

# Trophoblast Differentiation Defect in Human Embryonic Stem Cells Lacking PIG-A and GPI-Anchored Cell-Surface Proteins

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## SUMMARY

Pluripotent human embryonic stem (hES) cells can differentiate into various cell types derived from the three embryonic germ layers and extraembryonic tissues such as trophoblasts. The mechanisms governing lineage choices of hES cells are largely unknown. Here, we report that we established two independent hES cell clones lacking a group of cell surface molecules, glycosyl-phosphatidyl-inositol-anchored proteins (GPI-APs). The GPI-AP deficiency in these two hES clones is due to the deficiency in the gene expression of PIG-A (phosphatidyl-inositol-glycan class A), which is required for the first step of GPI synthesis. GPI-AP-deficient hES cells were capable of forming embryoid bodies and initiating cell differentiation into the three embryonic germ layers. However, GPI-AP-deficient hES cells failed to form trophoblasts after differentiation induction by embryoid body formation or by adding exogenous BMP4. The defect in trophoblast formation was due to the lack of GPI-anchored BMP coreceptors, resulting in the impairment of full BMP4 signaling activation in the GPI-AP-deficient hES cells. These data reveal that GPI-AP-enhanced full activation of BMP signaling is required for human trophoblast formation.

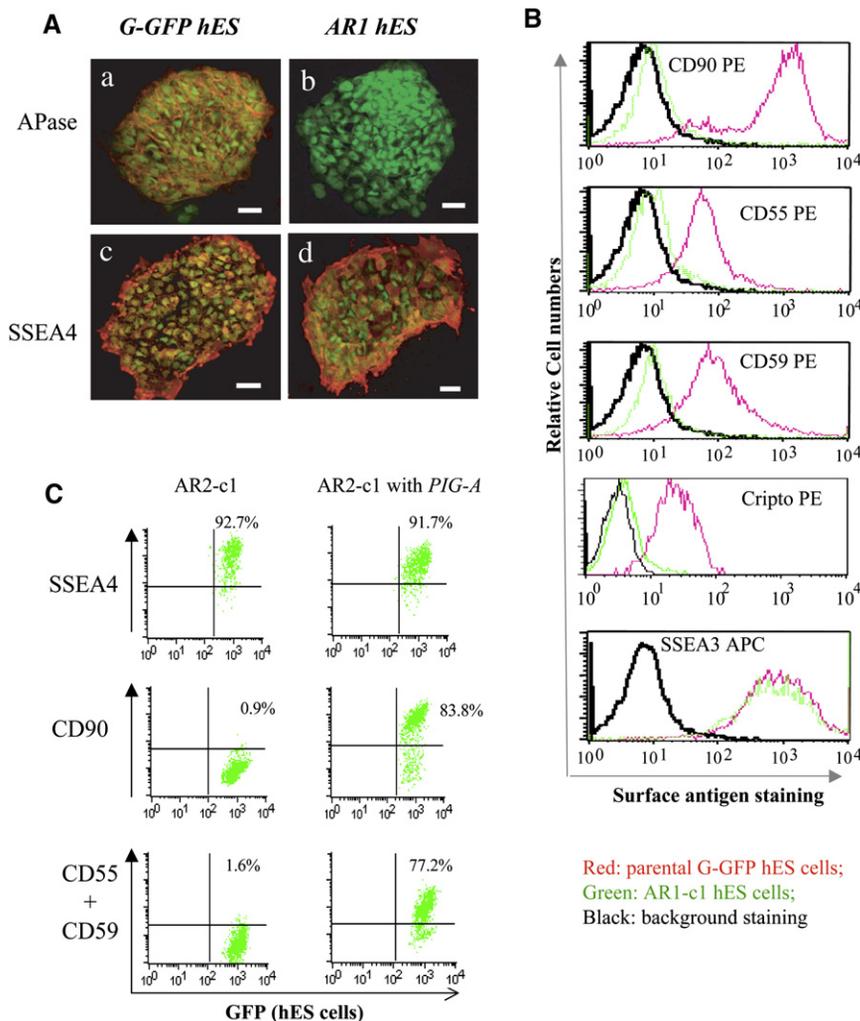
## INTRODUCTION

PIG-A is required for the first step in GPI anchor biosynthesis (Kinoshita et al., 1997). The human (X chromosome-linked) *PIG-A* gene is found mutated in hematopoietic stem cells (HSCs) of patients with paroxysmal nocturnal hemoglobinuria (PNH), a clonal disorder of the blood system that causes intravascular hemolysis, venous thrombosis, and bone marrow failure (Takeda et al., 1993; Luzzatto et al., 1997; Kinoshita et al., 1997; Dunn et al., 1999). Inactivation of PIG-A in HSCs results in the lack of

all GPI-APs, including two complement inhibitors CD55 and CD59; the lack of these two cell surface proteins explains the complement-mediated intravascular hemolysis associated with PNH. However, other clinical features of PNH, such as clonal expansion and the associated bone marrow failure, remain poorly understood (Kinoshita et al., 1997; Luzzatto et al., 1997; Dunn et al., 1999). Members of dozens of GPI-APs function as coreceptors, coligands, ectoenzymes, and cell adhesion molecules (Kinoshita et al., 1997; Minchiotti et al., 2000; Chesebro et al., 2005). The importance of the GPI anchor moiety in linking the protein to the cell membrane has been demonstrated for several GPI-APs (Minchiotti et al., 2000; Chesebro et al., 2005).

To establish a prospective experimental system for PNH, a somatic disease, mouse models have been established by disrupting the *Pig-a* gene in mouse ES (mES) cells (Dunn et al., 1996; Rosti et al., 1997; Keller et al., 2001). Although the *Pig-a* gene (also X-linked) is dispensable for the growth of undifferentiated mES cells in culture, the inactivation of the mouse *Pig-a* gene is embryonic lethal (Rosti et al., 1997; Keller et al., 2001). Conditional *Pig-a* null mice lacking GPI-APs in all the lineages of blood and immune cells were later achieved (Keller et al., 2001). However, these mice have a normal life span and do not recapitulate the PNH symptoms seen in human patients. Because of the current limited ability to expand human HSCs in culture that are required for selecting and expanding rare clones after stable genetic modification, it has been impossible to make a *PIG-A* null mutation by knocking out or down the *PIG-A* gene in normal human HSCs.

Our initial goal of this project was to make PIG-A-deficient hES cells that can be subsequently induced to differentiate into hematopoietic cells (Kaufman et al., 2001; Zhan et al., 2004; Lensch and Daley, 2006), which may serve as a novel genetic model for PNH. After trials with several methods, we established two independent clones of hES cells lacking the expression of the *PIG-A* gene and GPI-APs on hES cell surface. Although full characterizations of these GPI-AP-deficient hES cells such as differentiation to hematopoietic and other somatic lineages are still in progress, our data reveal an unexpected but critical role of GPI-APs in potentiating cellular signaling by bone morphogenetic protein 4 (BMP4) and trophoblast development of hES cells.



**Figure 1. Analyses of Two hES Cell Clones Lacking GPI-Anchored Proteins**

After aerolysin selection, G-GFP-derived hES cells that lacked GPI-APs were sorted and expanded. Six clones were obtained from two large-scale experiments. Two (AR1-c1 and AR2-c1) were further characterized since they can form undifferentiated colonies (Table S2). (A) Cell surface staining for alkaline phosphatase (APase). The AR1-c1 or its parental G-GFP hES cells (both constitutively expressing GFP) were cultured under a feeder-free condition and fixed before cell surface staining. Mouse monoclonal IgG recognizing either the APase (Aa and Ab) or SSEA-4 (Ac and Ad) was used as primary antibodies. Then, Alexa 555-conjugated antibodies recognizing anti-mouse IgG were used to light up detected antigens. The micrographic images of stained were superimposed with that of cellular GFP signals. While both APase and SSEA4 were detected on the cell surface of G-GFP cells (Aa and Ac), only SSEA-4 (Ad), but not the APase (Ab), was detected on AR1-c1 cells. (B) Flow cytometric analysis of other GPI-APs, such as CD90, CD55, CD59, and Cripto, using PE-conjugated-specific IgGs. The red lines represent staining profiles of the G-GFP control, whereas the green lines represent the AR1-c1 hES cells. The black lines represent the background staining (using irrelevant antibodies). Both hES cell types expressed a high level of surface marker SSEA3. (C) Similar analysis of the AR2-c1 hES cell clone before or after transduction by a lentiviral vector expressing a *PIG-A* transgene. AR2-c1 cells express SSEA4, but lack CD90, CD55, or CD59. After one round of transduction, ~80% AR2-c1 cells stably expressed GPI-APs.

## RESULTS

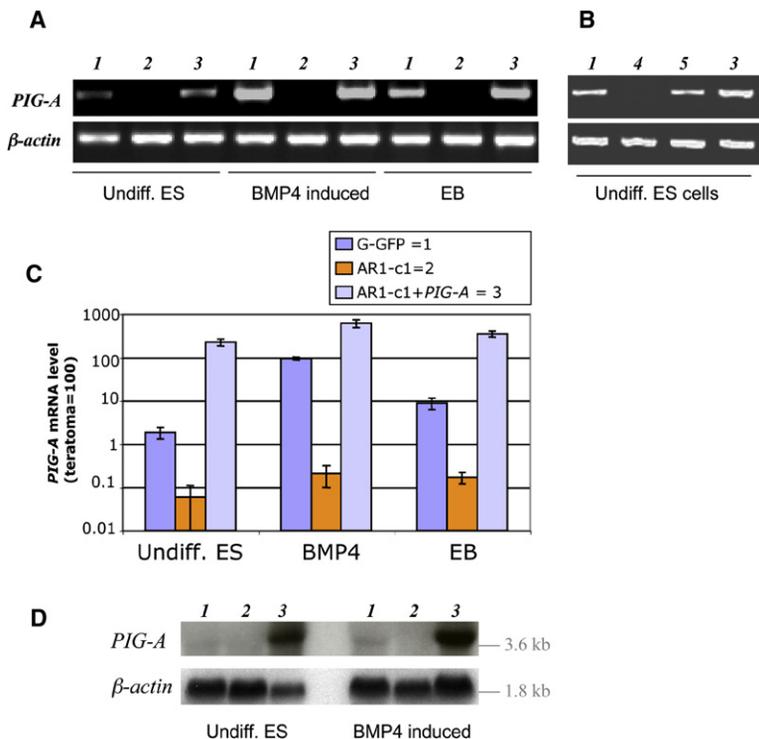
### Establishment of Clonal hES Cells Lacking GPI-APs

Consistent with previous studies, we found that several GPI-APs such as alkaline phosphatase (APase), CD90/Thy1, and Cripto are preferentially expressed on cell surface of undifferentiated hES cells (Figure 1). The mRNA expression profile of known GPI-AP genes in undifferentiated and differentiated hES cells is provided in Table S1 available online. We have attempted several approaches to knock out or down the X chromosome-linked *PIG-A* gene in XY hES cell line such as H1. The most successful approach to date was to use proaerolysin for counter selection of cells lacking GPI-APs. Proaerolysin is a bacterial toxin that uses GPI-APs as a cellular receptor. It is converted by cell surface proteases to aerolysin that potently kills mammalian cells normally expressing various GPI-APs (Brodsky et al., 1999; Hu et al., 2005). Cells lacking GPI-APs such as *PIG-A* null mutants escape aerolysin-mediated cell killing. We used the H1 hES cell population that had been transduced by a GFP-expressing lentiviral vector (Dravid et al., 2005; Zhou et al., 2007). After serial aerolysin selections of these GFP-expressing hES cells (called G-GFP thereafter), aerolysin-resistant (AR) hES cells lacking GPI-APs were obtained from two independent batch selections. By limit-

ing dilution, six independent AR hES cell clones were established (Table S2). Clones that lack GPI-APs but can be restored by *PIG-A* transgene expression were further expanded. One clone from each batch selection, AR1-clone 1 (AR1-c1) or AR2-c1, retained undifferentiated morphology after serial expansion and was characterized (Figure 1).

We found that both AR clones completely lack GPI-APs such as APase, CD90, CD55, CD59, and Cripto on cell surface (Figure 1), even though the protein portion was detected inside hES cells (Figure S1). Both AR hES clones can maintain undifferentiated morphology and express undifferentiated cell-surface markers such as SSEA3 and SSEA4 that are not GPI-APs (Figure 1). Both AR clones have a similar proliferation rate as compared to the parental G-GFP hES cells when cultured on feeder cells or under feeder-free conditions (data not shown). GPI-APs such as CD90, CD55, or CD59 can be restored by transduction of a lentiviral vector expressing the *PIG-A* coding sequence both in AR2-c1 (Figure 1C) and AR1-c1 cells (Zhou et al., 2007), indicating that the GPI-AP deficiency is due to the absence of *PIG-A*.

Once sufficient hES cells were obtained by expansion, both clones were confirmed to have a normal karyotype (Figure S2). The AR1-c1 clone is capable of maintaining a normal karyotype



**Figure 2. Analysis of *PIG-A* Gene Expression in Human ES Cells Lacking GPI-APs**

(A) Conventional RT-PCR of the *PIG-A* gene expression in undifferentiated ES cells (undiff. ES) and differentiated hES cells after BMP4 induction or embryoid body formation (EB) after 10 days. G-GFP hES cells (samples 1) and AR1-c1 (samples 2) clonal hES cells were analyzed side by side. In addition, we analyzed reconstituted AR1-c1 hES cells after transduction of *PIG-A* transgene that restored GPI-AP expression (sample 3). (B) Similar analysis of AR2-c1 hES cells (sample 4) and the AR2-c1 cells restored by the *PIG-A* transgene expression (sample 5). (C) Quantitative RT-PCR analysis of the *PIG-A* gene in AR1-c1 hES cells before and after differentiation. The relative level of *PIG-A* mRNA is first normalized by that of beta-actin and then by the level in teratoma (defined as 100), which was used as a common positive control in quantitative RT-PCR analyses. The mean and SD ( $n = 4$ ) were plotted in a log scale. The *PIG-A* mRNA in undifferentiated G-GFP cells is found at a low level (1% to 2% of teratoma), but elevated significantly after differentiation by BMP4 induction or EB formation (A, C, and D). Notably, the *PIG-A* mRNA level in undifferentiated AR1-c1 hES cells (sample 2) and AR2-c1 (sample 4) was much lower (~10 fold) than the control G-GFP cells by both assays in (A–C). The deficiency of *PIG-A* mRNA in AR1-c1 cells is more obvious after differentiation (A and C). (D) Northern blot of *PIG-A* mRNA in AR1-c1 cells confirmed RT-PCR data that the *PIG-A* deficiency is due to the lack of *PIG-A* mRNA.

over 2 years, although karyotypically abnormal cells occasionally arise. The AR2-c1 clone gained an extra Chromosome 12 in a small fraction (2/20) initially soon after clonal derivation. As happens often with wild-type hES cells after prolonged culture (Draper et al., 2004), mutated AR2-c1 cells that gain selective growth advantage soon took over the whole population and acquired additional mutations. Early-passage AR2-c1 cells with or without an extra chromosome 12 gave essentially the same results (see below). In this report, we present data obtained from karyotypically normal hES cells, mainly AR1-c1 hES cells, and G-GFP (H1) and H9 hES cells as controls.

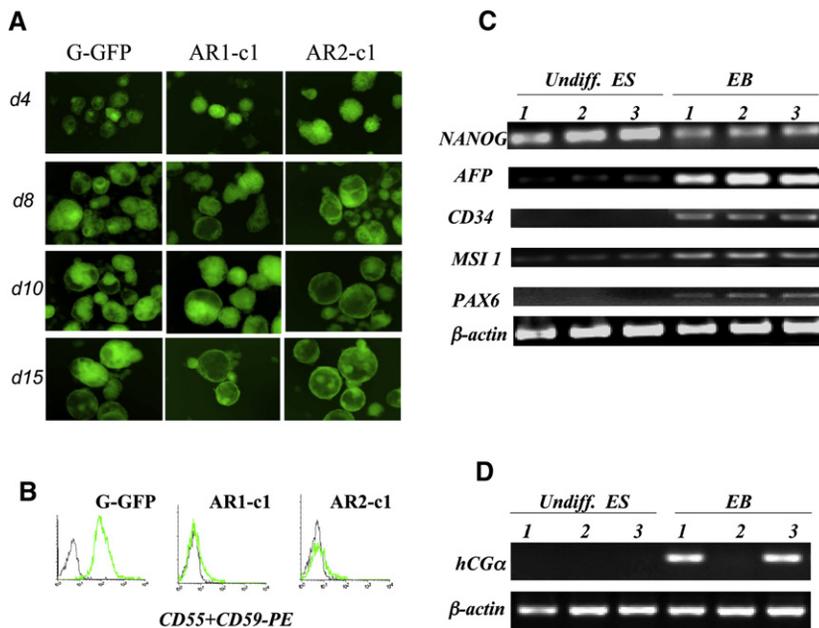
#### The GPI-AP-Deficient hES Cell Clones Lack *PIG-A* mRNA

We directly analyzed the *PIG-A* expression in normal and GPI-AP deficient undifferentiated hES cells cultured under a feeder-free condition with or without subsequent differentiation induction. As compared to somatic cell lines and differentiated (teratoma-derived) cells, undifferentiated normal (G-GFP) hES cells express a low level of *PIG-A* mRNA, as assayed by both conventional and quantitative RT-PCR analyses (Figure 2). Notably, *PIG-A* mRNA in AR1-c1 or AR2-c1 hES clone showed much a lower level than that in G-GFP hES cells (Figures 2A and 2B), consistent with the fact that the AR cells lack GPI-APs on cell surface. *PIG-A* mRNA levels in differentiated progeny after BMP4 induction or embryoid body (EB) formation was elevated significantly in the parental G-GFP cells, but remained low in AR1-c1 derivatives (Figure 2). Quantitative RT-PCR analysis using a different primer set revealed that the *PIG-A* mRNA level in the differentiated AR1-c1 cells is 450- and 52-fold lower than that in the parental G-GFP hES cells, after BMP4-induced differentiation or EB formation (Figure 2C). As expected, the AR1-c1 hES cells transduced

with a *PIG-A* expressing vector expressed *PIG-A* mRNA at a very high level before or after differentiation (Figures 2A–2C). Northern blot further confirmed the RT-PCR results: *PIG-A* mRNA of any size was not detected in AR1-c1 cells before the transgene restoration, whereas G-GFP cells expressed the wild-type (~3.6 kb) *PIG-A* transcript (Figure 2D). This is consistent with the fact that we did not detect any genetic mutation in all 6 exons and intron/exon junctions of the *PIG-A* gene in either AR hES clone after extensive sequencing (data not shown). The exact mechanisms of the observed *PIG-A* deficiency in the AR hES clones remain to be fully determined; nonetheless, we have obtained two *PIG-A* null hES clones lacking GPI-APs, permitting us to examine the roles of GPI-APs using hES cell-initiated developmental models.

#### Two GPI-AP-Deficient hES Clones Formed Morphologically Normal EBs and Hematopoietic Cells In Vitro

We next examined differentiation potentials of both AR1-c1 and AR2-c1 clones, in comparison with the parental G-GFP hES cells. Similarly to other normal hES cells, G-GFP hES cell aggregates cultured in suspension (with serum factors) developed into more structured (cystic) EBs. By day 8, enlarged EBs showed visible cavity. These cystic EBs are more evident by day 10–15 (Figure 3A). AR1-c1 and AR2-c1 both formed morphologically normal EBs at a rate similar to G-GFP hES cells (Figure 3A). Cells derived from EBs up to day 15 were further analyzed. The ubiquitously expressed GPI-APs, CD55 and CD59, remained absent in EB-derived cells from AR1-c1 and AR2-c1 clones (Figure 3B). This contrasts to previous studies that mES cells lacking *PIG-A* formed EBs poorly (Dunn et al., 1996).



**Figure 3. Embryoid Body Formation and Differentiation from the Parental and Two Independent PIG-A-/GPI-AP-Deficient hES Cell Clones**

(A) Three ES cell types were cultured in suspension as aggregates to form EBs. The morphology and numbers of EBs were monitored daily for 15 days. The EB morphology is better illustrated by constitutively-expressed GFP signal. The rate as well as morphology of EBs by three hES cell types was similar.

(B) Confirmation of the GPI-AP deficiency in EB-derived cells. Antibodies recognizing CD55 and CD59 were mixed and used to stain single-cell suspension.

(C) RT-PCR analysis of marker gene expression before and after EB formation at day 10. As expected, Nanog expression in G-GFP control cells (sample 1) is high in undifferentiated ES cells (undiff. ES) and visibly reduced after spontaneous differentiation by EB formation (EB). The gene expression of differentiated markers such as AFP (endoderm), CD34 (mesoderm), MSI 1, and PAX6 (ectoderm) were significantly elevated as compared to undifferentiated ES cells. AR1-c1 (sample 2) showed similar pattern to the G-GFP control, as did the AR1-c1 cells expressing *PIG-A* transgene (sample 3).

(D) RT-PCR analysis of a marker gene expression for trophoctoderm. *hCGα* expression from AR1-c1 hES cells (sample 2) failed to elevate after EB formation but was restored by *PIG-A* transgene expression. See more data by real-time quantitative RT-PCR in Figure S3.

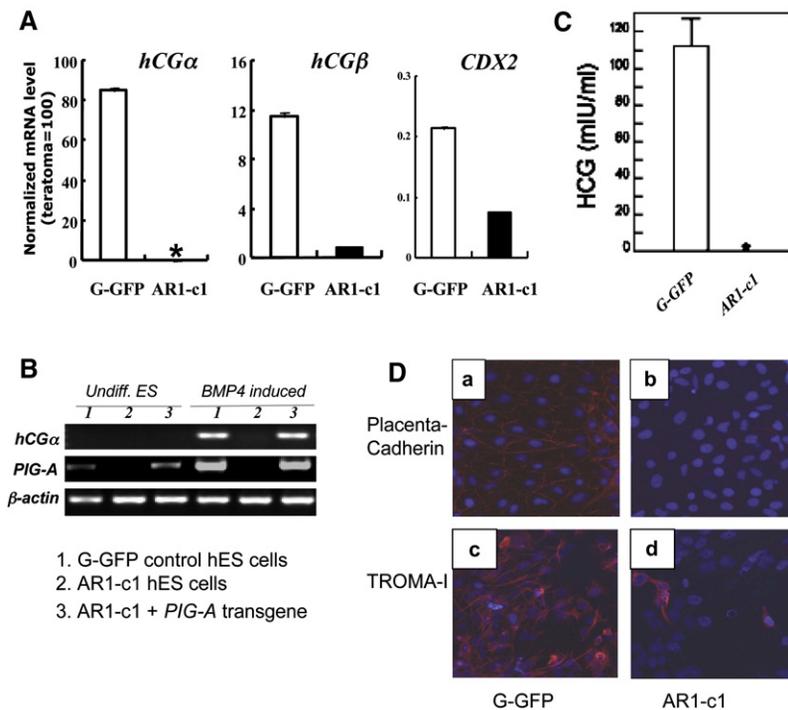
To examine the differentiation commitment to the 3 embryonic germ layers, we analyzed the acquired expression of lineage specific markers in EB-derived cells. The expression of AFP (endoderm), PAX6 and Musashi (MSI) 1 (ectoderm), and CD34 (mesoderm) marker genes within the EBs from AR1-c1 hES cells were comparable or moderately higher than within the G-GFP EBs (Figure 3C). Similar results were obtained using quantitative RT-PCR with different primer sets and detection probes (Figure S3). Although interesting differences were observed between two groups (such as levels of CD34 and NANOG expression), it appears AR1-c1 cells are, overall, competent in differentiation commitment to the three embryonic germ layers at this early stage. We further analyzed hematopoietic differentiation of AR1-c1 and AR2-c1 hES cells after EB formation (Figure S4). The level of produced CD34+ cells (hematopoietic and/or endothelial) or colony-forming hematopoietic progenitor cells from AR1-c1 clone appears comparable to the G-GFP control or the isogenic control where the *PIG-A* expression is restored by a transgene (Figure S4). Our results indicate that GPI-APs in hES cells are dispensable for entry to the 3 embryonic germ layers or the hematopoietic lineage commitment. The effects of GPI-APs on the progression along ectoderm and endoderm lineages are under investigation.

#### Defect in Trophoblast Differentiation from GPI-AP-Deficient hES Cells

Unlike mES cells, hES cells readily gave rise to trophoblasts after spontaneous or induced differentiation (Xu et al., 2002; Pera et al., 2004; Gerami-Naini et al., 2004). So, we also examined the expression of trophoblast or trophoctoderm (TE) markers from the same EBs (Figure 3). To our surprise, the expression of human chorionic gonadotropin alpha (*hCGα*), a TE marker,

failed to elevate after EB formation from AR1-c1 hES cells (Figure 3D). Similar results were obtained with the expression of CDX2 (Figure S3), a marker of TE lineage in early embryos (Strumpf et al., 2005; Niwa et al., 2000, 2005). The defect appears specifically due to the *PIG-A* and GPI-AP deficiency because the failure to turn on *hCGα* and CDX2 expression in AR1-c1 cells can be fully rescued by the *PIG-A* transgene (Figure 3D; Figure S3).

To confirm the link between defects of GPI-APs and trophoblast formation, we also used a second method of trophoblast formation from hES cells. The addition of exogenous BMP4 or a related family member to monolayer culture of hES cells that are cultured otherwise as undifferentiated cells (with feeder-derived factors and bFGF) rapidly induced morphological changes (Xu et al., 2002). Consistent with the previous report, we found that G-GFP hES cells differentiated into cells resembling trophoblasts when exogenous BMP4 (10–50 ng/ml) were added for 5 days. Nearly all the cells flattened after 7–10 days, and *hCG* hormone was readily detected as previously reported (Xu et al., 2002). Ten days after BMP4 induction of G-GFP hES cells, NANOG mRNA decreased and *hCGα* mRNA increased over 100-fold (Figure S3 and Figure 4A). However, *hCGα* gene expression in AR1-c1 hES cells failed to elevate after BMP4 treatment. Similar patterns were observed with other trophoblast markers such as *hCGβ* and CDX2 (Figure 4A). The deficiency of *hCGα* gene upregulation was rescued by the *PIG-A* transgene (Figure 4B). We further confirmed the deficiency of trophoblast formation at the protein level: the β subunit of secreted *hCG* in the AR1-c1 group was absent (Figure 4C). The lack of trophoblast formation in AR1-c1 cells after BMP treatment was further confirmed by the lack of induced placenta-cadherin (CDH3) and TROMA-1 that are normally accompanied with trophoblast



**Figure 4. BMP4 Induced Trophoblast Differentiation of Normal G-GFP hES Cells, but Not AR1-c1 hES Cells, Lacking GPI-APs**

The parental (G-GFP) and AR1-c1 hES cells were cultured under a feeder-free condition and treated with BMP4 (50 ng/ml) for 10 days. Then, cells were harvested for RT-PCR analyses (A and B) after the conditioned medium was collected and used to detect hCG hormone by ELISA (C). (A) Quantitative RT-PCR for trophoblast markers such as *hCGα*, *hCGβ*, and *CDX2*. \*Undetectable as in undifferentiated hES cells after 40 cycle PCR. Profiles of the gene expression of other markers (such as *NANOG*, *AFP*, *CD34*, and *PAX6*) after BMP4 induction are shown in Figure S3. (C) Conventional RT-PCR using a different primer set for *hCGα* (as in Figure 3D) and a specific primer set for *PIG-A* as in Figure 2A. RNA from undifferentiated ES cells (undiff. ES) or after BMP4 induction (BMP4 induced) from either G-GFP (sample 1) or AR1-c1 (sample 2) hES cells was used. In addition, we also included AR1-c1 derivatives in which a *PIG-A* transgene is expressed (sample 3) in this experiment. (D) Immunofluorescent staining for trophoblast markers placenta-cadherin (upper row, a and b) and TROMA-I (lower row, c and d) from the differentiated G-GFP (left) and AR1-c1 cells (right) 11 days after BMP4 induction. The presence of antigen is visualized by anti-mouse IgG conjugated with Alexa 595 (red) and nuclei were stained by DAPI (blue). While the trophoblast marker induction is obvious in the differentiated G-GFP cells, it is much lower in the GPI-AP-deficient AR1-c1 cell population.

differentiation (Niwa et al., 2005; Brulet and Jacob, 1982), as seen with G-GFP parental hES cells (Figure 4D).

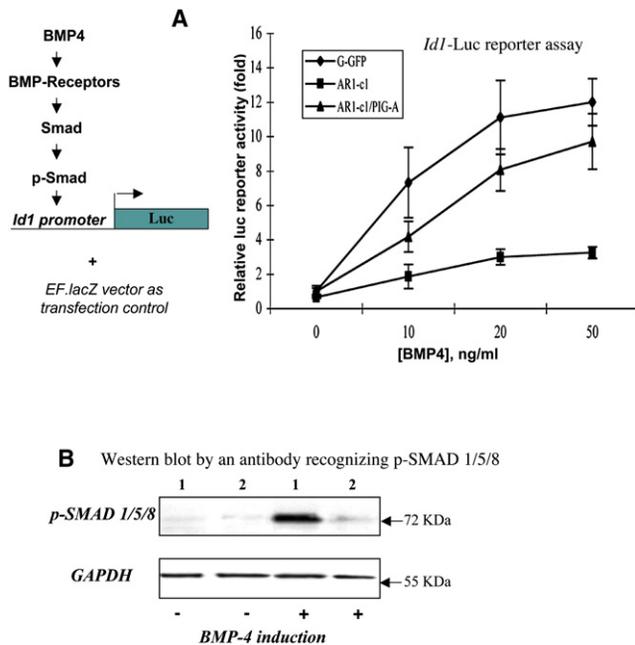
### BMP Signaling Is Impaired in AR1-c1 hES Cells Lacking GPI-APs

We next investigated underlying mechanisms of defective trophoblast formation from GPI-AP-deficient hES cells by employing the BMP4-mediated differentiation system. BMP4 functions through BMP receptor-mediated intracellular signaling by activating one or more members of SMAD family (SMAD 1, 5, and 8) and other targets (Zhang and Li, 2005). Two BMP receptors, type I and II, have been identified. Type II BMP receptors are primarily involved in ligand binding and activating a BMP type I receptor that has intrinsic serine/threonine kinase activity (Zhang and Li, 2005). One of three type I BMP receptors, *ALK2*, *ALK3* (BMPR-Ia), or *ALK6* (BMPR-Ib), subsequently activates (phosphorylates) SMAD 1, 5, or 8 and other targets and turns on downstream signaling cascades. In conjunction with other transcriptional factors, the BMP-activated SMAD complex modulates the expression of an array of genes including four members of *Inhibitor of differentiation* or *Id* genes (Hollnagel et al., 1999; Lopez-Rovira et al., 2002; Ying et al., 2003). We found that in hES cells the expression of human *Id* or *ID* genes (*ID1* to *ID4*), especially that of *ID1* and *ID2*, is indeed activated immediately by BMP4, but not by TGFβ (Figure S5). Our data extended the previous report with the *ID1* gene (Xu et al., 2005). The direct activation of *ID1* and *ID2* promoters by BMP4 signaling was further confirmed in both H1 and H9 hES cell lines by a Luciferase (Luc) reporter assay (Figure S5).

We next directly examined BMP4 signaling activation in AR1-c1 hES cells using two assays. In the first assay, the *Id1*-Luc reporter was transfected into either AR1-c1 or G-GFP control hES

cells followed by overnight BMP4 stimulation (Figure 5A). While the exogenous BMP4 stimulates the reporter activity up to ~12 fold in G-GFP cells in a dose-dependent manner, the stimulation on GPI-AP-defective AR1-c1 cells could reach only ~3 fold (Figure 5A). Notably, the *PIG-A* transgene expression in AR1-c1 cells largely restored the BMP4-mediated activation in this assay as in BMP4-induced trophoblast differentiation (Figure 5A). In the second assay, we measured earlier events of BMP signaling activation by western blot. We used a specific antibody recognizing the activated (phosphorylated) form of three highly related SMAD proteins (SMAD 1/5/8) that are direct targets of activated BMP receptors type I (BMPR-I) after BMP4 stimulation. Consistent with previous reports, phosphorylated forms of SMAD 1/5/8 were low in undifferentiated hES cells before BMP4 induction (Figure 5B). BMP4 induction drastically increased the level of phosphorylated forms of SMAD 1/5/8 in G-GFP cells, but not in AR1-c1 cells. Based on the results obtained from the two independent assays, we conclude that BMP signaling is defective in AR1-c1 cells, likely at the level of BMPR-I activation.

We also examined the requirement of BMP signaling for trophoblast differentiation from a different hES cell line H9 using a known BMP antagonist NOGGIN. As previously reported (Xu et al., 2002), NOGGIN blocked BMP4-induced trophoblast differentiation from H9 as well as H1 (G-GFP) hES cells in monolayer cultures. We next tested if NOGGIN could also prevent trophoblast differentiation following EB formation (in the absence of exogenous BMP). Ten days after EB formation from H9 hES cells, undifferentiated markers such as *NANOG* decreased, whereas lineage (differentiation) markers such as *CDX2*, *hCGα*, and *PAX6* increased (Figure S6). Increasing concentrations of NOGGIN resulted in a sharp reduction of *CDX2* and *hCGα* upregulation, but affected little on *NANOG* downregulation. In fact,



**Figure 5. BMP Signaling Activation Is Significantly Reduced in AR1-c1 hES Cells Due to the PIG-A and GPI-AP Deficiency**

(A) BMP4 signaling activation in G-GFP or AR1-c1 hES cells was measured by a Luciferase reporter (*Id1-Luc*) controlled by the *Id1* gene promoter. Six hours after transfection of the reporter plasmid, cells were stimulated with various concentration of BMP4 for 20 hr and harvested. The *Id1-Luc* activity was measured, normalized, and calculated relative to that of G-GFP without BMP4 stimulation (defined as 1). The normalized mean and SD ( $n \geq 5$ ) were plotted. In one experiment, the *Id1-Luc* activity in AR1-c1 hES cells that have been transduced with PIG-A transgene was also measured before and after BMP4 induction ( $n = 5$ ). Combined from two experiments ( $\Sigma n = 7$ ), the mean of the reconstituted AR1-c1/PIG-A cells is statistically greater than the AR1-c1 cells ( $p < 0.05$ ) and insignificant from G-GFP hES cells ( $p > 0.05$ ).

(B) Western blot to detect the phosphorylated (activated) form of three highly related SMAD proteins (1/5/8) without or with BMP4 induction. G-GFP (1) or AR1-c1 (2) hES cells were treated with BMP4 for 6 hr, and cell lysates were analyzed by western blotting, using purified specific antibodies recognizing the phosphorylated SMAD1/5/8. After stripping, the blot was reprobed by a specific antibody recognizing the housekeeping protein GAPDH.

the upregulation of PAX6 (an ectoderm marker) was further enhanced with increased concentrations of NOGGIN. Mesoderm marker upregulation following EB formation is less sensitive to NOGGIN blocking (Figure S6). Taken together, we show that in two distinct hES cell lines the dependency on BMP signaling for trophoblast formation is greater than some other lineages.

#### Overexpression of an Activated BMPR-I Gene in AR1-c1 hES Cells Restored BMP Signaling and Trophoblast Differentiation

Using the unique AR1-c1 (H1) hES cells, we examined if the BMP signaling as well as trophoblast differentiation could be restored by the overexpression of an activated form of BMPR-I in the deficient hES cells. We used an activated form of ALK3 (also known as BMPR-Ia) gene which contains a mutation (Q233D), rendering enhanced activation without or with less BMP ligands (Chen et al., 1998). We transfected AR1-c1 and G-GFP cells with such an ALK3 vector together with the *Id1-Luc* reporter, and treated cells

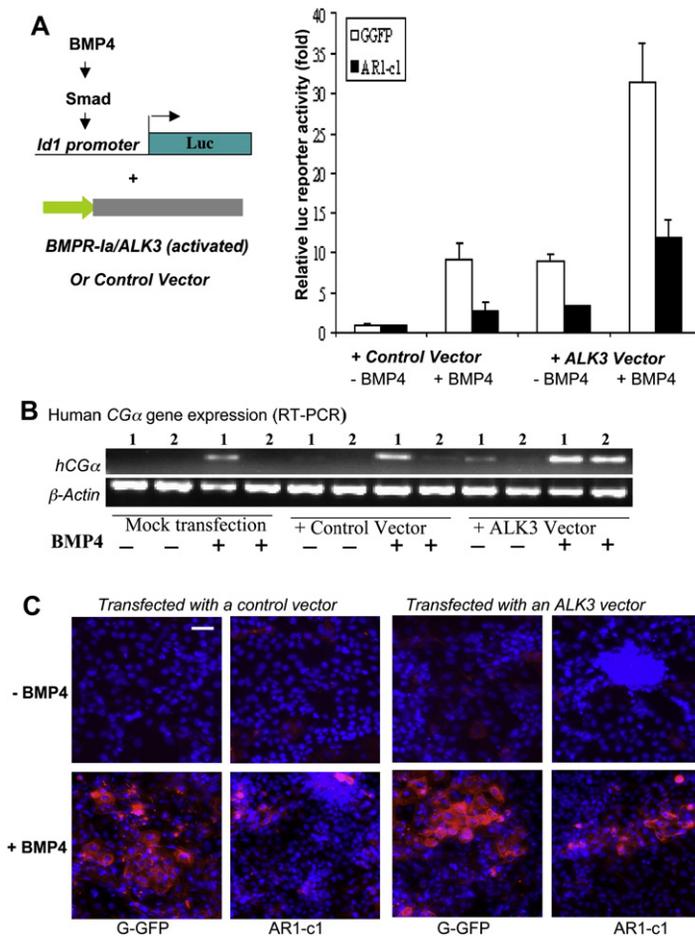
with or without BMP4 after transfection (Figure 6A). Twenty hours after BMP4 induction following cotransfection with the control plasmid, the BMP4 signaling measured as the *Id1-Luc* activation was as expected: BMP4 (50 ng/ml) stimulated the reporter activity by  $\sim 14$  fold in G-GFP cells, but only  $\sim 4$  fold in AR1-c1 cells as in Figure 5A. Transfection of the activated ALK3 gene to AR1-c1 cells moderately stimulated the reporter in the absence of exogenous BMP4. When BMP4 was added, however, the level of BMP4 activation in AR1-c1 cells with ALK3 transfection is comparable to that of G-GFP cells without transfection (Figure 5A) or transfected by a control plasmid (Figure 6A), even with a transfection efficiency of 40%–50%.

To test whether the overexpression of the activated ALK3 transgene in AR1-c1 cells can also restore trophoblast differentiation, we monitored upregulation of the *hCG $\alpha$*  gene expression (Figure 6B). After two rounds of transfection (at day 0 and 2) with the ALK3 or control vector, G-GFP or AR1-c1 cells treated with or without BMP4 were harvested at day 5. The levels of *hCG $\alpha$*  gene expression in the control vector-transfected cells were similar to the mock-transfected cells as expected: the *hCG $\alpha$*  gene expression was only turned on after BMP4 induction, and it was much weaker in AR1-c1 cells even after BMP4 induction. When AR1-c1 hES cells were transfected with the activated ALK3 gene, however, the BMP4-mediated *hCG $\alpha$*  gene upregulation is restored (Figure 6B). The presence of acquired TROMA-I protein, a trophoblast marker, further supported that trophoblast formation in AR1-c1 hES cells can be restored by the activated ALK3 gene transfection (Figure 6C). Similar results were observed with an activated form (Q203D) of ALK6 also known as BMPR-Ib (Chen et al., 1998; data not shown). Based on BMPR-I reconstitution assays, we conclude that one or more GPI-APs are critical for full activation of BMP4 receptors and human trophoblast differentiation.

#### Overexpression of Membrane-Bound DRAGON in AR1-c1 hES Cells Also Restored BMP Signaling and Trophoblast Differentiation

We then focused on a group of GPI-APs that are recently identified as accessory coreceptors for BMP2 and BMP4 (Samad et al., 2005; Babitt et al., 2005, 2006). This GPI-AP subfamily include 3 structurally related proteins called Repulsive Guidance Molecule a (RGMa), DRAGON (also called RGMb), and HFE2 (RGMc). It has been demonstrated that GPI-anchored (but not soluble) RGM proteins facilitate the binding of a BMP2 or BMP4 ligand to one or more forms of type II BMP receptors and potentiate the downstream BMP signaling (Samad et al., 2005; Babitt et al., 2005, 2006; Xia et al., 2007).

By RT-PCR, we confirmed the gene microarray data that DRAGON and, to a lesser extent, RGMa gene are expressed in all the hES cells used (Table S1; Figure S7A). Similar to previous reports with other cell types, soluble forms of DRAGON or RGMa proteins purchased did not activate BMP4 signaling in hES cells (data not shown). Among several approaches we tested, we observed that transgene expression of a modified DRAGON cDNA in AR1-c1 hES cells made functional constitution as did by the PIG-A transgene (Figure 7). Because the PIG-A deficiency results in a posttranslational defect for GPI-APs, transgene expression of the wild-type DRAGON cDNA (like the endogenous gene) could not produce a GP-anchored product on cell surface, even the precursor protein is made in cytoplasm. Therefore, we explored



**Figure 6. Transient Transfection of an Activated BMP-RI Gene Restored the BMP Signaling and Trophoblast Formation**

(A) G-GFP or AR1-c1 hES cells were transfected with an activated ALK3 gene or control vector, together with the Id1-luciferase reporter plasmid and the EF.lacZ as in Figure 5A. Six hours after transfection, cells were stimulated in the absence (–) or presence (+) of BMP4 (50 ng/ml) for 20 hr and harvested. The Id1-luciferase activity was measured, normalized by the lacZ activity, and calculated relative to the level without BMP4 stimulation (defined as 1) for either G-GFP or AR1-c1 hES cells. The normalized mean and SD values from 3 independent experiments were combined and plotted ( $\Sigma n = 8$ ). The difference of the ALK3-transfected, BMP4-activated AR1-c1 cells versus any of other 3 AR1-c1 groups is significant ( $p < 0.05$ ).

(B) Human *CGα* gene expression in AR1-c1 cells after ALK3 transfection and BMP4 stimulation. G-GFP (samples 1) or AR1-c1 (samples 2) hES cells in pair were transfected with the activated ALK3 gene or control vector at day 0 for 6 hours and treated in the absence or presence of BMP4 (50 ng/ml) for 2 days. Two days after, the same treatment was repeated. After a total of 5 days, various types of transfected cells were either harvested for RT-PCR (B) or fixed for immunostaining (C). Human *hCGα* gene expression was measured by RT-PCR as in Figure 3D and Figure 4B.

(C) TROMA-I staining of the double-transfected hES cells at day 5 as in (B). Scale bar, 50  $\mu$ m.

the reconstitution approach by making a cell-surface form of DRAGON using the coding sequence of the mature DRAGON domain fused to a transmembrane (TM) domain provided by an expression vector called pDisplay (Figure S7B). When overexpressed in 293T cells, the pDisplay vector encoding the fusion TM protein (DRAGON) indeed directed the cell surface expression as measured by flow cytometry (Figure S7) or immunostaining (data not shown). When the same vector was used to transfect AR1-c1 hES cells as we did previously with the ALK3 vector (Figure 6A), the DRAGON vector reconstituted the BMP4-mediated Id1-Luc activation (Figure 7A). Similarly, serial (transient) transfection of DRAGON vector in AR1-c1 cells resulted in the upregulation of trophoblast marker expression at day 5, such as *hCGα* by RT-PCR (Figure 7B) and TROMA-I by antibody staining (Figure 7C). Finally, we generated knockdown H9 hES cells that are stably transduced with a lentiviral vector expressing a small hairpin RNA targeting the DRAGON or *RGMA* gene (Figure S8). The H9 hES cells with reduced DRAGON or *RGMA* expression were impaired in BMP4-mediated Id1-Luc activation, *hCGα* upregulation, and acquired TROMA-I expression after BMP stimulation, a phenotype similar to AR1-c1 (H1) hES cells lacking all the GPI-APs.

## DISCUSSION

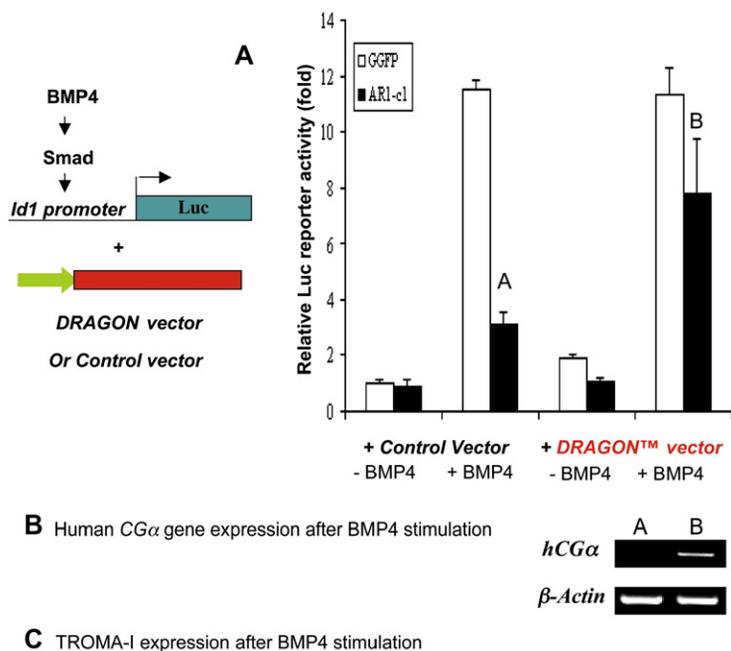
Specific gene inactivation in hES cells may provide novel genetic and developmental models for human diseases. Previously, two

reports described the inactivation of the X-linked *HPRT* gene in a XY hES cell line as a potential model for Lesch-Nyhan disease (Zwaka and Thomson, 2003; Urbach et al., 2004). Although these *HPRT* knockout hES cells indeed lacked the encoded enzyme, the biological or pathological consequences remain to be determined (Zwaka and Thomson, 2003; Urbach et al., 2004).

In contrast, the present study reports the establishment of hES cells lacking *PIG-A* gene expression, resulting in clonal hES cells deficient of a group of cell surface (GPI-anchored) proteins including APase, Cripto, and CD90. Our data demonstrate that GPI-APs are not essential for self-renewal of hES cells in culture, although they are preferentially expressed in undifferentiated hES cells.

PIG-A- and GPI-AP-deficient hES cells also appeared normal in the initial differentiation into derivatives of the three embryonic germ layers. Among mesoderm-derived lineages we examined, the production of CD34+ hematopoietic precursor cells in vitro from GPI-AP-deficient hES cells also appeared normal, thus allowing us to next examine the effect of PIG-A and GPI-AP deficiency on downstream steps of hematopoiesis. In addition, further neural cell development may require multiple GPI-APs based on previous mouse studies. The availability of hES cells lacking PIG-A and GPI-APs and their derivatives expressing an inducible PIG-A transgene may help us to study roles of various GPI-APs in the development of human cell types other than trophoblasts as demonstrated in this study.

We unexpectedly found that PIG-A-/GPI-AP-deficient hES cells failed to differentiate into trophoblasts. One of the prominent innovations in early mammalian embryogenesis is the formation of the trophoblast, the specialized tissue that subsequently forms trophic interface between the embryo and the mother. The first overt differentiation event in mammalian embryos is the formation of TE (the outer epithelial layer of the blastocyst), which goes on to



**Figure 7. Transient Transfection of a Transmembrane Form of DRAGON Restored the BMP Signaling Activation and Trophoblast Formation**

(A) G-GFP or AR1-c1 hES cells were transfected with the engineered DRAGON vector or the control pDisplay vector, together with the *Id1*-luciferase reporter plasmid and the EF.lacZ as in Figure 6A. Six hours after transfection, cells were stimulated in the absence (–) or presence (+) of BMP4 for 20 hr and harvested. The *Id1*-luc activity was measured, normalized by the lacZ activity, and calculated relative to the level without BMP4 stimulation (defined as 1) for each cell type. The normalized mean and SD values ( $n = 4$ ) were plotted. The difference between Samples A and B is significant ( $p < 0.05$ ). (B) Human CG $\alpha$  gene expression in AR1-c1 cells after DRAGON transfection and BMP4 stimulation. G-GFP (sample 1) or AR1-c1 (sample 2) hES cells in pair were transfected with the DRAGON or control vector at day 0 for 6 hours and treated in the absence or presence of BMP4 (50 ng/ml) for 2 days. Two days after, the same treatment was repeated. After a total of 5 days, various types of transfected cells were either harvested for RT-PCR (B) or fixed for TROMA-I immunostaining (C). Scale bar, 50  $\mu$ m.

develop into placenta. The cells isolated from TE, including trophoblast stem (TS) cell lines, can only commit to the cell fate of trophoblastic tissues. In contrast, the inner cell mass in the mouse blastocyst maintains pluripotency and later develops into epiblasts and then the three embryonic germ layers and other extraembryonic tissues. Numerous studies showed that mES cells, derived from the inner cell mass of mouse blastocysts, rarely form trophoblasts naturally. An exception is when the level of master transcriptional factors is altered by genetic manipulation, e.g., by enforced reduction of *Oct4* expression or *Cdx2* overexpression (Niwa et al., 2000, 2005; Zhang et al., 2006). It was recently reported, however, that some mES cell lines, but not others, can differentiate into trophoblasts (or TS cells) at a low level together with many mesodermal cells when cultured on collagen IV (Schenke-Layland et al., 2007). It remains to be determined whether hES cells can also be induced to trophoblasts or TS cells by culturing on collagen IV or under other conditions, in BMP-dependent or -independent manners.

It is now clear that most, if not all, hES cell lines readily form trophoblasts and other extraembryonic lineages after differentiation induction. With the BMP4 addition into a feeder-free culture condition for  $\geq 5$  days, undifferentiated hES cells cultured as a monolayer differentiate preferentially into trophoblasts

(Xu et al., 2002). This unique feature of hES cells helped us to discover that one or more GPI-APs are required for trophoblast differentiation. Mechanistically, our data reveal that GPI-AP-mediated high-level BMP activation is required for human trophoblast formation. It is of interest to determine whether this mechanism also holds true in other placenta-bearing mammals such as mice. It is possible that species difference may exist between humans and mice in this regard as seen between hES and mES cells (Ginis et al., 2004). For example, BMP signaling mediated by *Alk3* (*Bmpr-1a*) is required for derivation of mES cells (Qi et al., 2004). BMP4 also cooperate with LIF to maintain the proliferation of undifferentiated mES cells (Ying et al., 2003; Qi et al., 2004). However, adding BMP4 stimulates differentiation of hES cells under culture conditions that otherwise promote self-renewal of hES cells as shown here and in previous studies (Xu et al., 2002, 2005; David et al., 2005). Blocking endogenous BMP (and those made by feeder cells) by soluble BMP antagonists enhanced the self-renewal of hES cells (Pera et al., 2004; Xu et al., 2005). Thus, it appears that in undifferentiated hES cells BMP activation is kept at an inactive or low level. This is in a sharp contrast to mES cells where BMP signaling is required for their self-renewal (Ying et al., 2003; Qi et al., 2004). However, hES cells can respond to BMP stimulation acutely and differentiate.

It has been known that some cell-surface proteins (also called as type III receptors) are important to the signaling by some members of the TGF $\beta$ /Activin/Nodal/ BMP ligand superfamily, positively regulating ligand activities (Zhang and Li, 2005). For example, cell-surface protein  $\beta$ -glycan or CD105 (endoglin) functions as a coreceptor for TGF $\beta$ . Cripto, another GPI-AP, functions as a coreceptor for Nodal that utilizes activin receptors (Minchiotti et al., 2000). Very recently, DRAGON and related proteins RGM were identified as coreceptors for BMP2 and BMP4 (Samad et al., 2005; Babitt et al., 2005, 2006). Notably, DRAGON and other RGM members such as RGMa and RGMc (HFE2) are

GPI-APs. One possibility is that a membrane-anchored RGM protein, although not absolutely essential for BMP signaling, may help to sensitize all or some forms of type II BMP receptors (BMPRII, actRIIA, or ActRIIB) to the binding of a BMP ligand, thus enhancing or extending downstream BMP (type I receptor) signaling (Xia et al., 2007). Among these 3 RGMs, DRAGON/RGMb is expressed in mouse E2.5 preimplantation embryos and in later stages of embryos from E7 to E17 (Samad et al., 2005). DRAGON is also found expressed in human reproductive axis (Xia et al., 2005). We found that DRAGON and, to a lesser extent, RGMa genes are expressed in hES cells and postulated that the defect of GPI-AP-deficient (AR1-c1) hES cells in BMP signaling is primarily due to the lack of a DRAGON/RGM (GPI-AP) subfamily member that serves as a coreceptor for BMP4. To express a functional form of this subfamily on the surface of PIG-A-/GPI-AP-deficient AR1-c1 hES cells that are defective posttranslationally and lack all the GPI-APs, we made a novel DRAGON cell-surface protein by fusing the mature DRAGON domain with a transmembrane domain. As predicted, the overexpression of the engineered cell surface DRAGON protein (DRAGON<sup>TM</sup>) restored both high-level BMP activation and trophoblast differentiation (Figure 7) as did an activated type I BMP receptor (ALK3 or ALK6) gene vector (Figure 6). Conversely, knockdown DRAGON or RGMa gene expression in H9 hES cells (Figure S8) phenocopied the observation with AR1-c1 (H1) hES cells. Based on these data, we suggest that cell surface forms of DRAGON/RGM protein family play a critical role for high-level activation of BMP signaling in hES cells, which is in turn critical for trophoblast differentiation. How the members of DRAGON/RGM GPI-AP subfamily modulate or oppose related signaling such as those mediated by Activin/Nodal remains to be determined (Besser, 2004; James et al., 2005; Xia et al., 2007). Our approach of constructing a transmembrane form of GPI-APs such as DRAGON<sup>TM</sup> and reconstituting in AR1-c1 hES cells will likely help to delineate the first step of requirement for other GPI-APs during development using hES cell initiated developmental systems.

It is generally believed, based on mouse and other lower organism models, that mesodermal induction is dependent on BMP signaling (Hogan, 1996). However, mesodermal differentiation in AR1-c1 cells lacking GPI-APs appears normal (e.g., CD34 gene expression in Figure S3 and CD34<sup>+</sup> cell production in Figure S4), whereas trophoblast differentiation fails due to the reduced level of BMP signaling. One hypothesis is that GPI-anchored coreceptors such as DRAGON are only critical to the full activation of BMP signaling and, in turn, trophoblast or TE formation. This hypothesis then easily explains the fact that mesoderm induction was not affected in GPI-AP-deficient AR1-c1 hES cells. This hypothesis is also supported by gene array data that BMP ligands (such as *BMP4*) and downstream target genes (such as human *ID2* and *eHAND/Hand1*) are expressed significantly higher in human TE tissues versus inner cell mass (Adjaye et al., 2005) and also higher (such as *Id2*) in mouse TS cells versus mES cells (Tanaka et al., 2002). The generality of this novel hypothesis, such as in mice and other placental-bearing mammals, however, remains to be fully investigated. The present study highlights the excitement that hES cells provide an unprecedented and much-needed research tool for understanding the development and function

of human cells and tissues in normal and pathological processes.

## EXPERIMENTAL PROCEDURES

### Human ES Cell Culture

The H1 and H9 cell lines are obtained from WiCell Research Institute, Inc. (Wisconsin, MI) and propagated as previously described (Zhan et al., 2004; Dravid et al., 2005; Zhou et al., 2007). For the feeder-free culture and proaerolysin selection, undifferentiated hES cell clumps were passaged onto Matrigel (BD Biosciences, 1/30 dilution)-coated tissue plates and cultured in primary embryonic fibroblast (pMEF)-conditioned medium (Dravid et al., 2005). An H1 hES cell preparation (G-GFP, nonclonal) that stably expresses GFP after lentiviral vector transduction (Dravid et al., 2005; Zhou et al., 2007) was used as the starting cell population to select GPI-AP-deficient cells by proaerolysin selection. Cells were incubated with 500 nM proaerolysin (Protox Biotech, Victoria, Canada) at 37°C for 30 min and cultured under the feeder-free condition for one passage (5–7 days). The (pro-)aerolysin-resistant (AR) hES cells were expanded and monitored for the presence or absence of GPI-APs such as Thy-1/CD90 on cell surface. A detailed procedure of how we established two independent clones (AR1-c1 and AR2-c1) is described in Table S2. Gene transduction of AR hES cells by an inducible lentiviral vector (iDuet101/*PIG-A*) was previously described (Zhou et al., 2007). The karyotyping was conducted (300–400 bands, 20 mitotic spreads) as previously described (Zhan et al., 2004).

### Induced hES Cell Differentiation

Various hES cell lines cultured under the feeder-free condition were used for embryoid formation (in suspension, with 20% fetal bovine serum) as previously described (Zhan et al., 2004). For BMP4 induced differentiation, hES cells were cultured under the feeder-free condition (on Matrigel, serum-free and with pMEF-conditioned medium) in the presence of exogenous BMP4 (Xu et al., 2002). Unless otherwise indicated, 50 ng/ml BMP-4 (R&D Systems) was used. The supernatant was collected at day 10 for a human chorionic gonadotropin (hCG) ELISA (with an antibody specific to the hCG $\beta$ ) performed by Johns Hopkins Hospital. The BMP4-treated cells were analyzed subsequently by either RT-PCR or immunostaining.

### Immunostaining

Adherent hES cells before or after differentiation were fixed with PBS containing 4% paraformaldehyde for 20 min at RT. Following blocking of nonspecific binding with 4% goat serum, the cells were incubated with mouse monoclonal (IgG) antibodies recognizing alkaline phosphatase (TRA-2-49), SSEA-4 (MC-813-70), and TROMA-1 (Developmental Studies Hybridoma Bank, Iowa City, IA) or Cripto and placenta-Cadherin/CDH3 (both from R&D Systems). The stained cells were visualized by anti-mouse goat IgG conjugated with Alexa 594 or Alexa 555 (Invitrogen). For intracellular staining of TROMA-1 and Cripto, fixed cells were first permeabilized by treatment with 0.1% Triton X-100 and 1% BSA for 45 min. Flow cytometric (FACS) analysis was used as previously described (Zhou et al., 2007).

### Analysis of Gene Expression by RT-PCR

Total RNA isolated from various hES cell populations before and after induced differentiation was analyzed as before (Dravid et al., 2005). Primers used for semiquantitative PCR are listed in Table S3. For quantitative (real-time) RT-PCR, specific primers and probes were purchased from Applied Biosystems (Foster City, CA) and used as described before (Dravid et al., 2005). Total RNA isolated from normal hES cell-derived teratoma (consisting of mixed cell population) was used to normalize between various hES cell samples before or after differentiation. After normalization to the level of the  $\beta$ -actin gene, the expression level of each gene is plotted in a log scale relative to the level of the given gene expression in teratoma defined as 100.

### Northern Blot

RNA (10  $\mu$ m total) of undifferentiated and BMP4-induced hES cells were denatured, separated by electrophoresis, and transferred onto Hybond-N filter (Amersham Biosciences, Piscataway, NJ). After crosslinking RNA by UV

irradiation, the filter was hybridized with  $\alpha$ -<sup>32</sup>P-dCTP (Amersham Biosciences)-labeled *PIG-A* gene probe in ULTRAhyb Hybridization Buffer (Ambion, CA) overnight. After stripping, the filter was re-probed with the  $\beta$ -actin gene probe.

#### Western Blot

The protein extracts from hES cells  $\pm$  BMP4 induction (50 ng/ml for 6 hr) were made and separated by 8%–16% SDS-PAGE (Bio-RAD Laboratories, Hercules, CA) and were transferred onto Hybond-P membrane (Amersham Biosciences). Purified antibodies recognizing phosphorylated SMAD 1/5/8 (Cell Signaling Technology Inc.) were used (James et al., 2005). The purified rabbit antibody recognizes phosphorylated SMAD 1/5/8 at SMAD 1 (S463/465), SMAD 5 (S463/465), and SMAD 8 (S426/428). After stripping, the filter was re-probed with an antibody recognizing GAPDH, a housekeeping protein used as an internal control.

#### Construction and Use of a Vector Expressing a Transmembrane Form of DRAGON Protein

To express the DRAGON/RGMB molecule on the cell surface of AR1-c1 hES cells deficient in *PIG-A* and all the GPI-APs, we had to engineer a chimeric protein in which the mature form of DRAGON peptide (without the C-terminal region that should be cleaved by the GPI anchoring process) is fused to a transmembrane (TM) domain. The cDNA encoding the mature form of human DRAGON/RGMB (NP\_001012779, from the 87th to 452th amino acids, lacking the 26 amino acids at the C terminus) is cloned and inserted into the expression vector called pDisplay (Invitrogen) at the BglII and Sac II sites (Figure S7B). This allows in-frame fusion of DRAGON mature peptide to the TM domain of a PDGF receptor, resulting in a TM called DRAGON<sup>TM</sup>. The cell-surface expression of DRAGON from the pDisplay-based vector is shown in Figure S7.

#### Transient Transfection of hES Cells to Measure BMP4-Induced Signaling or Restoration

We used the reporter plasmid in which the luciferase gene is controlled by the BMP-responsive element from the *Id1* promoter (*Id120-Luc*) (Lopez-Rovira et al., 2002) or the *Id2* promoter (from Dr. Xiao-Hong Sun, Oklahoma Medical Research Foundation). Human ES cells plated on Matrigel-coated 24-well plates were transfected by lipofectamine 2000 as previously described (Dravid et al., 2005). In short, 0.6  $\mu$ g *Id120-Luc* plasmid and 0.2  $\mu$ g EF.lacZ plasmid were added into transfection mixture (0.1 ml) and added to 0.5 ml culture medium. Six hours after transfection, hES cells were treated with various concentrations of BMP4. After an additional 24 hr, transfected cells were lysed and normalized luciferase activities were measured (Dravid et al., 2005).

We also cotransfected AR1-c1 and G-GFP hES cells by a plasmid vector expressing an activated form of ALK3 (Q233D) or ALK6 (Q203D) gene (Chen et al., 1998) or from the pDisplay-based DRAGON vector. A mixture of 0.3  $\mu$ g of an expression vector and 0.3  $\mu$ g *Id120-Luc* reporter, plus 0.2  $\mu$ g EF.lacZ plasmid, was used ( $\Sigma = 0.8 \mu$ g per well). Human ES cells under the feeder-free conditions were transfected by either ALK3 or ALK6 expression vector at day 0 for 6 hr and then treated with or without BMP4 (50 ng/ml). BMP4-induced *Id1-Luc* activities were measured 20 hr after. For reconstitution of trophoblast formation in AR1-c1 cells, 0.8  $\mu$ g of the activated ALK3 or pDisplay-based DRAGON vector was used to transfect hES cells per well in 24-well plates. We repeated the procedure 48 hr later. By day 5, cells were either harvested to RT-PCR analysis for elevated expression of trophoblast genes or fixed for staining by TROMA-1.

#### Statistical Analysis

Data plotted are typically expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). Significance of differences was examined using the Student's *t* test (two-sided, unequal variance), or nonparametric Mann-Whitney rank sum test when sample sizes were smaller. It is considered significant if *p* value is  $\leq 0.05$ .

#### SUPPLEMENTAL DATA

The Supplemental Data include three tables and eight figures and can be found with this article online at <http://www.cellstemcell.com/cgi/content/full/2/4/345/DC1/>.

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