Biochemical and Biophysical Research Communications 431 (2013) 197–202

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Roles of CDX2 and EOMES in human induced trophoblast progenitor cells

Ying Chen [a,*], Kai Wang [a], Yun Guo Gong [b], Sok Kean Khoo [b], Richard Leach [a,c,*]

[a] Department of Obstetrics, Gynecology and Reproductive Biology, College of Human Medicine, Michigan State University, Grand Rapids, MI 49503, USA
[b] Genomic Microarray Core Facility, Van Andel Research Institute, Grand Rapids, MI 49503, USA
[c] Department of Obstetrics, Gynecology and Women's Health, Spectrum Health Medical Group, Grand Rapids, MI 49503, USA

ARTICLE INFO

Article history:
Received 20 December 2012
Available online 8 January 2013

Keywords:
CDX2
EOMES
Transcription factors
Human induced trophoblast progenitor cells

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. Trophoblasts stem (TS) cells, an *in vitro* model of trophectoderm 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 trophectoderm (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 trophectoderm 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.

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 trophectoderm (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 order 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

* Corresponding authors.
E-mail addresses: ying.chen@hc.msu.edu (Y. Chen), Richard.Leach@hc.msu.edu (R. Leach).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.135

human cardiomycytes [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 transducted 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 (TGFb-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, permeablized 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 Flours, invitrogen) for 45 min and nuclei counterstained with Hochest33342 (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.