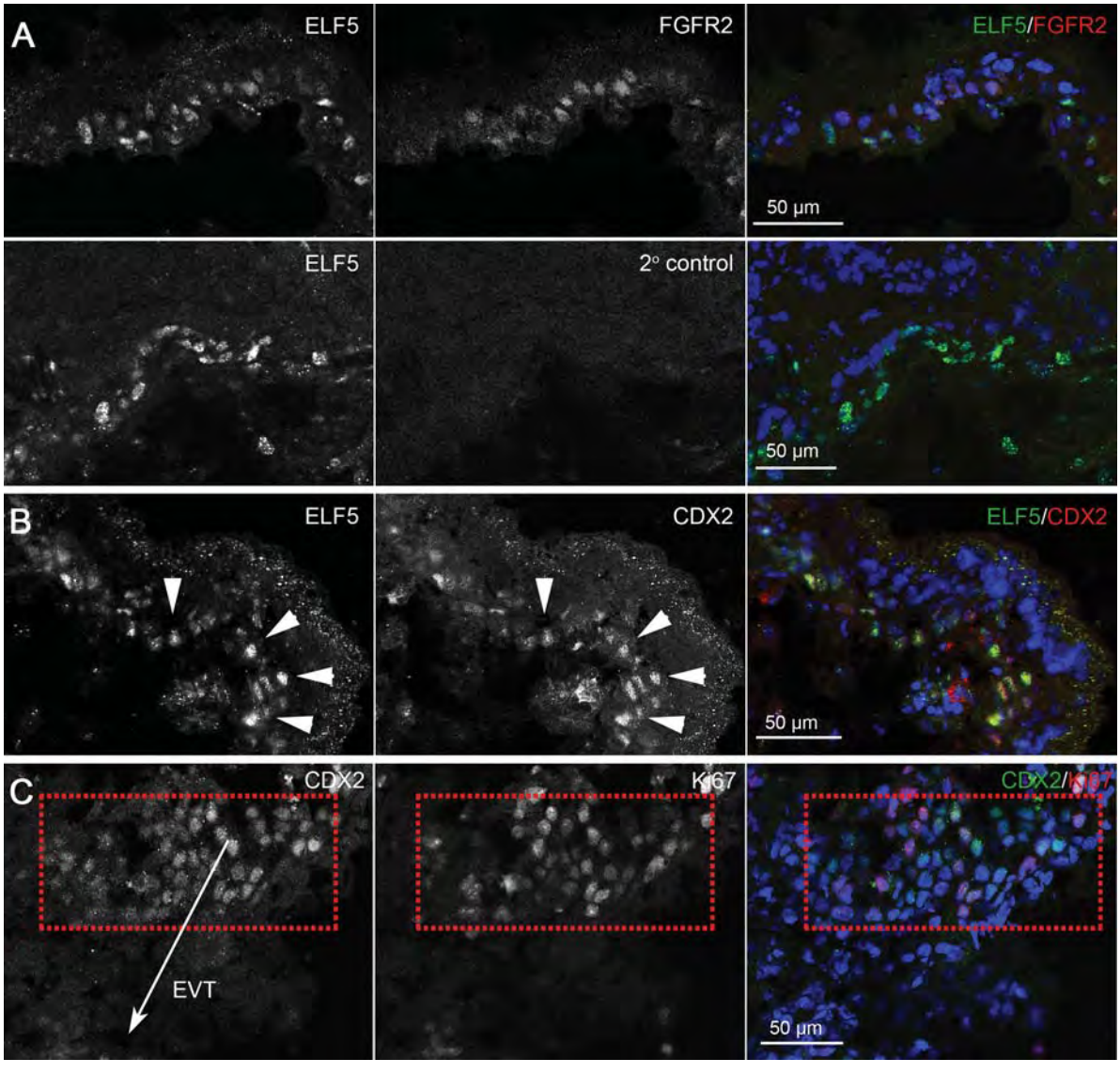

Figure 4. CDX2 identifies a subset of ELF5-positive cytotrophoblasts as a TS-like compartment that is regulated by FGFR2. (**A**) ELF5 co-localizes with FGFR2 in villous cytotrophoblasts as identified by confocal image analysis of double immunofluorescence stainings of 11 week placental sections. Since FGF signalling has been implicated in TS cell proliferation in mice and humans and can activate ELF5 expression in other tissues, FGF/FGFR2 may induce ELF5 expression within a putative TS cell niche in the human placenta. (**B**) Double staining of a 6 week placental section for ELF5 and CDX2. Larger groups of CDX2-positive cells are detected only in early gestation up to 8.5–9 weeks. CDX2 is mostly co-expressed with ELF5 (arrowheads). (**C**) Dual labelling of 6 week placental section for CDX2 and the proliferation marker Ki67. CDX2-expressing cytotrophoblasts preferentially stain positive for Ki67, indicating their high proliferation rate. CDX2 and Ki67 are restricted to the proximal end of cytotrophoblast cell columns (highlighted by the boxed area). The white arrows indicate the direction of progressive extravillous trophoblast (EVT) differentiation and migration.

$CDX2^+/ELF5^+$ double-positive villous cytotrophoblast cells were detected up to 13 weeks of gestation (Supplementary Material, Fig. S4).

We further reasoned that if this $CDX2^+/ELF5^+$ compartment represented an hTS cell-like population, these cells should be highly proliferative. In double labellings for CDX2 and Ki67, we observed that $CDX2^+$ trophoblasts preferentially stained positive for this proliferation marker (>55% of CDX2-expressing cells are $Ki67^+$ when compared with only 24% of CDX2-negative cytotrophoblasts, $P = 0.01$), indicative of the high mitotic activity of these cells (Fig. 4C and Supplementary Material, Fig. S3). Thus, the CDX2/ELF5-positive cytotrophoblast population may demarcate a proliferating, self-renewing hTS cell-like population in the human placenta.

Transcription factor circuits in human trophoblast

The observations that (i) *CDX2* expression correlates with high *ELF5* transcript levels in the first trimester, and that (ii) the CDX2 and ELF5 proteins are co-localized in a subset of villous cytotrophoblasts, suggested that CDX2 and ELF5 may co-activate each other. Because of the inaccessibility of first trimester placentas to obtain sufficient amounts of pure

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trophoblast material, we tested this hypothesis in chromatin immunoprecipitation (ChIP) experiments, first focussing on CDX2 binding to the *ELF5* promoter, using the *ELF5*-expressing JEG-3 and TCL-1 cell lines and the *ELF5*-negative TCL-2 cells as control. This analysis was further helped by the methylation profile of TCL-1 cells (Fig. 2E) that narrowed down the critical region for *ELF5* activation to 198 bp around its promoter containing a stretch of five unmethylated CpG dinucleotides. When scanned with transcription factor-binding motif search engines (TESS, Promo3.0), this sequence was indeed found to contain two conserved binding sites for caudal family homeodomain proteins like CDX2. In ChIP assays using CDX2 as the bait, the *ELF5* promoter was enriched in JEG-3 and TCL-1, but not in TCL-2 cells, indicating that CDX2 is bound to the *ELF5* promoter in cells where *ELF5* is unmethylated and expressed (Fig. 5A). The ability of CDX2 to bind to and activate the *ELF5* promoter was also supported by the correlation between higher *CDX2* and *ELF5* expression levels in JEG-3 cells when compared with TCL-1 cells (Fig. 6B and C). Thus, although CDX2 is not necessary for *ELF5* expression, it enhances its transcriptional activity.

Insights from the mouse have demonstrated that *Cdx2* and *Eomes* are genetically upstream of *Elf5*, but that ELF5 establishes a critical positive feedback loop to *Cdx2* and, in particular, *Eome*s to reinforce the activity of the trophoblast transcription factor circuit in a defined spatiotemporal window. To test whether such a cross-talk also exists in the human placenta, we performed anti-ELF5 ChIPs and assessed for enrichment of the *CDX2* and *EOMES* promoters. Our data showed that ELF5 binds to the *EOMES* promoter and also to the *CDX2* promoter, although with lower efficiency when adjusted to TCL-2 cells as the negative control (Fig. 5B). This low efficiency of ELF5 binding to the *CDX2* promoter is in line with the relatively small proportion of $CDX2^{+}$/$ELF5^{+}$ double-positive cells, and indicates that outside this presumptive stem cell niche the continuous activation of *CDX2* by ELF5 is interrupted by an as yet unknown factor or mechanism.

Cell lineage identity and *ELF5* regulation in stem cells

Having established that *ELF5* is epigenetically regulated and expressed in the human placenta and, together with CDX2, may demarcate an hTS cell population, an obvious question was how *ELF5* is regulated in hES cells and derived trophoblast cell lines. In a first step to address this point, we processed one hES cell line (Shef4) and a pool of two different hES-derived trophoblast cell lines (TrophShef4 and TrophH7) for bisulphite sequencing. Despite their capacity to differentiate into trophoblast, the *ELF5* promoter was fully methylated in hES cells, indicating that with regard to the *ELF5* epigenotype hES cells retain an embryonic lineage identity (Fig. 6A). Moreover, *ELF5* was also fully methylated in the hES-derived trophoblast sample (Fig. 6A).

A limited potential of trophoblast differentiation has been observed in all hES cell lines analysed to date, but different hES cell lines are known to vary in DNA methylation levels and can acquire epimutations during culture (26). Thus, to more thoroughly assess the epigenetic state of *ELF5*, and to correlate it with expression levels, we assessed six different

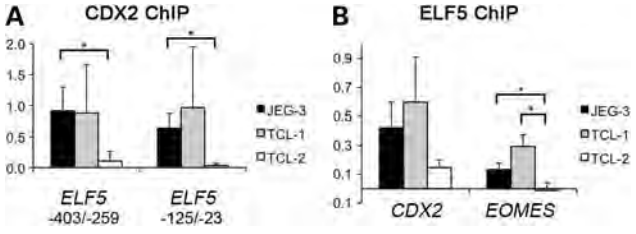

Figure 5. Inter-regulatory network of trophoblast transcription factors CDX2, EOMES and ELF5. (**A**) Chromatin immunoprecipitation assays show that CDX2 binds to the *ELF5* promoter region in JEG-3 and TCL-1 cells where *ELF5* is hypomethylated and expressed, but not in TCL-2 cells where *ELF5* is hypermethylated and repressed. (**B**) In turn, ELF5 binds to the *CDX2* and *EOMES* promoter regions in JEG-3 and TCL-1 cells where it is expressed, but not in TCL-2 cells from which it is absent, thereby establishing a transcriptional feedback loop between all three transcription factors. Binding to the *EOMES* promoter region was more consistent and is indicative of a more efficient, stronger interaction than with the *CDX2* upstream region, consistent with results observed in mouse trophoblast (9).

hES cell lines (Shef1, Shef4–7, H7), including one subclone with an abnormal karyotype (Shef5a) and two cytotrophoblast cell lines derived from them, TrophH7 and TrophShef4 (Fig. 6B–E). We compared these with the JEG-3, TCL-1 and TCL-2 cell lines and to an 8^{+4} week placental villus sample. In addition, a colorectal cancer cell line was included as positive control for *CDX2* (27). Five hES cell lines expressed some *ELF5* mRNA, albeit at extremely low levels that were >3.5-fold lower than in JEG-3 cells and 300-fold lower than in early placental tissue (Fig. 6B–D). In comparison, *CDX2* and *EOMES* transcripts were rather abundant in hES cells. Strikingly, however, all three trophoblast-associated transcription factors (*CDX2*, *EOMES* and *ELF5*) were completely absent from the hES-derived trophoblast cell lines (Fig. 6B and C). We chose three hES cell lines with different degrees of *ELF5* expression and both hES-derived trophoblast cell lines for bisulphite sequencing. In agreement with our initial analysis and corresponding to the very low abundance of *ELF5* transcripts, methylation levels at the *ELF5* promoter were high at >71% in all five samples with only minor and inconsequential differences between cell lines (Fig. 6E). Even in experiments designed to specifically enrich for unmethylated sequences, we could not detect any evidence for a putative small cell population that is hypomethylated at the *ELF5* promoter (Supplementary Material, Fig. S5).

hES cells have been shown to share more similarities with mouse epiSCs than with mES cells. We thus assessed *Elf5* methylation also in three independently derived epiSC lines and found that as in hES cells, the *Elf5* promoter is hypermethylated in this type of stem cell (Fig. 6F). Lastly, we assessed human iPS cells that are derived from somatic cells by transient overexpression of key pluripotency factors, namely c-MYC, KLF4, OCT4 and NANOG, which reprograms them into an hES-like state. We chose iPS cells derived from two different somatic cell types, fibroblasts and keratinocytes. Since *ELF5* is expressed in several epithelial cell types including skin (28), we reasoned that the gene locus may be more accessible to epigenetic reprogramming in keratinocytes when compared with other cell types. However, we found that *ELF5* was fully methylated in both iPS cell samples irrespective of the somatic cells' origin

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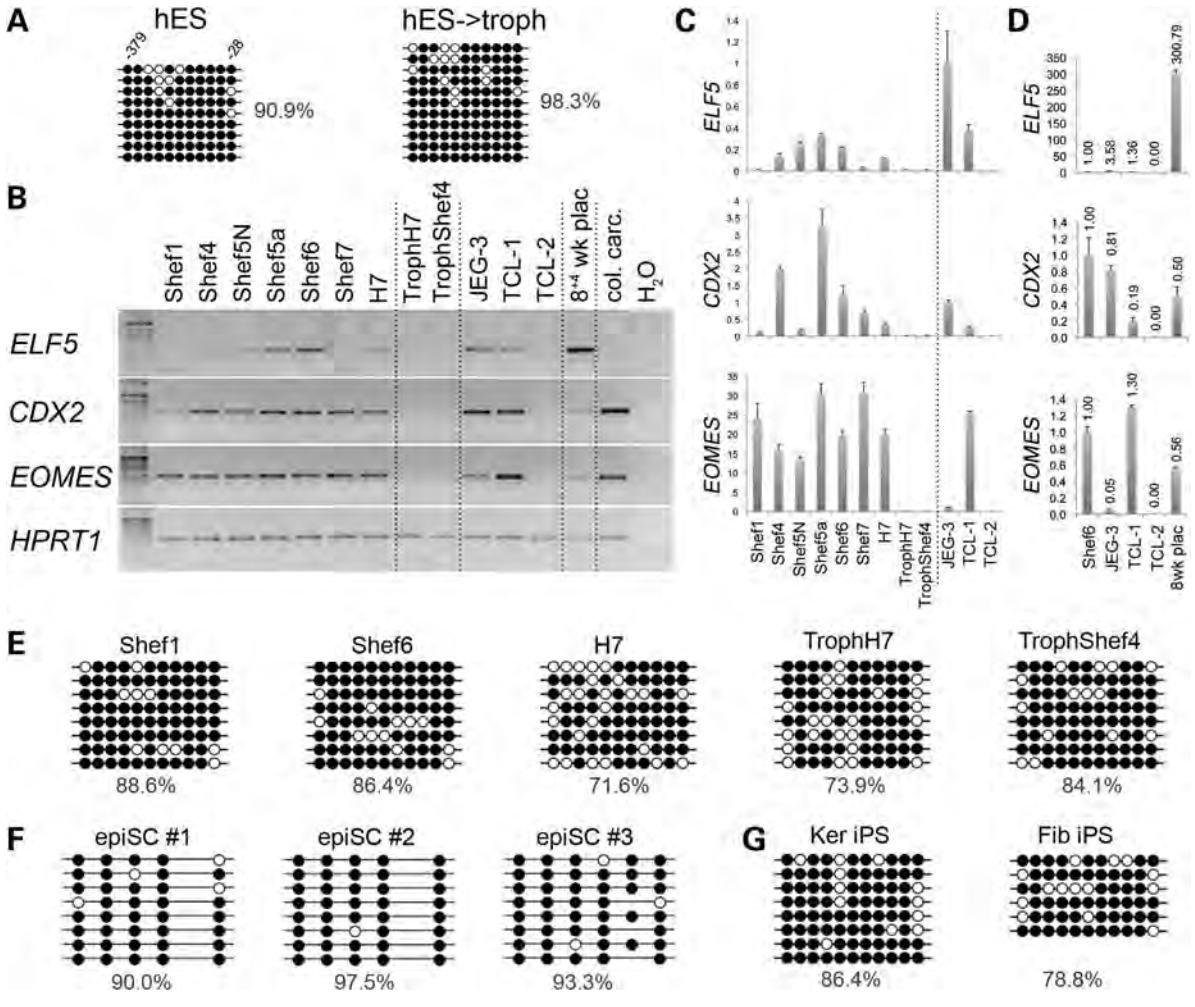

Figure 6. Trophoblast transcription factor expression and epigenetic regulation of ELF5 in human ES cells and derived trophoblast cell lines. (**A**) Initial bisulphite sequencing analysis of two pooled hES cell lines and derived trophoblast cells indicates a high degree of DNA methylation at the *ELF5* promoter despite the limited trophoblast differentiation potential. (**B**) RT–PCR and (**C**) qPCR analysis for trophoblast transcription factors *ELF5*, *CDX2* and *EOMES* on six different hES cells lines (Shef1, Shef4–7, H7), including one subclone with an abnormal karyotype (Shef5a), two derived cytotrophoblast cell lines (TrophH7 and TrophShef4), the JEG-3, TCL-1 and TCL-2 cell lines, an 8^{+4} week placenta for relative comparison of expression levels and a colorectal cancer cell line (DKO4) as positive control for *CDX2* expression (27). Colour-inverted photographs of ethidium bromide stained gels are shown. *ELF5* is detectable in some hES cell lines, albeit at very low levels. Higher expression levels of *CDX2* and *EOMES* may relate to their function within the embryonic lineage and is not directly indicative of trophoblast differentiation potential. Strikingly, in contrast to their expression in placenta, all three genes are absent from the hES-derived trophoblast cell lines. (**D**) Normalization of qPCR data to Shef6, one of the most highly *ELF5* expressing hES cell lines, in comparison with JEG-3, TCL-1 and TCL-2 cell lines as well as a first trimester placenta sample demonstrates the comparatively negligible amount of *ELF5* expression in hES cells that is approximately 300-fold less than in normal trophoblast *in vivo*. (**E**) Bisulphite sequencing analysis of the *ELF5* promoter in three different hES cell lines and two derived trophoblast cell lines shows relatively little epigenetic variability between different hES cell lines. Hypermethylation correlates with extremely low *ELF5* expression levels. (**F**) *Elf5* is also highly methylated in three independent mouse epiblast stem cell lines and (**G**) in two human-induced pluripotent stem cell lines derived from kereatinocytes and fibroblasts.

(Fig. 6G). Thus, hES cells, iPS cells and mouse epiSCs share the hypermethylated epigenetic state of *ELF5/Elf5*, and this methylation pattern is consistent with an embryonic lineage identity and inner cell mass origin of all three stem cell types. This pattern is in contrast to that in early placental trophoblast where *ELF5* is hypomethylated and expressed.

DISCUSSION

Here, we provide evidence for a conserved role of ELF5 in mice and humans as an epigenetically controlled lineage gatekeeper that provides the critical link in a TS cell-specific transcriptional circuitry. ELF5 is expressed in the human placenta throughout gestation, but is enriched in early gestation trophoblast samples, coinciding with the window of expression of CDX2. We also demonstrate that the TS cell factors *CDX2*, *EOMES* and *ELF5* establish a network of mutually interacting transcription factors akin to the pluripotency network in ES cells. This cross-talk establishes a TS cell compartment in the early human placenta that is characterized by a small number of CDX2 and ELF5 double-positive cytotrophoblast cells.

A key finding is the strict temporal and spatial restriction of CDX2 to relatively few cytotrophoblasts in the first trimester

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only. While ELF5 is able to activate *CDX2*, ELF5 alone is not sufficient for *CDX2* transcription. This interruption of the feedback loop is reflected by ELF5-positive, CDX2-negative cells in villous and EVT where expression of ELF5 is obviously disconnected from activating *CDX2*. At present it is not clear how this transition is regulated, but the exit from the presumptive $CDX2^{+}/ELF5^{+}$ stem cell niche to $CDX2^{-}/ELF5^{+}$ cells committed towards differentiation is again a conserved feature between both murine and human trophoblast (9). Possible mechanisms include the presence or the absence of additional factors that regulate TS cell proliferation, or post-translational modifications of individual transcription factors that may affect their function. Indeed, other transcription factors such as GATA3, ETS2 and TCFAP2c are important for the maintenance of the TS cell compartment in the mouse (29–33) and may be essential for human trophoblast (stem) cell proliferation as well (34–36). There is also evidence for the importance of post-translational modifications in regulating transcriptional networks. CDX2, for example, can be phosphorylated downstream of MAPK activation, and this modification targets the CDX2 protein for degradation in intestinal cells, thereby regulating its turnover (37). In fact, this mechanism could provide a neat auto-regulative control system to prevent trophoblast hyperproliferation. FGFR2 co-localizes with ELF5 and FGF signalling may be necessary for *ELF5* expression and thus for the establishment of the TS cell transcription factor circuit. At the same time, activation of this signalling cascade may prime CDX2 for degradation, and thereby restrict the TS cell self-renewal loop to a very limited cell population, which is precisely what we observe.

The small number of stem-like $CDX2^{+}/ELF5^{+}$ cytotrophoblasts may explain the notorious difficulty in deriving continuously proliferating trophoblast cell lines from the human placenta, and the inability to date to derive hTS cells from human blastocysts or early villous trophoblast. Our data suggest that the loss of proliferation and/or self-renewal is most likely due to the interruption of the positive transcriptional feedback loop among *CDX2*, *EOMES* and *ELF5*. Keeping this circuit active promises to be the key to the derivation of a self-renewing hTS cell line that fully recapitulates the differentiation potential of early trophoblast *in vivo*.

In addition to its role in TS cell maintenance, we also show that *ELF5* has an evolutionary conserved role in mice and humans as an epigenetically regulated gatekeeper to keep the embryonic and trophoblast lineages separate. Thus, *ELF5* is hypomethylated and expressed in trophoblast but hypermethylated and largely silent in cells of embryonic lineage origin. The very low *ELF5* transcript levels seen in some hES cell lines can be explained by epigenetic variability in a small fraction of cells in the hES cell population that may allow stochastic expression. The methylation state of *ELF5* is corroborated by recent genome-wide analyses of the human methylome by bisulphite sequencing and by immunoprecipitation and sequencing of methylated DNA, in which *ELF5* is hypermethylated in hES cells, lung fibroblasts and other somatic tissues but relatively hypomethylated in placenta (38,39). The unbiased detection of 5-methylcytosine residues by bisulphite sequencing further identified a large proportion of asymmetrical non-CpG methylation in hES cells but not in differentiated cell types (38). In this context, it is noteworthy that our bisulphite sequences of the *ELF5* promoter did not reveal any methylated cytosine residues outside the CpG context, and thus all DNA methylation in this region was confined to CpG dinucleotides. Methylation of *ELF5* in hES cells implies that the acquisition of this epigenetic mark occurs in cells of the inner cell mass at the blastocyst stage from which hES cells are derived. Our analysis of iPS cells also indicates that reprogramming of somatic cells by the four Yamanaka factors yields an hES-like state but does not proceed to reflect even earlier, pre-blastocyst developmental stages as judged by the epigenetic profile of *ELF5*.

Interestingly, *ELF5* methylation is also preserved in trophoblast cell lines that have been derived from hES cells by repeated rounds of β-HCG selection and culture conditions that promote TS cell self-renewal in the mouse (15). Thus this derivation procedure does not enrich for cells that contain a hypomethylated *ELF5* promoter, which would allow *ELF5* expression. The important conclusion from these results is that the hES-derived trophoblast cell lines are distinct from early human placental trophoblast where *ELF5* is unmethylated and expressed. Instead, hES-derived trophoblast-like cells may rather represent later stages of trophoblast differentiation. This view is supported by the fact that trophoblast differentiation from hES cells mostly results in post-mitotic syncytiotrophoblast cells, and derivation of cell lines often fails due to the low proliferative capacity of the emerging trophoblasts. Further, the lack of appreciable *ELF5* mRNA levels combined with the complete absence of *EOMES* [present study and (15)] and inconsistent *CDX2* expression [no expression in the present study, some expression reported previously in cell lines with high β-HCG levels (15)] is in line with the limited proliferative capacity of these cells and is indicative of a later developmental stage or an incomplete hES-to-trophoblast conversion. It is important to emphasize that expression of factors implicated in trophoblast differentiation alone is not proof of trophoblast conversion as CDX2, EOMES as well as ELF5 are also expressed in the embryo proper at later stages, and up-regulation of these genes may thus reflect differentiation within the embryonic lineage (40). Further, it has indeed been pointed out that the gene expression profile of hES-derived trophoblast cells only partially reflects that of endogenous placental trophoblast (14,15). Among the genes that are up-regulated is GATA3 that has recently been shown to induce formation of differentiated, post-mitotic trophoblast subtypes from mES cells (14,41). Thus the most likely scenario is that some stochastically expressed, BMP4-regulated transcription factors can induce a partial trophoblast differentiation programme in hES cells and activate some trophoblast-specific genes such as HLA-G and β-HCG. Critically, however, these cells do not undergo an epigenetic reprogramming to reflect the trophoblast lineage but retain their embryonic lineage-specific epigenetic signature at key loci such as *ELF5*. This is equally true for mouse epiSCs in which *Elf5* also remains highly methylated and that cannot form functional trophoblast derivatives *in vivo* (17).

The ability to derive hTS cells fully representative of the early trophoblast lineage, with self-renewing properties and the capacity to differentiate into all trophoblast subtypes of

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the mature placenta, will be essential for the study of early developmental processes in a developmental time window where human material is not available. The pathology of many, if not most, later-onset pregnancy-associated complications is believed to be based on trophoblast defects that occur much earlier in development, namely in the first trimester when trophoblast invasion and spiral artery remodelling lay the anatomical foundations to support fetal nutrition throughout the later gestational period. Our study provides insights into key factors and their epigenetic regulation that will help to establish 'true' hTS cell lines in the future. This will involve maintenance of the mutual activation of *CDX2* and *ELF5*, and protection of *ELF5* from *de novo* DNA methylation. Our study also highlights the intersection of autoregulatory control pathways that may be designed to prevent excessive trophoblast proliferation and trophoblastic tumour formation in the *in vivo* environment within the uterine bed.

MATERIALS AND METHODS

Human placental samples

Placental and decidual tissue samples were collected from normal first and early second trimester placentas using an ultrasound-guided chorionic villous sampling technique prior to surgical termination of pregnancy for psycho-social reasons, and from normal term pregnancies with informed written consent of the patients and permission from the Local Research Ethics Committees. Samples were either snap-frozen for RNA and DNA isolation, or natively embedded in cryoembedding medium for cryosectioning.

Cell lines

The JEG-3 choriocarcinoma cell line (42), term placenta trophoblast-like cell line TCL-1 (43,44) and first trimester mesenchymal-like cell line TCL-2 cells were grown in RPMI 1640 medium with glutamine (Invitrogen) containing 20% fetal bovine serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 50 U penicillin and 50 μg/ml streptomycin (Invitrogen). Human ES cell lines were derived under appropriate ethical and patient consent according to local and national guidelines (45), and are summarized in Aflatoonian *et al*. (46). hES-derived cytotrophoblast cell lines were isolated by repeated β-HCG selection and grown in conditions as described for mouse TS cell maintenance consisting of 20% fetal bovine serum in RPMI 1640 containing glutamine, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 50 U penicillin, 50 μg/ml streptomycin, 25 ng/ml bFGF (Sigma) and 1 μg/ml Heparin with 70% of the medium being pre-conditioned on mouse embryonic fibroblasts (13,15). Murine epiblast stem cells were grown under standard conditions in DMEM-F12 (Invitrogen), 20% knockout serum replacement (KSR), 5 ng/ml FGF2 (R&D Systems), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM L-glutamine and non-essential amino acids (both from Invitrogen) (17).

Expression analysis

Total RNA was isolated from placental samples using Trizol® reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription (RT) was carried out with 2 μg of total RNA and 500 ng oligo $d(T)_{15}$ primers (Promega) in the presence of 200 U RevertAid H-minus M-MuLV (MBI Fermentas). An aliquot of 0.25–1 μl of cDNA was used for standard RT–PCR. For qPCR analysis, cDNA was diluted 1:40 and 5 μl used per reaction. qPCRs were performed at least in triplicate for each sample with SYBR Green Jump Start *Taq* Ready Mix (Sigma) on a Bio-Rad CFX96 real-time thermal cycler. Data were normalized to *GAPDH* and *HPRT1* yielding similar results. For PCR conditions and primers, see Supplementary Material.

Bisulphite DNA sequencing

An amount of 1–2 μg of genomic DNA was processed for bisulphite conversion using the EpiTect Bisulfite Kit (Qiagen) following the manufacturer's instructions. Ten per cent of the eluted DNA was used for PCR amplification of the −432/−3 bp and +6/+427 bp regions around the ELF5-2b transcriptional start site, spanning all 29 CpG dinucleotides in this sequence stretch. PCR products were cloned into the pGEM-T Easy Vector System (Promega) and sequenced.

Immunostaining

Cryosections of placental villi from a total of 18 samples between 6 and 17 weeks of gestation and of decidual biopsies from three samples between 8 and 11 weeks of gestation were cut at 15 μm and fixed with ice-cold methanol/acetone for 10 min. Tissues were blocked with phosphate-buffered saline, 0.5% bovine serum albumin (Sigma), 0.1% Tween-20 and normal serum depending on the antibody used. Antibodies and dilutions were: anti-ELF5 1:100 (Santa Cruz Biotechnology), anti-SPINT1 1:100 (Santa Cruz Biotechnology), anti-ITGA5 1:200 (Santa Cruz Biotechnology), anti-Ki67 1:200 (Millipore), anti-CK7 1:200 (DAKO), anti-FGFR2 1:200 (Santa Cruz Biotechnology) and anti-CDX2 1:100 (BioGenex). Incubations were done for several hours at room temperature or at 4°C overnight for CDX2 and ELF5. Detection was carried out with Alexa fluorophor-conjugated secondary antibodies diluted 1:500. Nuclear counterstaining was performed with 4′,6-diamidino-2-phenylindole dihydrochloride or bis-benzimide (both from Sigma). Images were taken at an Olympus BX41 epifluorescence microscope and a Zeiss 510 Meta confocal microscope at optimal pinhole/optical thickness settings.

Chip assays

Cells from three to four T175 flasks were trypsinized and formaldehyde cross linked according to a standard protocol (47). Cross-linked chromatin was sonicated to a fragment size of <1 kb. For each immunoprecipitation reaction, 50 μg of chromatin was pre-cleared and incubated overnight at 4°C with 5 μg of anti-ELF5 antibody (Santa Cruz Biotechnology), anti-CDX2 antibody (BioGenex) or control antibody bound to Protein G sepharose beads (Amersham). Bound, unbound and input fractions were analysed by qPCR for *ELF5*, *CDX2* and *EOMES* promoter regions, and normalized against mock

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control. ChIPs were performed at least in triplicate from independent samples.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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