Biochemical and Biophysical Research Communications 436 (2013) 313–318

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

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

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ARTICLE INFO

Article history:
Received 3 May 2013
Available online 4 June 2013

Keywords:
H19
Trophoblast
ES cell
Cdx2
Oct3/4
PARP-1

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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http://dx.doi.org/10.1016/j.bbrc.2013.05.100

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

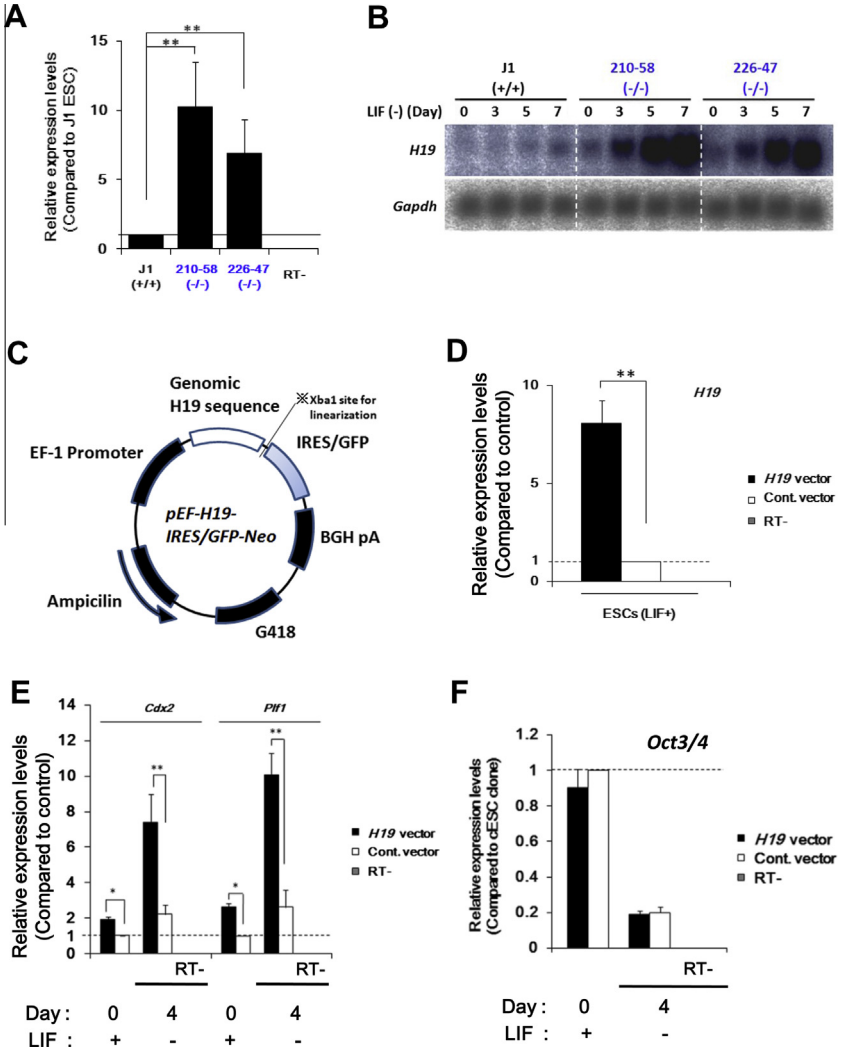

Fig. 1. Stable overexpression of *H19* activated expression of trophoblast related genes. (A and B) Upregulation of *H19* expression in *Parp-1*$^{-/-}$ ESCs. The expression level was analyzed by real-time PCR (A) and northern blot (B). The expression level was normalized using the level of *Gapdh* ($^{**}P < 0.01$). RT- means without reverse transcription. (C) The vector construct for constitutive *H19* overexpression. (D) The *H19* overexpression in the *H19* vector-transfected clone. (E) Enhancement of trophoblast marker gene expression in the *H19*-overexpressing ESC clones. The ESC clone showed enhanced expression of trophoblast marker genes (*Cdx2* and *Plf1*), and these expression levels were higher at day 4 (D4) than in the control ESC clone (E). LIF withdrawal induced differentiation in the *H19* vector- or control vector-transfected ESC clones at day 4 (D4), but *Oct3/4* expression levels did not change under *H19* overexpression (F). Expression levels were analyzed by real-time PCR and were normalized using the expression level of *Gapdh* ($^{*}P < 0.05$ and $^{**}P < 0.01$, respectively).

trophoblast giant cells [22,23]. The *Oct3/4* level was not affected in the *H19*-transduced clone, confirming that *Oct3/4* is not regulated by the *H19* (Fig. 1F). Taken together, a forced expression of the *H19* triggered commitment to trophoblast lineage in ESCs under differentiation condition by LIF withdrawal.

To exclude potential bias through the cloning process in the above experiments, which used a stable clone, we transiently overexpressed the *H19* in a wild-type ESC clone, and cultured under differentiation conditions after LIF withdrawal (Fig. 2A and B). Although there was no difference in the expression level of *Fgf5*, a marker of PE, three days after differentiation induction (Fig. 2D), expression of the trophoblast marker genes, *Cdx2, Tpbpa* (trophoblast specific protein α) and *Plf1* were enhanced in the *H19*-transduced population (Fig. 2D). These results confirmed that under differentiation pressure by LIF withdrawal in ESCs, forced expression of the *H19* acts as a trigger for induction of expression of the *Cdx2*, a main transcription factor required for commitment to trophoblast lineage.

3.2. Exogenous induction of the H19 leads to the expression of a trophoblast key regulator gene Cdx2, even under Oct3/4 overexpression

In the present study, we demonstrated that expression of the *Cdx2* is activated in undifferentiated ESCs by a forced expression of the *H19*, which could trigger trophoblast lineage commitment. It has been suggested that Oct3/4 could repress the *Cdx2* induction during ICM differentiation [2]. This led us to clarify whether the *H19* could override Oct3/4 suppressive pressure on *Cdx2* transcription or not. ZHTc6 ESC clone was employed in this experiment, in which exogenous *Oct3/4* is regulated under the control of the Tc responsive element (Tet-OFF system) [4] (Fig. 3A). Two days after Tc withdrawal in the presence of LIF, increased expression of *Oct3/4* slightly upregulated the *Fgf5* (fibroblast growth factor 5) but did not repress the *Cdx2* in ZHTc6 ESCs (Fig. 3B and C, block 3), which assured the Oct3/4 accumulation by Tc withdrawal and indicated that Oct3/4 upregulation is not sufficient to suppress

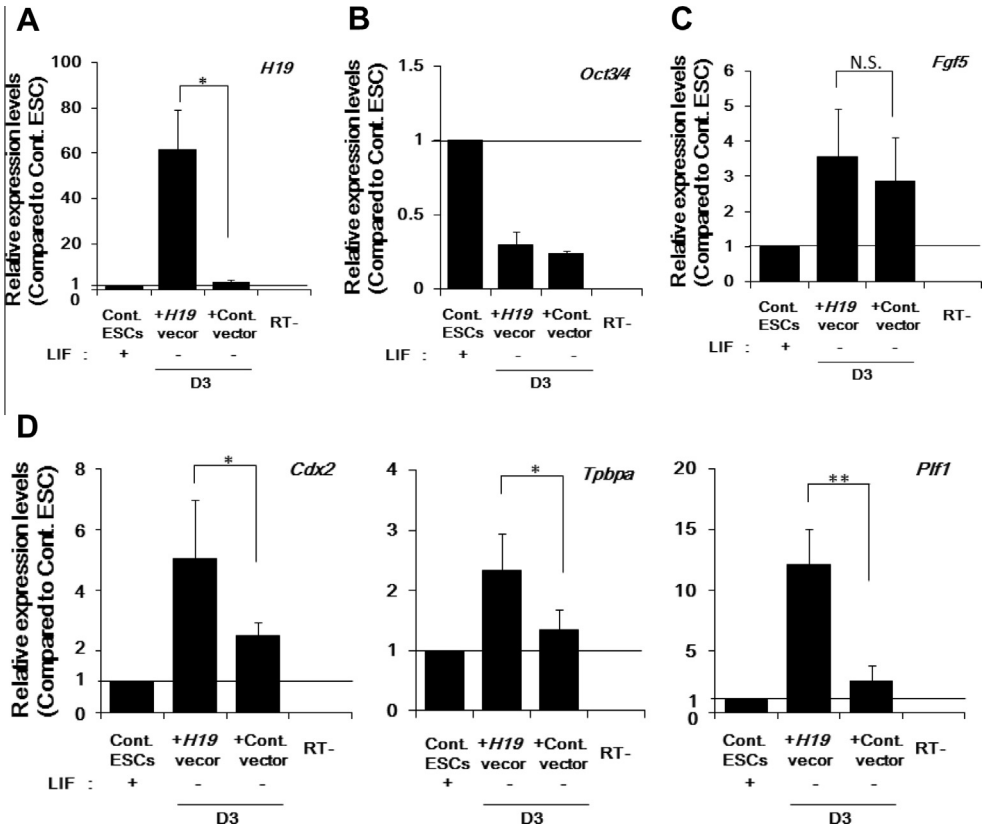

Fig. 2. The transient expression of exogenous *H19* enhanced trophoblast lineage differentiation during ESC differentiation. (A and B) Control ESC clones transfected with *H19* or control vector were induced to differentiate by LIF withdrawal. (C and D) Three days after differentiation (D3), trophoblast marker genes, *Cdx2*, *Tpbpa* and *Plf1*, were upregulated in *H19* vector-transfected ESCs (D), whereas no difference was observed in the levels of *Fgf5* between the *H19* vector- and control vector-transfected ESCs (C). Expression levels were analyzed by real-time PCR and were normalized using the expression level of *Gapdh* ($^{*}P < 0.05$ and $^{**}P < 0.01$, respectively). RT- means without reverse transcription.

the *Cdx2* at this period. The *Oct3/4* overexpression system successfully worked; the *Fgf5* expression is further augmented on day 6, which corresponds to 4 days after control vector transfection, indicating that the PE differentiation was further enhanced (Fig. 3B–D, block 5). On the other hand, expression of the *Nanog* (an epiblast marker) and *T* (a mesoderm marker) was not significantly altered (Fig. 3B and C, blocks 5 and 6) indicating that the PE differentiation is the main differentiation direction in this system. Of note, two days after Tc withdrawal, the *Oct3/4* forced expression repressed the *H19* expression prior to *Cdx2* suppression in the control vector transfected cells (Fig. 3C, the upper panel, block 3). Six days after Tc withdrawal, the *Oct3/4* forced expression reduced the *Cdx2* expression accompanying the attenuated level of *H19* expression (Fig. 3D, block 5). Importantly, forced expression of the *H19* to Oct3/4 overexpressing cells led to increased expression of *Cdx2* expression (Fig. 3D, block 6). Taken together, these results suggest that the *H19* expression is able to enhance the *Cdx2* expression even under the negative pressure of Oct3/4 on *Cdx2* in differentiating ESCs.

4. Discussion

In this study we demonstrated that the *H19* induction triggers trophoblast lineage commitment independently on Oct3/4 level in mouse ESCs cells. We showed that the expression of *Cdx2*, a key molecule for control of trophoblast differentiation, is induced by the *H19* expression. Our study also supported the notion that the *H19* upregulation in *Parp-1*$^{-/-}$ ESCs, which we previously observed, could activate the cascade of trophoblast lineage commitment by contributing to induction of the *Cdx2* expression (Fig. 4). Furthermore, during germ cell tumor formation, perturbed expression of the *H19* and trophoblast differentiation could be observed and affects malignancy of the tumors in some cases [1], suggesting that the trophoblast differentiation during tumorigenesis may be also initiated by the *H19* expression.

The role of non-coding RNA *H19* in trophoblast lineage commitment and differentiation regulation has not been fully elucidated. The *H19* expression depends on the DNA methylation state of the imprinting control region (ICR) located between the *H19* and *Igf2* (insulin-like growth factor 2) genes. A recent report demonstrated that *Dnmt1* deficient ESCs differentiated into trophoblast lineage [17]. *Dnmt1* deficiency induced DNA demethylation of the ICR and led to high level of the *H19* expression [18]. Although mouse ESCs do not generally differentiate into trophoblast lineage, it is suggested that the barrier for trophoblast lineage commitment in ESCs might be just dependent on epigenetic silencing.

The *H19* locus is paternally imprinted, and expressed only from the maternal allele [24]. The transcriptional product from the *H19* locus is a capped and polyadenylated non-coding RNA of 2.3 kb. This RNA is co-regulated with eight other imprinting genes co-organizing an imprinting gene network and is spliced and generates microRNA-675 but does not encode any protein [24]. In mouse models, the *H19* deficiency increases fetal weight and increases the frequency of teratoma formation [25]. Furthermore, the engineered expression of the *H19* could lead to complete parthenogenesis development [24]. These reports suggest the *H19* function as an

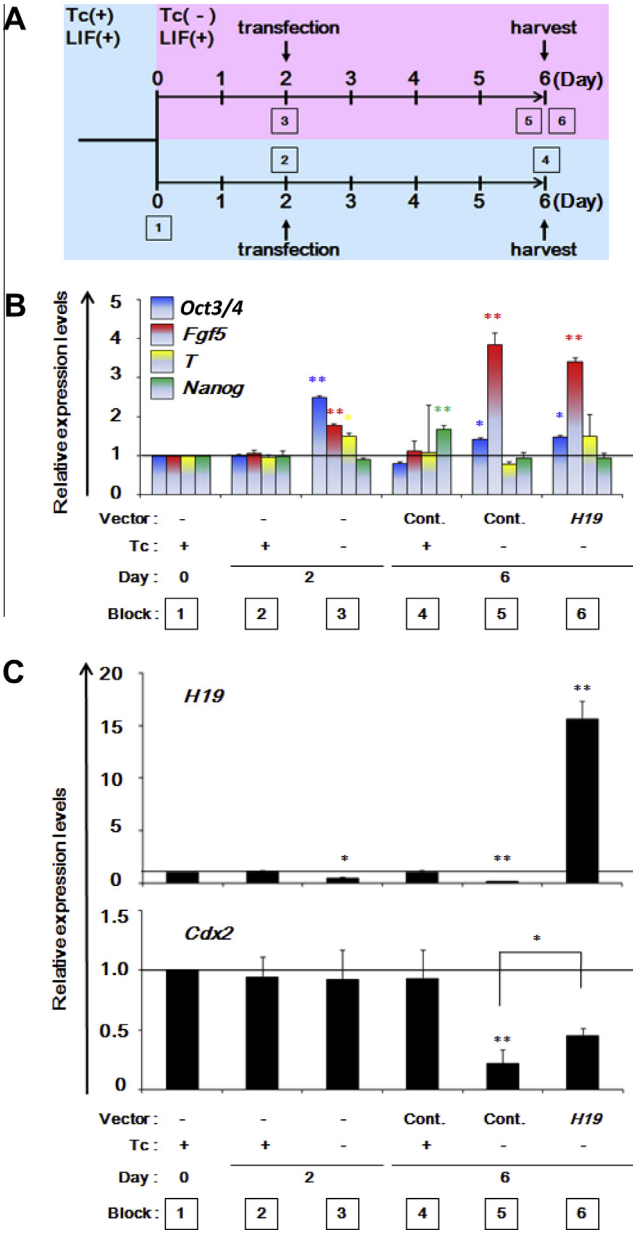

Fig. 3. The effect of *H19* overexpression in the presence of Tc-dependent Oct3/4 accumulation. (A) Experimental procedure. Each squared number indicates the RNA extraction points of the data shown in B and C. (B and C) Enhanced *Cdx2* transcription by *H19* overexpression in Oct3/4 accumulation conditions. Two days after Tc withdrawal in the presence of LIF, corresponding to the time when *Fgf5* transcription was increased (B), *H19* or control vectors were transfected into ZHTc6 ESCs (see A). Four days after transfection (the day 6 after Tc withdrawal), *H19* overexpression caused the augmented expression of *Cdx2* (C, block 5 and 6). The relative expression ratios of each gene (to the level at day 0) were shown after normalization to *Gapdh*. Unlinked asterisks indicate a significant difference in the expression level compared to that of ESCs (block 1) ($^{*}P < 0.05$ and $^{**}P < 0.01$, respectively).

important regulator of cell growth and differentiation. Further mechanistic role of the *H19* function in trophoblast lineage regulation should be elucidated.

In this study we also showed that forced expression of the *H19* even under a high level of Oct3/4 led to trophoblast lineage possibly acting as a cascade activator of trophoblast lineage by overriding the Oct3/4 action on the *Cdx2* in ESCs. Oct3/4 and Cdx2 are known to interact and regulate their levels each other. The main reported pathway of trophoblast lineage commitment is Oct3/4 down-regulation that leads to the *Cdx2* induction, which acts as a

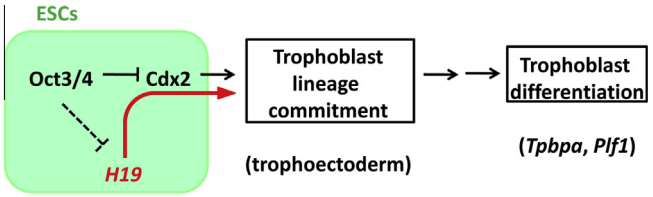

Fig. 4. A model; the *H19* upregulation could drive trophoblast lineage commitment in mouse ESCs. ESCs do not differentiate into trophoblast lineage because of the suppression of the *Cdx2* expression by Oct3/4. Forced expression of the *H19* is able to induce the *Cdx2* transcription (red letters and an arrow), which is critical for trophoblast lineage commitment of ESCs. Endogenous expression of the *H19* is downregulated by Oct3/4 (dotted lines) in ESCs. Forced expression of the *H19* drives *Cdx2* transcription even under the negative pressure by a high level of Oct3/4. This ectopic context model of ESCs suggests that *H19* upregulation might therefore contribute to induction of trophoblast lineage during tumorigenesis and pathogenesis of other reproductive system diseases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

key transcription factor to induce Eomes, Mash-2, mSna, and Hand1 cascade. Our study suggested that the *H19* overexpression is able to promote trophoblast lineage commitment even under the suppressive pressure by Oct3/4. It is of question whether this Oct3/4 independent trophoblast lineage commitment by the *H19* could be involved in the differentiation control of trophoblast lineage in normal development and aberrant induction of trophoblast lineage during tumorigenesis and other pathogenesis of the reproductive system diseases.

Acknowledgments

This work was supported by Grants-in-Aid for Third Term Comprehensive 10-Year Strategy for Cancer Control (10103833), a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan (19-9), a Grant-in-Aid for Scientific Research on Priority Areas from the ministry of Education, Science, Sports, and Culture of Japan (22300343), a Grant-in-Aid for the National Cancer Center Research and Development Fund (23-RI-14).

We appreciate the suggestions and help provided by H. Ogino, M. Maeda, T. Ochiya, H. Nakagama, and T. Sugimura. We thank H. Sasaki for kindly providing us with 5-10-A cosmid, and T. Sugimoto and H. Niwa for kind donation of ZHTc6 ESCs.

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