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The *H19* induction triggers trophoblast lineage commitment in mouse ES cells

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ABSTRACT

Trophoblast lineage differentiation is properly regulated to support embryogenesis. Besides normal developmental process, during germ cell tumor formation or development of other reproductive system diseases, unregulated trophoblast differentiation is also observed and affects the pathogenesis of the diseases. During normal embryogenesis, cell fate of late-stage blastcyst is regulated by a reciprocal repression of the key transcriptional factors; Oct3/4 dominancy inhibits *Cdx2* expression in inner cell mass (ICM) and leads them to epiblast/primitive ectoderm but Cdx2 dominancy in trophectoderm (TE) leads them to trophoblast lineage. In contrast during early blastcyst stage, the *Cdx2* expression is restricted in TE and not present in ICM, although Oct3/4 signaling does not inhibit the *Cdx2* expression in ICM, implying that some factors could be inactivated leading to the suppressed *Cdx2* expression in ICM of early blastcyst.

ES cells (ESCs), which are derived from ICM, could be a unique model to study trophoblast differentiation in an ectopic context. We previously showed that poly(ADP-ribose) polymerase-1 (*Parp-1*) deficient ESCs highly expressed non-coding RNA *H19* and could differentiate into trophoblast lineage. The expression of *H19* is known to start at pre-blastcyst stage during mouse development, and the gene shows high expression only in trophoectoderm (TE) at blastcyst stage. However, its role in trophoblast differentiation has not been clarified yet. Thus, we hypothesized that the *H19* activation may act as a trigger for induction of trophoblast differentiation cascade in mouse ESCs. To investigate this issue, we asked whether a forced *H19* expression drives ESCs into trophoblast lineage or not. We demonstrated that the *H19* induction leads to trophoblast lineage commitment through induction of the *Cdx2* expression.

We also showed that the expression of *Cdx2* is induced in ESCs by forced *H19* expression even under a high level of Oct3/4, which could act as a suppressor for *Cdx2* expression. It is thus suggested that the *H19* induction promotes trophoblast lineage commitment against the repression pressure by Oct3/4 in differentiating ESCs. Taken together, this study suggests that the *H19* expression is able to function as a cascade activator of trophoblast lineage commitment possibly by overriding the Oct3/4 action in ESCs.

1. Introduction

Trophoblast lineage differentiation is properly regulated during embryogenesis. Besides normal developmental process, during development of germ cell tumors or other reproductive system disorders, unregulated trophoblast differentiation is also observed and malignancy of the tumors and pathogenic properties of the diseases are affected by the presence of trophoblasts [1]. The regulation mechanism of trophoblast differentiation during normal embryonal development and pathogenesis of the diseases, including tumorigenesis has not been fully elucidated.

Abbreviations: ESCs, embryonic stem cells; ICM, inner cell mass; PE, primitive ectoderm; TE, trophectoderm; Parp-1, poly(ADP-ribose) polymerase 1; Plf1, proliferin 1; Dnmt1, DNA methyltransferase 1; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Tc, tetracycline; Zeo, zeocin; LIF, leukemia inhibitory factor; *Cdx2*, caudal-type homeobox protein 2; Fgf5, fibroblast growth factor 5; Tpbpa, trophoblast specific protein α; Igf2, insulin-like growth factor 2.

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Trophoblast differentiation is initiated with *Tead4* and *Klf5* activation in the outer layer of morulae, which specifies them to trophoblast lineage and induces genes for maintenance of trophoectoderm (TE) commitment in blastcyst stage [2]. However, one of the most significant regulations to define the TE and epiblasts has been suggested as a reciprocal expression pattern of *Cdx2* (caudal-type homeobox protein 2) in TE and Oct3/4 in epiblasts, which is established during the period of blastcyst stage. Previous reports demonstrated the repressive interaction between Oct3/4 and Cdx2 [2]. Furthermore, perturbed expression of the two genes induces the activation of TE-related genes in inner cell mass (ICM) cells/ES cells (ESCs) or that of ICM-related genes in TE cells during differentiation [3,4]. Accumulating *in vivo* studies show that; while Oct3/4 signaling does not inhibit *Cdx2* expression in ICM of early blastcyst, *Cdx2* expression is restricted in TE, implying that some factors could be inactivated or absent, that leads to suppression of the *Cdx2* expression in ICM [5].

We previously observed poly (ADP-ribose) polymerase-1 (*Parp-1*) deficient ESCs could differentiate to trophoblast lineage, while normal ESCs could not [6–8]. Mouse ESCs have been established from the ICM of blastocysts [9,10], which starts differentiation during mouse embryogenesis into the epiblast and primitive ectoderm (PE) on embryonic day 4.5; however, generally they do not give rise to trophectoderm (TE) derivatives [11,12]. The placenta of *Parp-1*$^{-/-}$ mouse also shows an increase in trophoblast giant cell number and a decrease in spongiotrophoblast number [8]. Parp-1 is involved in the regulation of transcription [13,14] and chromatin remodeling [15] through poly(ADP-ribosylation) of proteins and interaction with proteins. The absence of Parp-1 alters transcription of particular genes and induces trophoblast differentiation. *Parp-1*$^{-/-}$ ESCs shows early and enhanced expression of extraembryonic/trophoblast differentiation-associated gene, the *H19*, a non-coding RNA gene, and a homeobox transcription factor *Cdx2* gene upon ESC differentiation, and later a trophoblast specific gene, *Plf1* (proliferin 1) [16] is induced in *Parp-1*$^{-/-}$ ESCs. *Dnmt1* (DNA methyltransferase 1) deficient ESCs also differentiate into trophoblast lineage [17], accompanying induction of the *H19* expression [18].

The *H19* gene expression is initially activated at 2-cell stage, but from blastcyst stage, it is highly restricted in TE including primary trophoblasts and ectoplacental cone, although its role in trophoblast differentiation has not been clarified yet [19]. We hypothesized that the *H19* expression may induce trophoblast differentiation cascade and investigated here using ectopic context model of ESCs in this study. Our study showed that the *H19* gene expression initiates trophoblast differentiation commitment in mouse ESCs.

2. Materials and methods

2.1. Cell culture

Wild-type J1 ESC clones and *Parp-1*$^{-/-}$ 210–58 and 226–47 ESC clones were used in this study [6]. These ES clones were cultivated as previously described [16]. Briefly, ES cells were cultured in the Dulbecco's Modified Eagle's Medium (Invitrogen) containing 20% fetal bovine serum (Thermo Fisher Scientific), non-essential amino acids (Invitrogen) and leukemia inhibitory factor (LIF), ESGRO (Millipore) on gelatin-coated dishes (AGC Techno Glass). For differentiation, ESCs were transferred to the differentiation condition 1 day after vector transfection and were cultured for 3 days. ZHTc6 ESCs were cultured as previously described [4]. Culture medium was supplemented with tetracycline (Tc, 40 ng/mL, Sigma) and zeocin (Zeo, 100 μg/mL, Invitrogen). For selection of Oct3/4 positive cells, ZHTc6 ESCs were inoculated in medium including Zeo, at least for 2 weeks. Exogenous *Oct3/4* induction in ZHTc6 ESCs was achieved by Tc withdrawal for 2 days in LIF containing condition.

2.2. Forced expression of the H19 in mouse ESCs

A 3170 bp fragment of the mouse *H19* (−252 to +2918 base from the transcription initiation site) was purified by digestion of cosmid 5-10-A [20]. The *Cyp7a1* enhancer/promoter in the pCyp7a1-GFP-Neo vector [21] was replaced by the *EF-1a* promoter fragment of the pEF/myc/nuc vector (Invitrogen) to yield pEF-GFP-Neo. Then, the *H19* fragment was inserted under the *EF-1a* promoter sequence. The resulting plasmid pEF-*H19*/GFP-Neo (Fig. 3A) was linearized with XbaI (Takara Bio) and then transfected into the ESC line J1 by using Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol in serum-free condition. For establishment of stable cell-lines, transfected clones were selected by growth in the presence of 175 μg/mL G418 (Invitrogen). Following 9 days of selection, 24 colonies were collected. From them, ten ESC lines that proliferated with piled-up morphology were selected, and one clone showing a strong *H19* expression was used in the present study. For transient expression experiments, non-linearized *H19* vector (pEF-H19/GFP-Neo) or control vector (pEF-GFP-Neo) was transfected into control ESC clone or ZHTc6 ESCs as described above.

2.3. Northern blot analysis

RNAs of *H19* (2.3 kbps) and *Gapdh* (1.6 kbps) were probed as previously described [16]. The membrane was exposed to a Fuji Imaging Plate (Fuji Film), and the radioactivity was analyzed using BAS-2500 Bio-imaging analyzer (Fuji Film).

3. Results

3.1. H19 overexpression enhanced expression of trophoblast marker genes after LIF withdrawal in ESCs

Previously we reported that the *H19* gene expression is increased in undifferentiated ES cells and also further augmented in the differentiation condition under LIF removal by semi-quantified RT-PCR [16]. When analyzed by quantitative RT-PCR, we found that under undifferentiated condition in the presence of LIF, the increased expression level of the *H19* was found to be 6–10 folds in *Parp-1*$^{-/-}$ ESCs (Fig. 1A). Northern blot analysis also confirmed that the expression of full-length form of the 2.3 kb *H19* RNA is upregulated in *Parp-1*$^{-/-}$ ESCs in the presence of LIF and further augmented after LIF withdrawal (Fig. 1B). We thus hypothesized that the functional *H19* may act as a trigger and induce trophoblast differentiation cascade in mouse ESCs.

To investigate this issue, we asked whether a forced upregulation of *H19* expression promotes the commitment of ESCs to trophoblast lineage or not. As shown in Fig. 1C, we transfected wild-type J1 ESCs with an *H19* overexpression vector to obtain ESCs constitutively overexpressing the *H19* transgene. Real-time RT-PCR analysis confirmed the establishment of cells expressing the *H19* transgene (Fig. 1D) and enhanced expression of *Cdx2* in the ESCs (Fig. 1E, ESCs). Four days after differentiation condition induced by withdrawal of LIF, the *H19*-transduced clone showed an increased expression of the trophoblast marker genes, *Cdx2* and *Plf1*, but not the *Oct3/4* gene (Fig. 1E and F), compared to the control vector-transduced clone. The *Cdx2* is a marker gene for TE at an early stage of trophoblast differentiation, and induction of *Cdx2* is known to be sufficient for differentiation of ESCs into the TE [3]. The *Plf1* is a late stage marker of terminally differentiated