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The APC/C activator Cdh1 regulates the G2/M transition during differentiation of placental trophoblast stem cells

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ABSTRACT

Differentiation of placental trophoblast stem (TS) cells to trophoblast giant (TG) cells is accompanied by transition from a mitotic cell cycle to an endocycle. Here, we report that Cdh1, a regulator of the anaphase-promoting complex/cyclosome (APC/C), negatively regulates mitotic entry upon the mitotic/endocycle transition. TS cells derived from homozygous *Cdh1* gene-trapped ($Cdh1^{GT/GT}$) murine embryos accumulated mitotic cyclins and precociously entered mitosis after induction of TS cell differentiation, indicating that Cdh1 is required for the switch from mitosis to the endocycle. Furthermore, the $Cdh1^{GT/GT}$ TS cells and placenta showed aberrant expression of placental differentiation markers. These data highlight an important role of Cdh1 in the G2/M transition during placental differentiation.

1. Introduction

The endocycle, also known as the endoreplicative cycle, is a well-conserved process that occurs in both plants and animals. This specific type of cell cycle is mechanistically unusual in that it bypasses several controls that are fundamental to the regulation of the mitotic cycle [1]. During the endocycle, cells undergo repeated rounds of DNA replication without completing cell division, leading to polyploidy [2].

A good example of the endocycle can be seen in *Drosophila*. *Drosophila* follicle cells, which comprise the egg chamber, divide mitotically until mid-oogenesis and then uniformly exit the mitotic cycle to enter the endocycle thereafter [3]. When a cell transits from the mitotic cycle to the endocycle, the block of mitotic entry is an essential step. Cdh1 (also known as Fzr) activates the anaphase-promoting complex/cyclosome (APC/C) to inhibit entry into mitosis via destruction of mitotic cyclins. Cells of Cdh1 loss-of-function fly mutants are unable to enter the endocycle [4,5].

In contrast to *Drosophila*, the endocycle in mammals is infrequent. Such rare cases can be seen in the rodent placenta, where differentiation of trophoblast stem (TS) cells to trophoblast giant (TG) cells is contingent upon the endocycle. A recent study indicated that the cyclin-dependent kinase (Cdk) inhibitor p57 inhibits mitotic entry for endocycle initiation in TS cells [6]. Following FGF4 deprivation to induce differentiation, most, but not all, $p57^{-/-}$ TS cells are unable to increase DNA content to more than 4 N [6]. However, the DNA content in placental TG cells of *p57* homozygous knockout mice is not significantly reduced compared to wild-type TG cells [7,8], suggesting that a p57-independent regulator of the mitotic/endocycle switch exists in mammals.

Several groups have generated Cdh1 deficient mice and found that their placental TG cells show significantly reduced ploidy compared with their wild-type counterparts [9–11], indicating the involvement of Cdh1 in the mammalian endocycle, as in *Drosophila*. Nonetheless, it is unclear how Cdh1 regulates the endocycle in TS cells. To clarify this issue, we established *Cdh1* gene-trap (GT) TS cells and examined the role of Cdh1 in the regulation of mitotic entry at the mitotic/endocycle transition.

2. Materials and methods

2.1. Cdh1 gene-trap mice

$Cdh1^{+/GT}$ mice (C57BL/6 background) were established and maintained as described previously [11]. All animal experiments were approved by the Animal Ethics Committee of Keio University.

Abbreviations: APC/C, anaphase-promoting complex/cyclosome; TS cell, trophoblast stem cell; TG cell, trophoblast giant cell; GT, gene-trap; F4H, FGF4 and heparin; CM, conditioned medium.

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2.2. Isolation and maintenance of TS cells

TS cells were derived from E3.5 blastocysts and were established and cultured as described previously [12,13]. In brief, after mating with *Cdh1*$^{+/GT}$ male mice, a pregnant female *Cdh1*$^{+/GT}$ mouse was sacrificed at 3.5 dpc and the uterus was removed. Blastocysts were flushed out of the uterus with M2 medium (Sigma) using a 26 gauge needle. Each blastocyst was cultured on feeder cells in TS medium (RPMI-1640 (Sigma) supplemented with 20% fetal calf serum (Nichirei, Tokyo, Japan), 100 μM 2-mercaptoethanol (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1× antibiotic–antimycotic (Invitrogen), and 1×F4H (25 μg/ml FGF-4 (Peprotech) and 1 μg/ml Heparin (Sigma)). When the outgrowths reached a visible size, cells were trypsinized to dissociate and transferred to colony formation cultures. TS colonies were cultured on feeder cells in 70 CM (7:3 mixture of TS medium conditioned by mouse embryonic fibroblasts (MEFs) and fresh TS medium) containing 1.5×F4H until appropriate cell densities were attained, and colonies were then maintained in TS medium containing 1×F4H. For feeder-free cultures, TS cells were maintained in gelatin-coated dishes in 70 CM containing 1×F4H.

2.3. Immunocytochemistry

Immunofluorescence staining was performed as described previously [14] using antibodies to E-cadherin (1:500 dilution; BD), or Ser^{10}-phosphorylated histone H3 (1:100 dilution; Cell Signaling). Sections were observed with a Fluoview laser-scanning confocal microscope (IX70, Olympus) equipped with a 60× objective (numerical aperture, 1.25). The size of the nuclei (represented by the number of pixels) in Hoechst 33342–stained cells was determined using the ImageXpress and Metamorph software (Molecular Devices).

2.4. Western blotting

TS cells were cultured in the absence of F4H and collected at the indicated times after F4H deprivation. Cells were washed in phosphate buffered saline and lysed by incubation for 15 min in lysis buffer (0.5% NP-40, 25 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, and complete protease inhibitor cocktail (EDTA free; Roche)). After centrifugation of the lysates at 14,000*g* for 20 min, the supernatant was subjected to SDS gel electrophoresis, Western blotting, and immunodetection using the indicated antibodies. Antibodies used in this study were as follows: Cyclin B (GNS1, Santa Cruz), Cyclin A (C-19, Santa Cruz), Cdh1 (DH01, Neomarkers), Cdc20 (H7, Santa Cruz), Skp2 (GP45, Zymed), Aurora A (clone4, BD), p27 (C-19, Santa Cruz), p57 (E-17, Santa Cruz), α-tubulin (B5-1-2, Sigma), PL-1 (AB1288, Chemicon) and Cdk4 (Ab-2, Neomarkers).

2.5. In situ hybridization

In situ hybridization was performed on sections of staged placentas as described previously [15], with some modifications in the signal detection by using a DIG-labeled RNA probe (Roche). All plasmids used for the preparation of riboprobes were kindly provided by G. Leone [16]. Digoxigenin-UTP-labeled antisense and control (sense) riboprobes were synthesized from plasmids using SP6 or T7 RNA polymerase and the MAXIscript *in vitro* transcription kit (Ambion).

2.6. Quantitative analysis of gene expression

Total RNA was extracted from TS cells using an RNeasy Minikit (Qiagen) and was subjected to reverse transcription (RT) using PrimeScript (Takara). Real-time PCR was performed in a Thermal Cycler Dice apparatus (Takara) using SYBR Premix Ex Taq (Takara). All primers used to amplify trophoblast marker genes have been described previously [17]. Primers for the murine Cdh1 and glyceralehyde-3-phosphate dehydrogenase (GAPDH) genes were obtained from Takara. Relative mRNA levels were calculated by normalization of cycle threshold (C_t) values of the target gene to those of the reference genes (GAPDH and ubiquitin).

2.7. Flow cytometry

Asynchronously growing TS cells were stained with propidium iodide and collected using a FACSCalibur apparatus (BD) combined with the CellQuest software (BD). Cell-cycle profiles were analyzed using the FlowJo software (Version 7.2.5; Tree Star, Inc.).

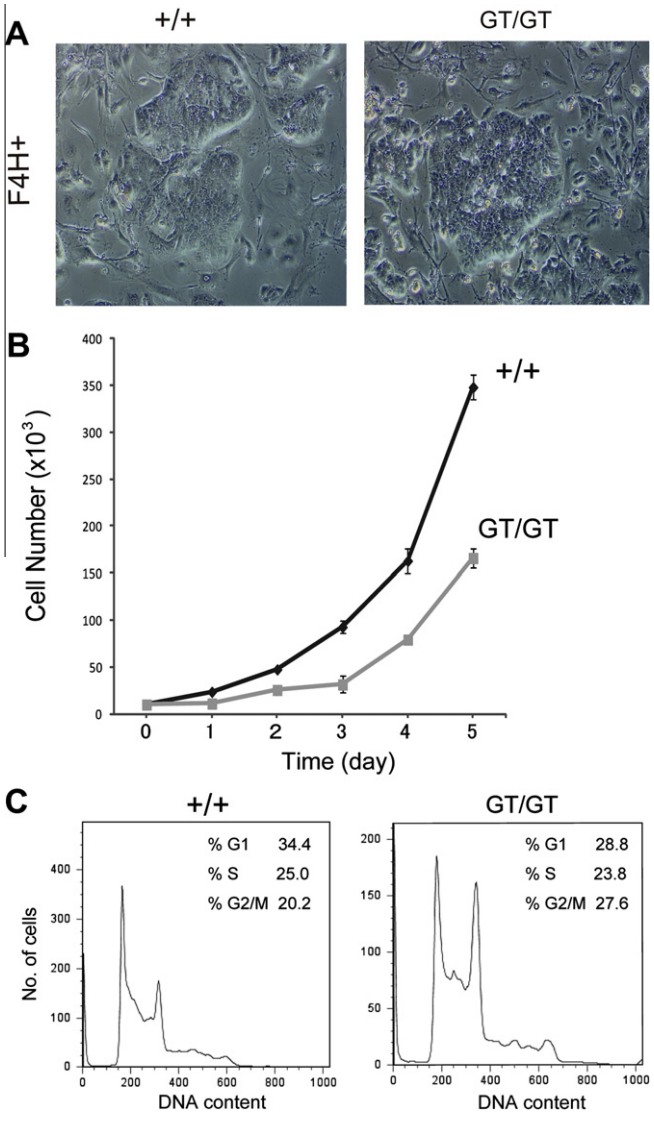

Fig. 1. Characterization of *Cdh1*$^{GT/GT}$ TS cells. (A) Establishment of *Cdh1*$^{GT/GT}$ TS cells. TS cells of the indicated *Cdh1* genotypes were cultured on feeder cells in the presence of FGF4/Heparin (F4H) (undifferentiated TS cells), and were then examined by phase-contrast microscopy. Original magnification, 40×. (B) Cell proliferation of undifferentiated TS cells with the indicated *Cdh1* genotypes was analyzed by seeding 1×10^5 cells in 70 CM medium containing F4H onto feeder-free dishes (gelatin-coated) and determining the cell number every 24 h. (C) Flow cytometric analysis of TS cells with the indicated *Cdh1* genotypes stained with propidium iodide. The fraction of cells in each stage of the cell cycle is presented.

2.8. Statistical analyses

Quantitative data are presented as means ± standard deviation (SD). Differences in nuclear size were determined by the Wilcoxon–Mann–Whitney nonparametric test using the JMP7 software (SAS Institute, Inc). Differences in the percentage of mitotic cells were evaluated using the two-tailed Student's *t* test. A value of $P < 0.05$ was considered significant.

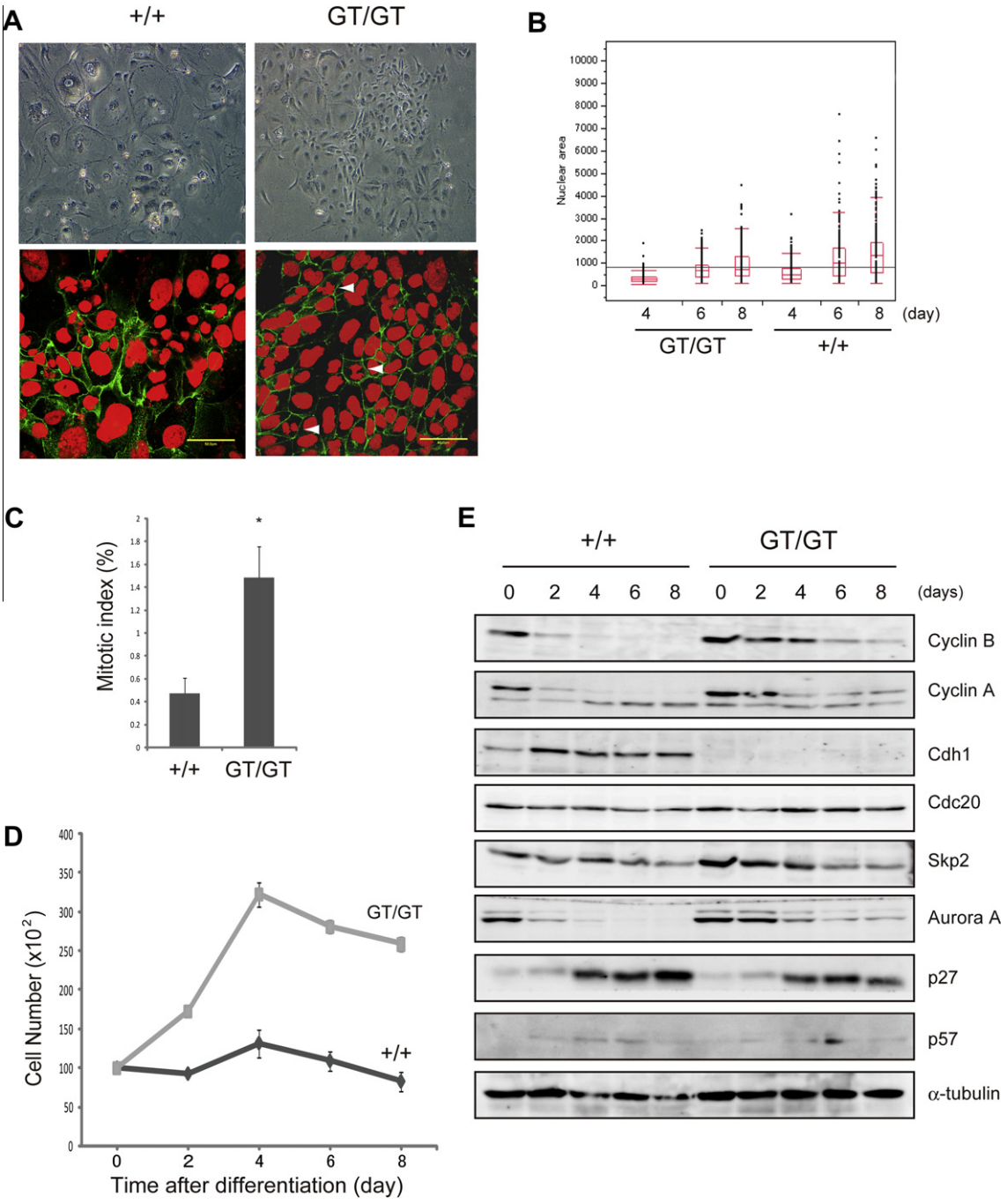

Fig. 2. Precocious mitotic entry and impaired endoreplication in *Cdh1*$^{GT/GT}$ TS cells after the induction of differentiation. (A) Loss of nuclear enlargement in *Cdh1*$^{GT/GT}$ TS cells. Upper panels: TS cells of the indicated *Cdh1* genotypes were cultured on feeder cells in the absence of F4H for 5 days and were then examined by phase-contrast microscopy (original magnification, 40×). Lower panels: TS cells cultured for 3 days in the absence of F4H were stained with propidium iodide (red) and antibodies to E-cadherin (green). Arrowheads indicate mitotic cells. Scale bars, 50 μm. (B) TS cells were cultured for the indicated times in the absence of F4H, and were then fixed and stained for DNA using Hoechst 33342. The size of over 200 nuclei (represented by the number of pixels on the *y* axis) was determined for each sample; bars on the dot plot denote the 10th and 90th percentiles, and each box indicates the first and third quartiles. The bar in each box shows the median value. Data are representative of two independent experiments. (C) The mitotic index of TS cells cultured for 5 days in the absence of F4H was measured by immunostaining with antibodies to phosphorylated histone H3. Data are means ± SD for over 800 cells scored for each genotype in two independent experiments. $^{*}P < 0.05$. (D) Growth curves for wild-type and *Cdh1*$^{GT/GT}$ TS cells after the induction of differentiation. Data are means ± SD from three independent experiments. (E) Immunoblot analysis of the indicated proteins in TS cells cultured in the absence of F4H for the indicated times after induction of differentiation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Characterization of *Cdh1*$^{GT/GT}$ TS cells

Previous analysis of *Cdh1* homozygous GT (*Cdh1*$^{GT/GT}$) placentas revealed frequent thrombus formation in the labyrinth layer [11]. Another striking pathology in Cdh1-deficient placentas is a lack of giant nuclei in TG cells. The TG cells also contained significantly fewer genome copies (N) than their wild-type counterparts (wild-type, 100–1500 N; Cdh1$^{-/-}$, <100 N) [9,11].

To elucidate the molecular mechanisms by which Cdh1 regulates the endocycle in TG cells, we established *Cdh1*$^{GT/GT}$ TS cells from E3.5 blastocysts, as previously described [12]. TS cells have the potential to give rise to all differentiated trophoblast cell subtypes, including TG cells, and to reconstitute the placenta in chimeric animals [12]. Both wild-type and *Cdh1*$^{GT/GT}$ TS cells manifested a similar epithelial sheet morphology when cultured in TS medium supplemented with a combination of fibroblast growth factor 4 and heparin (F4H) (Fig. 1A). The proliferative capacity of undifferentiated TS cells was examined. *Cdh1*$^{GT/GT}$ TS cells proliferated at a slower rate when compared with wild-type TS cells (Fig. 1B), indicating that Cdh1 is required for TS cell division, as previously shown in MEFs [9,10]. Flow cytometric analysis of the cell-cycle profile of TS cells revealed that Cdh1 ablation resulted in an increase in the proportion of cells in the G2/M phase and a decrease in the fraction of cells in the G1 phase (Fig. 1C), a phenotype similar to that of Cdh1-deficient MEFs, with the exception of S phase. Only a minor decrease in the number of S phase *Cdh1*$^{GT/GT}$ TS cells compared to wild-type cells was detected [9,18] (Fig. 1C).

3.2. Precocious mitotic entry of *Cdh1*$^{GT/GT}$ TS cells after endocycle induction

Whether the *Cdh1*$^{GT/GT}$ TS cells recapitulate the pathological changes seen in *Cdh1*$^{GT/GT}$ placentas was examined next. In the absence of F4H, wild-type TS cells ceased to proliferate as a part of the mitotic/endocycle switch and differentiated into cells with giant nuclei (Fig. 2A and D). By contrast, the ability of *Cdh1*$^{GT/GT}$ TS cells to form structures with giant nuclei was markedly impaired (Fig. 2A). To evaluate the extent of polyploidy after various times in culture in the absence of F4H, the area occupied by the nuclei in these cells was measured (Fig. 2B). The ploidy of *Cdh1*$^{GT/GT}$ TG cells was significantly reduced compared with that of wild-type cells ($P < 0.0001$), indicating that Cdh1 plays an important role in the mammalian endocycle and that our *in vitro* system closely resembles the *in vivo* situation [9,11].

Upon the mitotic/endocycle switch, mitotic entry is inhibited in wild-type TS cells. However, mitotic cells were frequently observed

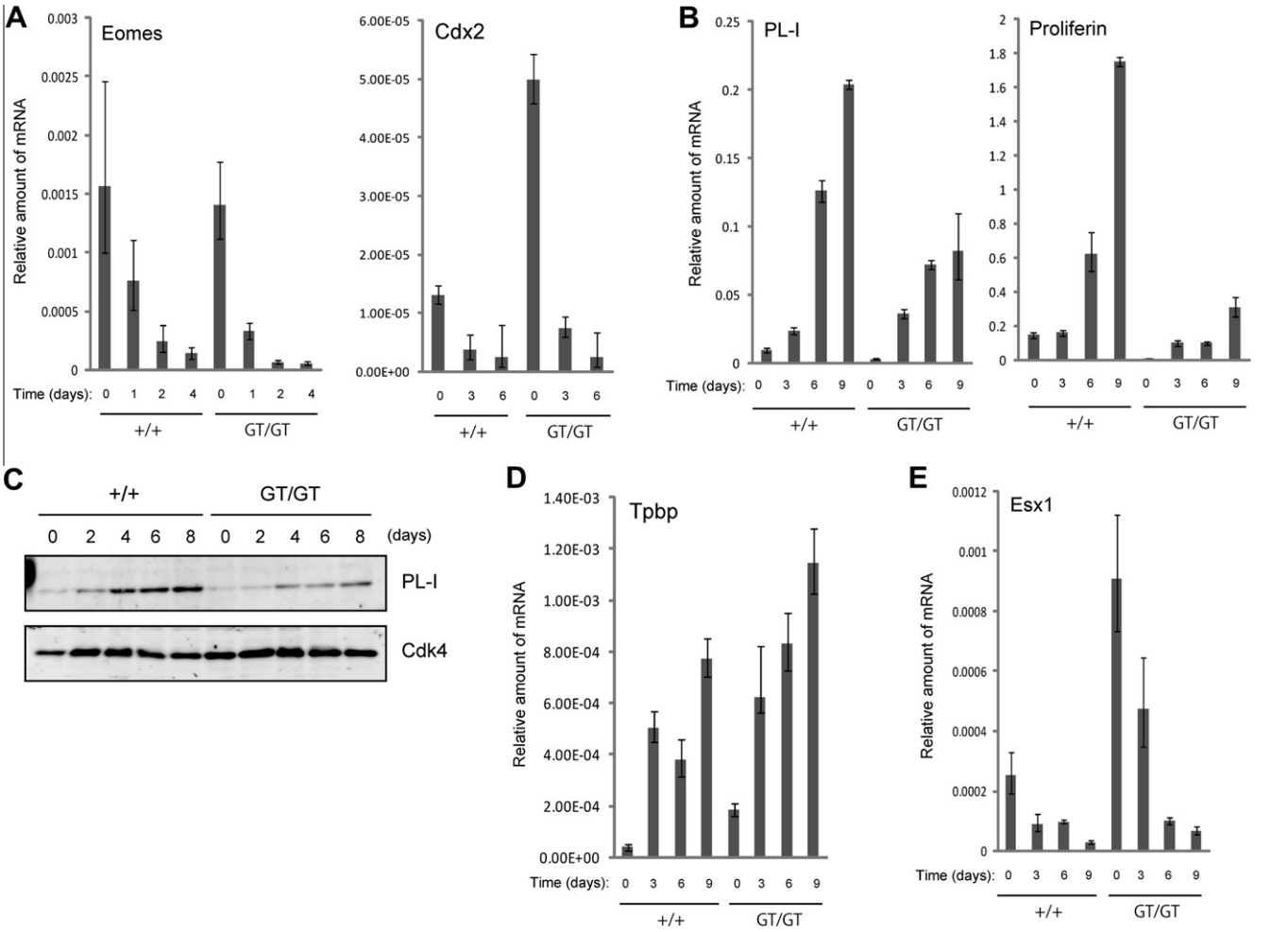

Fig. 3. Aberrant differentiation of *Cdh1*$^{GT/GT}$ TS cells into TG cells. (A–E) Wild-type and *Cdh1*$^{GT/GT}$ TS cells were incubated in the absence of F4H for the indicated times, and total RNA was isolated and subjected to quantitative RT-PCR analysis of mRNAs for Eomes or Cdx2 (A), PL-1 or Proliferin (B), Tpbp (D), or Esx1 (E). Data were normalized to the amount of ubiquitin mRNA and are represented as means ± SD from at least three independent experiments. (C) Immunoblot analysis of PL-1 protein levels in cells incubated in the absence of F4H for the indicated times. Expression of Cdk4 was used as a protein loading control.

in *Cdh1*$^{GT/GT}$ cells even after 5 days of F4H deprivation (Fig. 2A and C), and *Cdh1*$^{GT/GT}$ cells typically did not stop proliferating after F4H deprivation (Fig. 2D). These results strongly suggest that *Cdh1*$^{GT/GT}$ TS cells fail to switch to the endocycle and continue to undergo precocious mitotic entry.

In *Drosophila*, Cdh1 regulates the mitotic/endocycle switch by mediating the destruction of mitotic cyclins [4,5]. To address whether such a mechanism is conserved in mammals, the expression of mitotic cyclins and other Cdh1-targets was examined in TS cells after F4H depletion. Mitotic cyclins (Cyclin A and Cyclin B) accumulated in *Cdh1*$^{GT/GT}$ cells during the early stages of induction of TS cell differentiation (Fig. 2E, first and second rows). Other targets of Cdh1, such as Skp2 and Aurora A [19,20], also accumulated in *Cdh1*$^{GT/GT}$ cells (Fig. 2E, fifth and sixth rows). Contrary, the expression of p27 in *Cdh1*$^{GT/GT}$ cells was slightly reduced when compared with that in wild-type cells, which might be due to accumulation of Skp2, a ubiquitin ligase that targets p27 [21] (Fig. 2E, seventh and fifth rows). These results suggested that the accumulation of mitotic cyclins (and possibly a decrease in the CDK inhibitor) led to precocious mitotic entry in *Cdh1*$^{GT/GT}$ cells.

3.3. Abnormal differentiation of *Cdh1*$^{GT/GT}$ TS cells

To determine whether the abnormal proliferative potential of *Cdh1*$^{GT/GT}$ TS cells affects their differentiation into TG cells, RT-PCR and real-time PCR analyses were used to examine the expression of differentiation markers characteristic of the different trophoblast cell lineages. Culture of both wild-type and *Cdh1*$^{GT/GT}$ TS cells in the absence of F4H resulted in loss of mRNAs encoding the stem cell markers eomesodermin (Eomes) and Cdx2 (Fig. 3A). By contrast, up-regulation of the mRNAs for the TG cell differentiation markers placental lactogen-1 (PL-1) and proliferin (Plf-1) was markedly suppressed in *Cdh1*$^{GT/GT}$ cells compared to wild-type cells (Fig. 3B). Consistent with this difference in mRNA abundance, the amount of PL-1 protein in wild-type cells was much greater than that of *Cdh1*$^{GT/GT}$ cells (Fig. 3C). By contrast, expression of the spongiotrophoblast marker Tpbp (also known as 4311) was greater in *Cdh1*$^{GT/GT}$ cells compared to wild-type cells (Fig. 3D). The level of transcripts encoding the labyrinth layer marker Esx1 was greater in *Cdh1*$^{GT/GT}$ cells at early time points compared to wild-type cells (Fig. 3E). These results indicate that Cdh1 deficiency results in abnormal TG cell differentiation *in vitro*. In contrast with previous observations that showed a separation of endoreplication and differentiation in TG cells [17,22], loss of Cdh1 affected both processes.

To confirm the data obtained by RT-PCR and real-time PCR analyses of the expression of trophoblast differentiation marker genes, *in situ* hybridization was performed to evaluate TG cell differentiation *in vivo*. Transcripts for the TG cell differentiation marker proliferin showed a cytoplasmic staining pattern in cells with large nuclei in wild-type placentas (Fig. 4A), consistent with previous observations [23]. By contrast, the staining was almost completely absent in *Cdh1*$^{GT/GT}$ placentas (Fig. 4B), which suggested that the *in vivo* differentiation of TG cells was compromised in the gene-trapped animals.

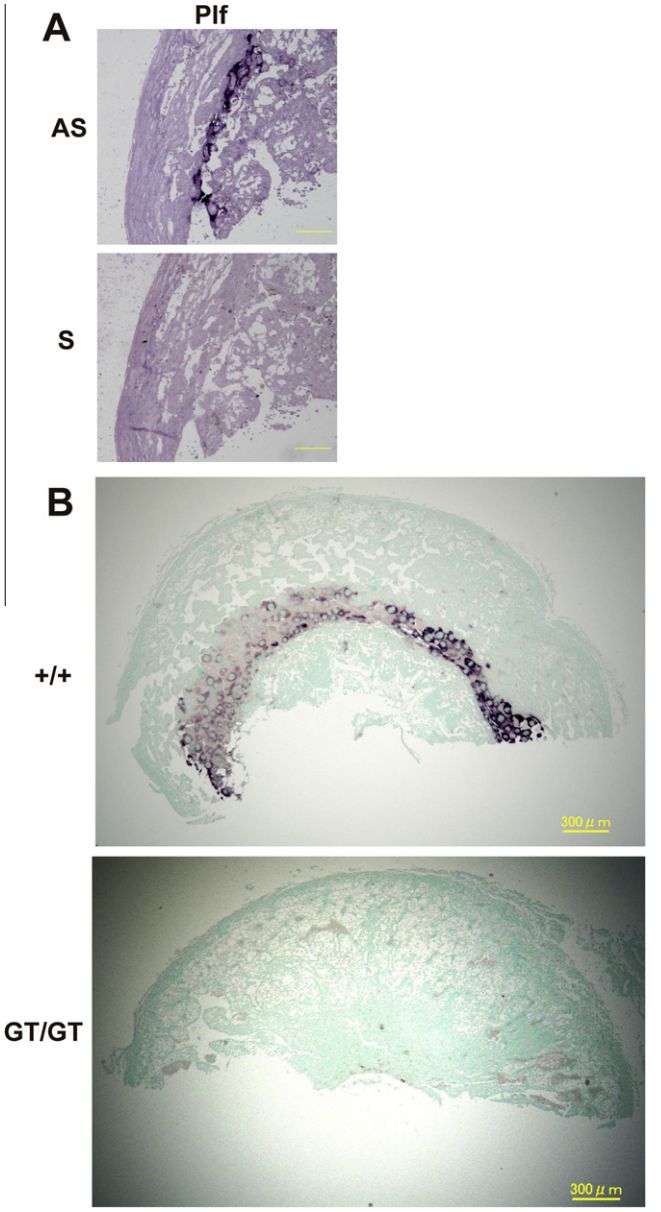

Fig. 4. Reduced expression of a TG cell specific marker in the placenta of *Cdh1*$^{GT/GT}$ embryos. *In situ* hybridization analysis of proliferin mRNA in sections of E12.5 (A) and E10.5 (B) placenta. (A) Panels show staining of wild-type placenta with antisense (AS, upper panel) or sense (S, lower panel) probes. (B) Upper and lower panels show wild-type and *Cdh1*$^{GT/GT}$ placentas (E10.5) stained with the antisense probe. The counterstain was methyl green. Scale bars, 300 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In the present study, Cdh1 was shown to function in the mitotic/endocycle transition in TS cells. *Cdh1*$^{GT/GT}$ TS cells could not block mitosis when differentiated into TG cells by F4H deprivation, resulting in endocycle failure (Fig. 2C and D). Cdh1 suppressed mitotic entry via degradation of mitotic cyclins (Fig. 2E). Similarly, *Drosophila Cdh1* mutant follicle and nurse cells could not switch from the mitotic cycle to the endocycle as a result of accumulation of mitotic cyclins [4,5]. Therefore, the role of Cdh1 in the mitotic/endocycle transition is well conserved from fly to mammals.

Cdk1 activity is required for entry into mitosis [24]. To prevent segregation of damaged and/or improperly replicated chromosomes, Cdk1 activity is strictly regulated by several distinct but highly conserved mechanisms [25–27]. Recent studies indicate that Cdh1 plays a role in mitotic entry as a part of the DNA damage-induced G2 checkpoint [28,29]. Upon double-strand DNA breaks, the Cdc14B phosphatase is released from the nucleolus into the nucleoplasm, where Cdc14B dephosphorylates and activates Cdh1 to target the polo-like kinase1 (PLK1) for degradation. Since PLK1 antagonizes ATR/Chk1/Cdc25A signaling, Cdh1 enhances the Chk1-mediated Cdk1 inhibition after DNA damage in G2 [29].

Therefore, Cdh1 is involved in the G2/M transition in DNA-damaged cells. Given that Cdh1 participates in mitotic exit via Cyclin B degradation (Cdk1 inhibition) in the unperturbed mitotic cell cycle, Cdh1-mediated signaling pathways control Cdk1 activity not only at mitotic exit but also at mitotic entry.

Importantly, Cdh1-mediated regulation of the G2/M transition is not restricted to TS cell differentiation or the DNA damage checkpoint of somatic cells. In mammals, oocytes are arrested at the dictyate prophase I, equivalent to the G2 phase [30]. Oocytes resume the cell cycle to enter meiotic metaphase I after a peri-ovulatory luteinizing hormone surge. Cdh1 promotes Cyclin B degradation to inhibit precocious meiotic entry before a hormonal cue [30]. Collectively, Cdh1 has many physiological roles that are well correlated with the control of the G2/M transition via its regulation of Cdk1 activity.

Cdk1 suppression is required for the mitotic/endocycle transition and TS cell differentiation. Ullah et al. reported that the CDK1 inhibitor p57 can induce TS cells to differentiate into TG cells, which showed that suppression of Cdk1 is an essential step in TS cell differentiation [6]. In this study, we could not clearly detect the reduced levels of p57 in *Cdh1*$^{GT/GT}$ TS cells after F4H deprivation (Fig. 2E, eighth row). However, mitotic cyclins were significantly accumulated in these cells (Fig. 2E, first and second rows), suggesting Cdk1 can be inhibited by another means. Indeed, we found *Cdh1*$^{GT/GT}$ TS cells and placenta showed defective expression of placental differentiation markers (Figs. 3 and 4). Thus, post-translational regulation of mitotic cyclins comprises an important mechanism for controlling Cdk1 activity during TS cell differentiation.

In summary, our data revealed a conserved role of Cdh1 in the mitotic/endocycle transition. Cdh1 regulated the G2/M transition to facilitate a switch from mitosis to the endocycle and also participated in TS cell differentiation during placental development.

Acknowledgments

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