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

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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
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 altered (10–12). Conversely, trophoblast stem (mTS) cells are multipotent and can be derived from the early embryo. In the mouse, pluripotent TS cells have some potential to differentiate into the trophoblast lineage, 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 (POUSF1), 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 differential 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 (ELF5-2bΔex3/4, Ensembl ELF5_203) and thus the SAM/Pointed domain, a widespread domain in signalling and nuclear proteins, but it retains the ets consensus
that formed on an extended set of placental samples and revealed purpose, quantitative RT–PCR (qPCR) analysis was performed to determine ELF5 expression is regulated with gestational age. For this analysis, DNA methylation increased to 40–50% in second 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 syncytiotrophoblast. 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 cytotrophoblasts, 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 trophoblasts.
(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

**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.
Figure 3. Immunofluorescence localization of ELF5 to cytотrophoblasts in the human placenta. (A) Overview of 11 week placental villous cross-section shows ELF5 localization to nuclei of villous cytотrophoblasts, but absence from nuclei of the overlying syncytiotrophoblast layer. Cytотrophoblasts 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 cytотrophoblast marker SPINT1 (also known as HAI-1) shows that every ELF5-positive nucleus resides within the cytотrophoblast 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 cytотrophoblast (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 decidua bed.
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
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, Eomes 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, and found that as in hES cells, the 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).

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 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 8th 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 >10-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 epicSCs than with mES cells. We thus assessed Elf5 methylation also in three independently derived epicSC 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 iPSCs 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 iPSC 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 iPSC cell samples irrespective of the somatic cells’ origin.
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 trophoblast cells.

A key finding is the strict temporal and spatial restriction of CDX2 to relatively few cytотrophoblasts in the first trimester.
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+ 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 TS 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
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 auto-regulatory 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 national and local guidelines (45), and are summarized in Aflatoonian et al. (46). hES-derived cytrophoblast 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
control. ChIPs were performed at least in triplicate from independent samples.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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