



Roles of CDX2 and EOMES in human induced trophoblast progenitor cells

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ABSTRACT

Abnormal trophoblast lineage proliferation and differentiation in early pregnancy have been associated with the pathogenesis of placenta diseases of pregnancy. However, there is still a gap in understanding the molecular mechanisms of early placental development due to the limited primary trophoblast cultures and fidelity of immortalized trophoblast lines. Trophoblast stem (TS) cells, an *in vitro* model of trophoblast that can differentiate into syncytiotrophoblasts and extravillous trophoblasts, can be an attractive tool for early pregnancy research. TS cells are well established in mouse but not in humans due to insufficient knowledge of which trophoblast lineage-specific transcription factors are involved in human trophoblast (TE) proliferation and differentiation. Here, we applied induced pluripotent stem cell technique to investigate the human trophoblast lineage-specific transcription factors. We established human induced trophoblast progenitor (iTP) cells by direct reprogramming the fibroblasts with a pool of mouse trophoblast lineage-specific transcription factors consisting of *CDX2*, *EOMES*, and *ELF5*. The human iTP cells exhibit epithelial morphology and can be maintained *in vitro* for more than 2 months. Gene expression profile of these cells was tightly clustered with human trophoblast but not with human neuron progenitor cells, mesenchymal stem cells, or endoderm cells. These cells are capable of differentiating into cells with an invasive capacity, suggesting extravillous trophoblasts. They also form multi-nucleated cells which secrete human chorionic gonadotropin and estradiol, consistent with a syncytiotrophoblast phenotype. Our results provide the evidence that transcription factors *CDX2* and *EOMES* may play critical roles in human iTP cell generation.

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1. Introduction

It is well accepted that aberrant trophoblast proliferation and differentiation are two of the major causes of placenta-associated diseases, but the pathogenesis of these diseases are still largely unknown. The molecular mechanisms of human trophoblast lineage proliferation and differentiation are difficult to study due to the existing ethical (use of human embryos) and practical (use of <6-week placenta) issues. Trophoblast stem (TS) cells, which represent trophoblast (TE) *in vivo*, can be a useful tool for the study of trophoblast lineage proliferation and differentiation *in vitro* [1]. However, unlike mouse TS cells which are well established and extensively studied, established human TS cell line does not exist. Numerous studies have been attempted to use human embryonic stem (ES) cells or 1st trimester placenta (8–12 week) to generate human TS cells [2–10]. Other studies have focused on analyzing transcriptomes between human inner cell mass (ICM) and TE or differentiation of human ES cells into trophoblasts over time in or-

der to identify the transcription factors involved in human trophoblast lineage commitment and differentiation [11–16]. It has been shown that mouse TS cells and human TE share similar lineage transcription factors. However, applying similar culture conditions which are effective in mouse ES cells/blastocysts differentiation into TS cells are ineffective for human ES cells, indicating the existence of different transcription factor loops/pathways between humans and mice. Thus, there is an urgent need to identify human trophoblast lineage-specific transcription factors and generate viable human TS cell lines to advance reproductive research.

Induced pluripotent stem (iPS) cell technique is the direct reprogramming of fibroblasts into various cell types via transduction with different groups of lineage-specific transcription factors [17]. iPS technique shows promise in clinical applications; for example, dopaminergic neurons, cardiac cells, and hematopoietic cells have been successfully generated directly from fibroblasts using this technique [18–20]. iPS technique has also been proven to be a useful tool to investigate the biofunction of transcription factors; over-expression of *POU5F1* in mouse TS cells can lead to generation of mouse ES cells, suggesting *POU5F1* as a critical transcription factor in ES cells [21]. A similar study identified three transcription factors as a group of critical loop for induction of

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human cardiomyocytes [19]. Therefore, it is rational to use this strategy to examine the transcription factors required for establishing human trophoblast cells directly from the fibroblasts. In this study, we transduced the well-documented mouse trophoblast lineage-specific transcription factors: caudal-type homeobox transcription factor 2 (*CDX2*) [22–24], eomesodermin (*EOMES*) [25,26], and E74-like factor 5 (*ELF5*) [27–29], which are known to maintain mouse TS cells pluripotency and lineage specificity as a loop. *CDX2*, *EOMES*, and *ELF5* are also found expressed in human 1st trimester placental trophoblast [2,10]. Additionally, we included two oncogenes: MYC avian myelocytomatosis viral oncogene homolog c (*C-MYC*) and Kruppel-like factor 4 (*KLF4*), which are essential for cell proliferation and transformation [17]. The iTP cells generated in this study represent a useful tool for the study human trophoblast lineage-specific transcription factor biofunction.

2. Materials and methods

2.1. Generation of human induced trophoblast progenitor (iTP) cells

Human lenti-virus constructs for *CDX2*, *ELF5*, *C-MYC*, *KLF4* (Open Biosystems) and *EOMES* (home-made) were used to generate iTP cells from human fetal fibroblasts (IMR90, ATCC). 1.25×10^5 fibroblasts were transduced for 24 h in a mixture of 5 viral genes with fibroblast medium: DMEM supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% non-essential amino acids (NEAA), 1% penicillin, and 1% streptomycin. After 96 h, cells were passaged onto in-activated CF-1 mouse embryonic fibroblast (Millipore) in fibroblast medium which was replaced with human iTP medium (mouse TS medium [1] modified as follows): RPMI1640 supplemented with 20% FBS, 1% L-Glutamine, 1% sodium pyruvate, 0.5% penicillin, 0.5% streptomycin, 0.1 mM beta mercaptoethanol (2-ME), 5 ng/ml Activin A, 2 ng/ml Transforming Growth Factor beta-1 (TGFβ-1), 1 μg/ml heparin, and 25 ng/ml Fibroblast Growth Factor-4 (FGF-4). iTP colonies were manually picked approximately 25–30 days after viral transduction and mechanically passaged every 5–7 days. Two iTP colonies which passaged over 2 months were chosen for further analysis.

2.2. Differentiation of iTP cells

iTP cells were cultured in human iTP medium (–FGF4, –Activin A, –TGFβ1 and –heparin) in the presence of 1.0 μM cAMP (Sigma) for 7 days. The media was refreshed every other day and cells were analyzed on day 7.

2.3. ELISA assay

Supernatant of day-7 differentiated iTP cell culture was collected to detect hCG and estradiol using ELISA assays (R&D), following manufacturer's instructions. The spectral readouts were performed at 490–560 nm and each sample was analyzed in duplicates.

2.4. Invasion assay

2×10^4 iTP cells were seeded above transwell chambers with or without Matrigel-coating (BD Biosciences.). Fibroblasts (IMR-90) were used as a control. The medium used in upper well was: RPMI1640 with 0.1% BSA, 1% sodium pyruvate, 1% L-glutamine, and 0.1 mM 2-ME while in bottom well: RPMI1640 with 20% FBS, 1% sodium pyruvate, 1% L-glutamine, and 0.1 mM 2-ME. 20–22 h later, cells were stained with 0.09% Crystal violet solution. Non-invading cells on the upside of the transwell were carefully removed by cotton swab. Cells on the lower surface of the membrane

were then counting the number of invaded cells (5 fields) and photographed (Nikon software).

2.5. qRT-PCR (quantitative real-time PCR)

Total RNA was isolated with PicoPure RNA isolation kit (Molecular Devices) and first strand cDNA synthesis was performed with Superscript II (Invitrogen). qRT-PCR was performed using SYBR green PCR master Mix (Applied Biosystems). Gene names and the primers were summarized in Table S1.

2.6. Immuno-histochemistry

Cells were fixed 10 min in 4.0% paraformaldehyde, permeabilized with 0.3% Triton X-100 in PBS for 15 mins, and then blocked in 0.1% triton X-100 with 5% BSA for 30 min. Following primary antibody incubation for 2 h at room temperature (or 4 °C overnight), cells were then incubated with secondary antibodies (Alexa Fluors, invitrogen) for 45 min and nuclei counterstained with Hoechst33342 (Sigma). Imaging and analysis were performed using a Nikon 2000 inverted microscopy and imaging system (CRI). Primary antibodies used in this study were CK7 (1:100, Dako) and HLA-G (1:50, Abcam).

2.7. Gene expression microarray and analysis

To investigate the dynamic transcriptome changes during the transition from fibroblast to iTP cells, we compared total RNA from iTP cells with control fibroblasts using the human genome 4×44 K gene expression chip (Agilent Technologies, CA) according to manufacturer's instructions and primary data were collected from an Agilent Scanner. Probe intensities were extracted using Agilent Feature Extraction Software (v9.5). The signals of each array were normalized by median centering of ratios with fibroblast as a reference. Approximately 25,700 probes could be detected and were included in the analysis. All the statistical calculations were performed on logarithmic values of signals to the base 2. Statistical significance of differentially-expressed genes between iTP1 or iTP2 versus fibroblast was evaluated by two-tailed one-sample *t*-tests. Global expression profiles of fibroblast and iTP cell lines were examined using hierarchical clustering analysis by Cluster 3.0 (<http://www.falw.vu/~huik/cluster.htm>).

To compare the transcriptomes of iTP (based on Agilent expression chip) with previously published TE database studies based on the Affimetrix Human Genome U133 Plus 2.0 (GSM706172, GSM706171, GSM706170, GSM706169, GSM706168), neuron progenitor cells (NPC) (GSM335938, GSM335941), endoderm cells Endo (GSM1032057) and mesenchymal stem cells (MSC) (GSM878100, GSM878101), we included the IMR90 cells in both Agilent (our data) and Affimetrix (GSM713542, GSM713543) expression microarrays in the comparison as background. We then used GeneSpring software (Agilent Technologies) to pick up the “present” gene expression in all checked cell type in both Agilent and Affimetrix arrays. Affimetrix raw data (.CEL file) and Agilent raw data (.TXT) was loaded into software, background subtracted, and the signals were normalized by MAS5 or percentile shift method, respectively. After transferring probe-based data into gene-based data by “none” in Affimetrix and “quantile” method in Agilent data, we did the cross-section of “present” gene in each cells as the “common” expressed gene list ($n = 3711$). Then, log fold change of “common gene” in each cell type vs. IMR90 in their own array type were used to demonstrate the similarity among cell types by hierarchical clustering analysis using Cluster 3.0 software.

2.8. Statistical analysis

We used Student's *t*-test to compare relative expression of genes in iTP1, iTP2 and IMR90 (donor fibroblasts). *P* < 0.05 is taken as statistically significant.

3. Results

3.1. Characterization of human iTP cell lines

Human iTP cell lines were successfully established from human fetal fibroblasts (IMR90) by lenti-viral transduction with a pool of transcription factors (*CDX2*, *EOMES*, *ELF5*, *KLF4*, and *c-MYC*) (Fig. 1A and B). Four iTP cell lines showed similar growth and morphological characteristics and two (iTP1 and iTP2) were further characterized in detail. 25–30 days after initial transduction, the donor fibroblasts were reprogrammed into iTP cells which formed distinct colonies composed of small round cells with increased nuclear/cytoplasm ratio. The iTP cell lines maintained normal karyotyping throughout the lenti-viral transduction and differentiation process (Fig. S1). Quantitative real time PCR showed highly-expressed endogenous *CDX2*, *EOMES*, and *ELF5*, with expression of transduced viral transcripts *CDX2* slightly detected as well (Fig. 2A and B). Compared with donor fibroblasts, iTP cell lines also showed significantly high expression of several mouse TS cell genes such as GATA binding protein 3 (*GATA3*) and tea domain family member 4 (*TEAD4*), as well as pluripotent stem cell genes such as forkhead box D3 (*FOXD3*) and telomerase reverse transcriptase (*TERT*) (Fig. 2A). The cells were also positively stained for CK7, a known trophoblast marker [30] (Fig. 1C).

3.2. Epigenetic signature of iTP cells

Since *ELF5* is unmethylated in mouse TS cells, we sought to investigate the epigenetic regulation of *ELF5* in iTP cells. Our results show that *ELF5* has similar hypermethylation patterns in donor fibroblast IMR90 and iTP cells (Fig. S2).

3.3. Transcriptome changes from transition of fibroblasts to iTP cells

We performed global gene expression analysis by microarray and identified significant differentially-expressed genes between fibroblasts and iTP cells. Cluster analysis revealed that our two iTP cell lines formed the same cluster with each other while were a sub-cluster from donor fibroblasts (Fig. 3A). Selected highly-expressed trophoblast lineage genes are listed (Fig. 3B). Our transcriptome analysis shows that human iTP cells is more tightly clustered with human TE than NPC, MSC, or Endo cells (Fig. 3C), indicating iTP cells are in the trophoblast lineage.

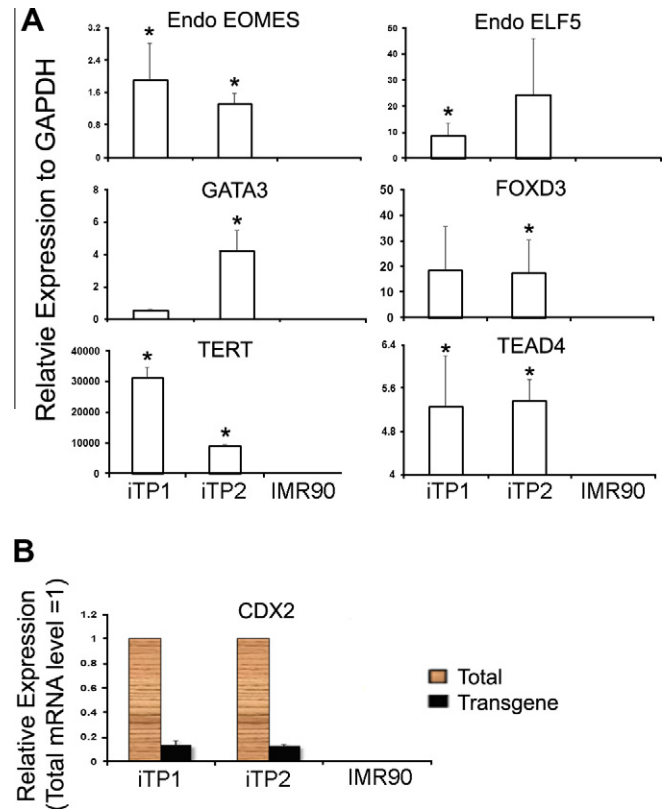


Fig. 2. iTP cells express stemness and trophoblast lineage transcripts. (A) Endogenous *EOMES* (*P* < 0.05) and *ELF5* were highly-expressed in the iTP cells. iTP cells expressed pluripotent stem cell-associated transcripts: *FOXD3* and *TERT* (*P* < 0.05) and mouse TS cell-associated transcripts: *TEAD4* (*P* < 0.05) and *GATA3*. The expression level relative to fibroblast = 1. (B) *CDX2* was highly-expressed while viral *CDX2* is slightly detectable in iTP cells.

3.4. Trophoblast cells differentiation from iTP cells

To determine whether iTP cells are functionally similar to human trophoblast progenitor cells, we evaluated the ability of iTP1 cells to differentiate into trophoblast cell subtypes: invasive trophoblasts and syncytiotrophoblasts. After 7 days in differentiation culture, multi-nucleated cells were formed (Fig. 4A) and the secretion of estradiol and hCG, two placenta-specific hormones, could be detected in the supernatant (Fig. 4B), suggesting formation of syncytiotrophoblasts. iTP1 cells were also grown on Matrigel-coated transwell (BD) to assess their invasive capacity [31]. We found a higher percentage of invasion in iTP1 cells vs. control fibroblasts (44.0% vs. 26.1% at 20 h, *n* = 3) (Fig. 4C), with invading cells staining positively for HLA-G (invasive trophoblast marker) (Fig. 4D), suggesting the formation of extravillous trophoblasts.

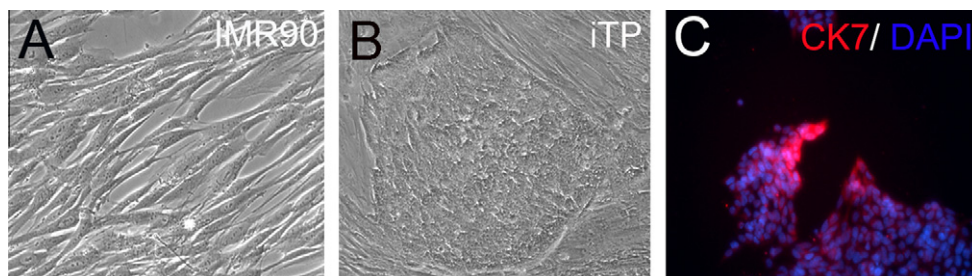


Fig. 1. Induced trophoblast progenitor (iTP) cells established from human fibroblasts. (A) Human fetal fibroblasts (IMR90). (B) iTP1 cell line generated from IMR90 after transduction with 5 viral genes (*CDX2*, *EOMES*, *ELF5*, *c-MYC* and *KLF4*). (C) Expression of CK7 in iTP cells. The original picture is 200×.

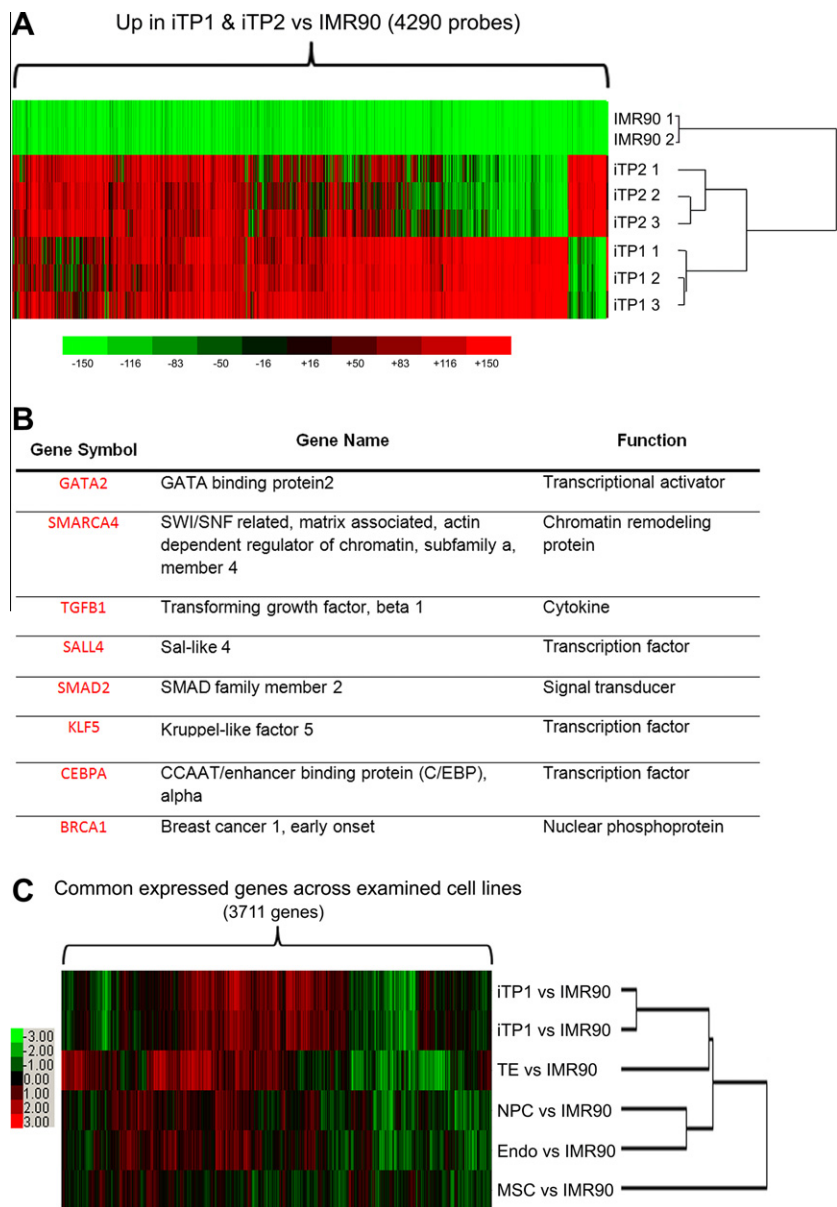


Fig. 3. Transcriptome profiling of iTP cells. (A) Heat map of upregulated 4290 probes with 2-fold change or more ($P < 0.05$) in replicates of iTP1 or iTP2 vs. fibroblasts (IMR90). iTP1 cells and iTP2 cells are tightly clustered when compared with fibroblasts. (B) Eight TS cell-related genes are highly-expressed in iTP cells. (C) Heat map of 3721 transcripts which commonly exist across all examined cells showed that iTP1 cells and iTP2 cells clustered more tightly with human trophoctoderms (TE) as compared with human neuron progenitor cells (NPC), mesenchymal stem cells (MSC) or endoderm cells (Endo).

4. Discussion

In this study, we generated human iTP cells to elucidate the trophoblast lineage-specific transcription factors required for human trophoblast lineage proliferation and differentiation. Here, we used 3 transcription factors (*CDX2*, *EOMES*, and *ELF5*) which were previously shown to form a transcription loop in mouse trophoblast lineage [32,33]. *CDX2* is critical for generating mouse TS cells; mouse ES cells could be transformed into TS cells solely by over-expressing *CDX2* [23,34] whereas *CDX2* null blastocysts failed to implant due to aberrant trophoblast function [24]. Further, it has been shown that *CDX2* null embryos were arrested during the early blastocyst stage, indicating that *CDX2* also plays a critical role in TE formation [22]. Similarly, *EOMES* was also previously shown to be required for mouse TS cell line maintenance [25] and *EOMES* null blastocysts displayed defective trophoblast differentiation [26].

ELF5 is hypomethylated in both mouse inner cell mass and TE cells [35] as well as TS cells, and is highly-expressed in mouse TS cells, suggesting *ELF5* as a trophoblast lineage gate keeper [27]. In addition, previous studies have shown that mouse ES cells, in the presence of bFGF growth factor, could be transformed into epiblast stem cells, which is equivalent to human ES cells in terms of morphology, transcriptome, epigenetic, and pluripotent biofunction [36,37]. Therefore, we applied this same strategy to culture human iTP cell line in mouse TS medium. To our knowledge, this is the first time that *CDX2* and *EOMES* have been shown to play important bio-functional roles in human trophoblast lineage.

This study shows that our iTP cells express high levels of endogenous *CDX2* and *EOMES*, which is consistent with data obtained from mouse TS cells. Since the role of *ELF5* in human placental trophoblast is unclear [2,10], we examined *ELF5* DNA methylation in our iTP cells and found similar hypermethylation pattern with do-

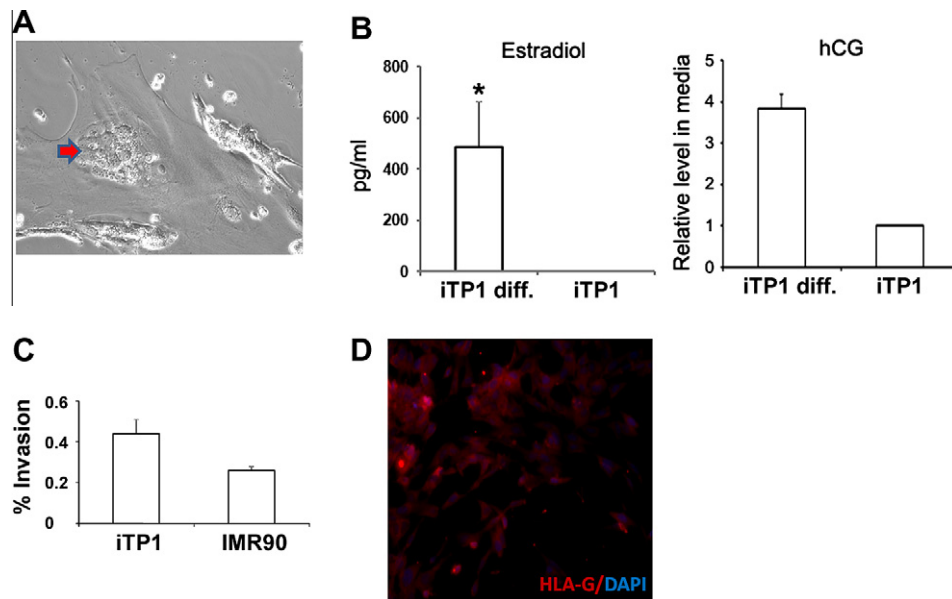


Fig. 4. iTP cells can differentiate into trophoblast subtypes. (A) Formation of multi-nucleated trophoblast cells (red arrow). (B) Estradiol and hCG levels detected from day-7 culture medium. ($n = 3$). (C) iTP cells showed higher % of invasion compared with donor fibroblasts ($n = 3$). (D) Invasive cells were stained with HLA-G (red) with Hoechst 33342 fluorescence as nuclear counterstaining (blue). The original picture is 200 \times .

nor fibroblasts, with endogenous *ELF5* expressed. Our data and others suggest that, although *ELF5* plays an important role in trophoblast lineage specificity in mouse, its role in human trophoblast lineage requires further investigation.

Besides the three trophoblast lineage-specific transcription factors mentioned above, *TEAD4* and *GATA3* were also highly expressed in our iTP cells. *TEAD4* is the earliest known trophoblast lineage transcription factor whose expression coincides with TE and inner cell mass (ICM) separation. *TEAD4* null embryos cannot form blastocoel cavities but their ICM is able to establish ES cells [38,39]. It has also been shown that *GATA3* is expressed in TE at mouse blastocyst stage and its expression pattern is parallel with that of *CDX2* [40,41]. Our study further shows that these iTP cells are tightly clustered with human TE but not with other three germ layer cells. Taken together, these data indicate that iTP cells are of trophoblast lineage. Furthermore, *FOXD3* and *TERT* are highly-expressed in our iTP cells. Reports have confirmed that *FOXD3* is required for trophoblast progenitor cells self-renewal and multi-differentiation [42], whereas *TERT* is required for trophoblast progenitor cell self-renewal [43]. These data further suggest that our iTP cells have retained the progenitor status.

To investigate the biofunctions of our iTP cells, we performed differentiation studies and found multi-nucleated cells and hormone secretion, indicating syncytiotrophoblast formation. Further studies revealed the more invasive potential of iTP cells when compared with donor fibroblasts. Although these iTP cells have slightly viral transcript *CDX2* expression, the differentiation capacity was not affected. Taken together, our data are consistent with previous findings on human trophoblast progenitor cells [3,10,44].

We conclude that *CDX2* and *EOMES* play critical biofunctional roles in generation of human iTP cells, whereas the role of *ELF5* requires further investigation. The results of this study can be further applied to generate reporter cell lines (i.e. *CDX2* reporter cell line) for efficient screening of human trophoblast lineage-specific transcription factor loops/pathways as well as therapeutic application to study the pathogenesis of placenta-associated diseases. In addition, we introduce a proof-of-concept that human iTP cells can be directly trans-differentiated from fibroblasts; this approach pro-

vides a new alternative to establish human trophoblast progenitor *in vitro*, instead of using human embryonic stem cells and 1st trimester placenta.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2012.12.135>.

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