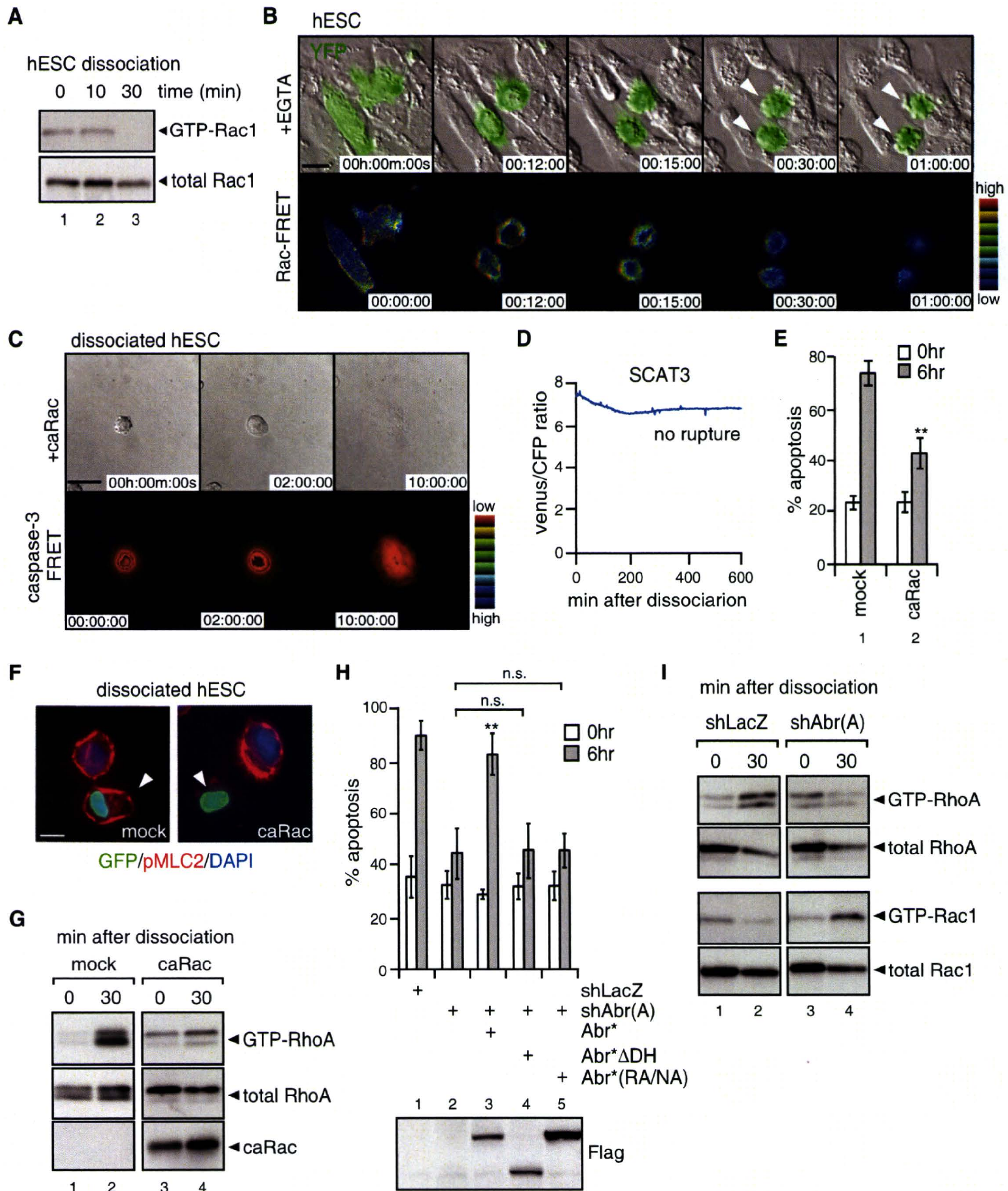


**Figure 6. Rho-High/Rac-Low State Plays a Key Role for Induction of Myosin Hyperactivation**

(A) Measurement of Rac activity in dissociated hESCs by pull-down assay.

(B) Snapshots of EGTA-treated hESC expressing the Rac-Raichu FRET probes (identified as a YFP-positive cell). White arrowheads indicate blebbing cells. The scale bar represents 10 μm.

(C and D) Effects of a constitutive active form of Rac1 (caRac, Rac1V12) on blebbing and apoptosis in dissociated hESCs. Snapshots of dissociated hESCs expressing the SCAT3 probes together with caRac (C) The scale bar represents 20 μm. The time course of the mean Venus/CFP ratios over the whole cell was shown (D).

(E) Apoptosis assay in caRac-expressing hESCs. **$p < 0.01$ in t test ($n = 3$).

(F) Decreased MLC2 phosphorylation in dissociated hESC expressing caRac (white arrowheads indicate as a GFP-positive cell, green). The scale bar represents 10 μm.

(G) Rho activity in mock- or caRac-transfected hESC before and after dissociation.

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which potentially contributes to the Rho-high/Rac-low state. Abr is required for both the dissociation-induced myosin hyperactivation and the apoptosis in hESCs. However, given that *Abr* is expressed both in hESCs and mESCs (data not shown), just the presence or absence of Abr in the cell is unlikely to determine the differential vulnerability of the mammalian ESCs, suggesting that the upstream regulation of Abr's function, rather than its expression, plays a key role. How the function of Abr is differentially regulated in attached and dissociated cells at the molecular level is an open question for future investigation.

### Possible Biological Roles of the ROCK/Myosin Hyperactivation and Apoptosis

In vivo, the cells in the ICM of the preimplantation embryo are not polarized and form a simple cell mass with no epithelialization (Rossant and Tam, 2009). After implantation, the pluripotent cells derived from the ICM become epithelialized and form an epiblast tissue with a clear apico-basal polarity, including the appearance of a basement membrane and tight junctions (Krtolica et al., 2007). During this process, cells that do not contribute to the epiblast undergo apoptosis, forming a cavity in the center.

One possible role of the dissociation-induced apoptosis is the quality control of the epiblast tissue formation in the postimplantation embryo, in other words, elimination of those ICM-derived cells that fail to be incorporated into the epiblast cell sheet. Another intriguing possibility is that the hyperactive state of the ROCK/myosin system in vivo is related more to morphogenesis rather than to cell survival. The process by which the ICM (a simple cell mass) is reshaped into the epiblast (a cell sheet) involves a dramatic 3D rearrangement of cells that requires high cell motility. The hyperactivity of the ROCK/myosin system may enable the epiblast-stage cells to prepare to undergo rapid cell movement. Consistent with this idea, recent studies have identified a novel type of Rho-ROCK-dependent blebbing (or myosin hyperactivation) that is used as a driving tool for directed migration of cells in 3D culture (Sanz-Moreno et al., 2008; Charras and Paluch, 2008).

Finally, another stimulating open question regarding the cell-state-specific ROCK/myosin hyperactivation is whether this phenomenon is limited to the early embryonic cells of mammalian species. In the *Xenopus* embryo, the inner layer cells of the blastula animal cap are equivalent to mouse ICM cells. They are pluripotent and form the animal pole roof lining the blastocoel, but do not have evident apico-basal polarity (e.g., no basement membrane). These cells become epithelialized during early gastrulation upon their fate specification into the ectoderm lineage. Importantly, the dissociated *Xenopus* gastrulae ectodermal cells also exhibit blebbing (Johnson, 1976) in a ROCK-dependent manner (Movie S6), implying the possibility that the "hyperactivation"-ready nature of epiblast/early-ectoderm cells has a profound biological role across species in the reproducible formation of the first-born epithelial structure in vertebrate ontogeny.

## EXPERIMENTAL PROCEDURES

### Cell Culture

The hESCs (KhES-1, KhES-3) were used in accordance with the hESC guidelines of the Japanese government. Five hiPSCs (gift from Y. Nakamura and S. Yamanaka) were also tested and similar observations were made. Undifferentiated hESCs and its subline 1 were maintained as described previously (Hasegawa et al., 2006: Watanabe et al., 2007). Additional details are in the Supplemental Experimental Procedures.

### Plasmids and Transfection

PCR-amplified cDNAs was sequenced and subcloned into the *pCAG-IP* or *pCAG-IG* expression vector. FRET probes for Caspases (SCAT3 and SCAT9) and Rho proteins (Rho-Raichu and Rac-Raichu) were kindly provided by M. Miura (University of Tokyo) and M. Matsuda (Kyoto University), respectively. The pSIREN RNAi system (Clontech) was used for knocking down the expression of specific genes. The transfection of hESC with cDNA- or shRNA-expression plasmids was performed with the FuGENE HD transfection reagent (Roche). Additional information was in Supplemental Experimental Procedures.

### Biochemical Analyses

For the evaluation of apoptosis, cells were stained with fluorescence-conjugated Annexin-V/Propidium lodide (Biovision) and flow cytometric analysis was performed with FACSAria (BD Biosciences). Immunostaining and colony formation assay was performed as described previously (Watanabe et al., 2007; Eiraku et al., 2008). The Rho/Rac activity was evaluated with a GST pull-down assay with MLB solution, Rhotekin-RBD, and Pak1-RBD (Upstate). For the detection of protein expression, cell lysates were made in RIPA lysis buffer. For dissection of protein-protein interaction, cell lysates were made in NT lysis buffer and immunoprecipitation was performed as described before. The cell lysates and immunoprecipitates were analyzed by SDS-PAGE and sequential western blotting. Antibodies used in this study were shown in Supplemental Experimental Procedures.

### Live Imaging

For the live single-cell imaging, dissociated cells were seeded onto a Matrigel-coated 35 mm glass-bottom dish. The recording was started at 15 min after the first contact with the dissociation reagent. For the EGTA experiments, cell clumps maintained on a feeder layer or a Matrigel-coated 35 mm glass-bottom dish for a few days were used for imaging. In this case, the recording was started when EGTA was added to the culture medium. For confocal observation, the images were collected with a CSU-X1 unit (Yokogawa) configured with an IX81-ZDC microscope (Olympus). The ratiometric analyses were performed with MetaMorph 7.5 (Molecular Devices).

### Statistical Analysis

Error bars shown in the figures represent standard deviations and n in the legends is the number of experiments. Statistical significance (two-sided) was tested by a Student's t test for two-group comparison, and by the one-way ANOVA for multiple-group comparison (for Annexin-V staining analysis, samples at the 6 hr point were analyzed unless otherwise mentioned) with a post-hoc Tukey's (among all groups) or Dunnett's test (versus control) with the Prism 4 program (GraphPad).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and seven movies and can be found with this article online at doi:10.1016/j.stem.2010.06.018.

(H) No significant restoration of dissociation-induced apoptosis by expression of RNAi-resistant Abr mutants (Abr*) lacking the GEF domain (ΔDH) or GAP activity (RA/NA) in Abr-depleted hESC. Abr* was used as a positive control. The bottom panel shows a western blot against the amino-terminal Flag tag. Dunnett's test (n = 3) versus lane 2 (among Abr-depleted cell groups) is shown. n.s., not significant; $^{**}p < 0.01$.
(I) Pull-down assay for Rho and Rac activity in Abr-depleted hESC before or after dissociation (lanes 3 and 4).
The bars in the graphs represent standard deviations. See also Figure S6 and Movie S7.

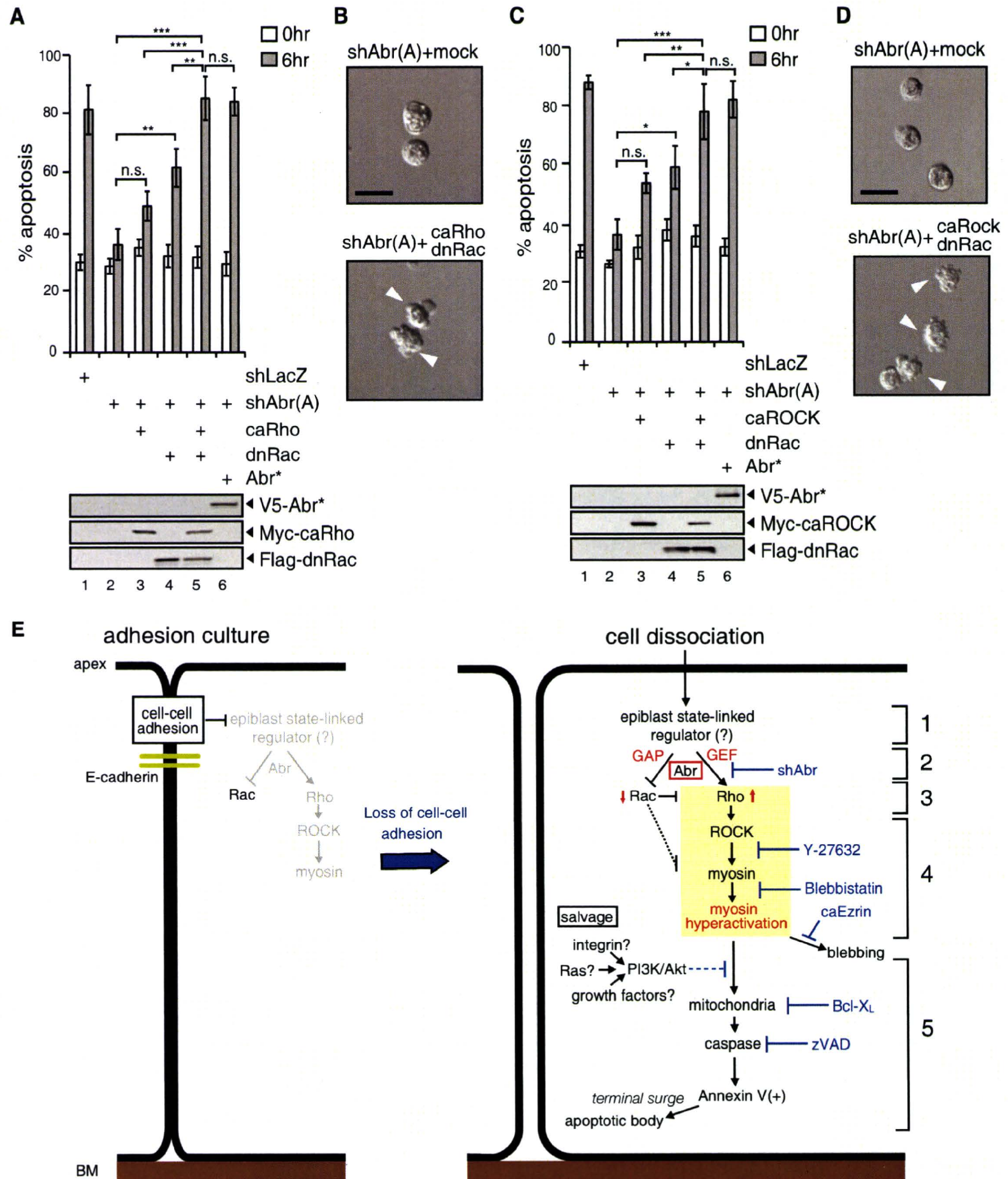


**Figure 7. Rho-High/Rac-Low State Responsible for the Dissociation-Induced hESC Apoptosis**

(A–D) Rac inhibition contributes to ROCK-dependent blebbing and apoptosis induction. The sensitivity to dissociation-induced blebbing and apoptosis were fully restored in Abr-depleted hESCs when dominant negative forms of Rac1 (dnRac and Rac1N17) were coexpressed with constitutive active forms of RhoA (caRho, RhoAV12) (A) or caROCK (ROCK1-Δ3) (C) (compare lanes 5 and 6; Annexin-V staining). Snapshots of dissociation culture of mock- or caROCK/dnRac or caRho/dnRac-transfected hESCs from which Abr was depleted are shown (C and D). Arrowheads indicate blebbing cells. The scale bar represents 20 μm. The bottom panel shows a western blot with an antibody against the amino-terminal tags. Tukey's test (n = 3) among all groups is shown. n.s., not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

(E) The molecular pathway of dissociation-induced hESC apoptosis contains at least five regulatory steps in the cascade: (1) Desuppression of epiblast state-linked regulator by dissociation; (2) Rho-GEF/Rac-GAP function of Abr; (3) generation of Rho-high/Rac-low state; (4) ROCK-dependent myosin hyperactivation; and (5) actomyosin-dependent apoptosis induction via mitochondria.

The bars in the graphs represent standard deviations. See also Figure S7.

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# Role of *SOX2* in maintaining pluripotency of human embryonic stem cells

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**Human embryonic stem cell (ESC) pluripotency is thought to be regulated by several key transcription factors including *OCT4*, *NANOG*, and *SOX2*. Although the functions of *OCT4* and *NANOG* in human ESCs are well defined, that of *SOX2* has not been fully characterized. To investigate the role of *SOX2*, we modulated the level of *SOX2* expression in human ESCs. Reduction of *SOX2* expression in human ESCs induced trophectodermal and partial endodermal differentiation. Interestingly, *CDX2*, a typical trophectoderm-associated gene, was not up-regulated. In contrast, using the Tet-on gene inducible system, *SOX2* over-expression in human ESCs induced trophectoderm differentiation accompanied by increased *CDX2* expression. Additionally, *SOX2* over-expression resulted in an increase in CGα-positive cells, which marks later stage trophectoderm development, rather than placental lactogen-positive cells. Thus, over-expression as well as repression of *SOX2* expression in human ESCs resulted in their differentiation into the trophectoderm lineage. Our data show that *SOX2* plays an important role in the maintenance of pluripotency of human ESCs and possibly, trophoblast development.**

## Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass (ICM) of blastocysts (Evans & Kaufman 1981; Martin 1981). ESCs have the ability to maintain self-renewal and to differentiate into all types of cells. Derivation of human ESCs (Thomson *et al.* 1998) could provide material for clinical applications in regenerative medicine and drug discovery in the future. In addition, human ESCs are expected to provide clues to human embryo development in culture. Thus, identification of the mechanisms governing ESC self-renewal, differentiation, and proliferation is important for these studies.

Comparison to mouse ESCs is often used to understand human ESCs. However, various differences exist between mouse and human ESCs, including the molecular mechanisms of self-renewal. For example, the LIF/STAT3 pathway is involved in mouse ESC self-renewal (Niwa *et al.* 1998), but is dispensable in human ESCs (Daheron *et al.* 2004; Humphrey *et al.* 2004). Moreover, BMP4 signaling is required for the maintenance of self-renewal in mouse ESCs by inhibiting neural differentiation in serum-free medium (Ying *et al.* 2003). However, the addition of BMP4 to human ESCs promotes primitive endoderm or trophectoderm differentiation (Xu *et al.* 2002; Pera *et al.* 2004).

Even with these differences, both mouse and human ESCs may still have similar molecular mechanisms to maintain an undifferentiated state. Chromatin immunoprecipitation assays combined with genome-wide location methodologies suggest that in both human and mouse ESCs, *OCT4*, *SOX2*, and *NANOG* have common target sites within the regulatory regions of many genes. These include both active, highly expressed genes (including *OCT4*, *SOX2*, and *NANOG* themselves) and inactive genes, such as developmental regulators, that maintain the pluripotent state (Boyer *et al.* 2005; Loh *et al.* 2006). Indeed, repression of *OCT4* in mouse and human

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ESCs induces trophectoderm differentiation (Niwa *et al.* 2000; Hay *et al.* 2004; Matin *et al.* 2004; Zaehres *et al.* 2005; Babaie *et al.* 2007); but when over-expressed, ESCs differentiate into endoderm or mesoderm cells (Niwa *et al.* 2000; Rodriguez *et al.* 2007). Repression of *NANOG* in mouse ESCs induces endoderm differentiation (Chambers *et al.* 2003; Mitsui *et al.* 2003), whereas its repression in human ESCs also induces both endoderm and trophectoderm differentiation (Hyslop *et al.* 2005; Zaehres *et al.* 2005). Over-expression of *NANOG* promotes stabilization of the undifferentiated state in ESCs. Mouse ESCs can maintain an undifferentiated state in the absence of LIF (Chambers *et al.* 2003; Mitsui *et al.* 2003), whereas in human ESCs it enables feeder-free growth or growth without conditioned medium from feeder cells (Darr *et al.* 2006). Repression of *Sox2* in mouse ESCs induces trophectoderm differentiation (Masui *et al.* 2007), whereas over-expression of *Sox2* in mouse ESCs induces non-specific lineage differentiation, neuronal differentiation or massive cell death (Mitsui *et al.* 2003; Zhao *et al.* 2004; Kopp *et al.* 2008). Furthermore, *Sox2*-deficient mice are defective in the maintenance of the ICM/epiblast and trophoblast development (Avilion *et al.* 2003), leading to the conclusion that *SOX2* may have an important role in trophoblast development as well as in ESC maintenance. However, the role of *SOX2* in human ESCs is not fully understood.

Recently, Fong *et al.* (2008) reported that a reduction in *SOX2* expression in hESCs resulted in the loss of the undifferentiated stem cell state accompanied by increased expression of trophectoderm markers (Fong *et al.* 2008). However, they used mouse embryonic fibroblasts (MEFs) as feeder cells for the assay, thus possible indirect effects of mouse MEFS cannot be excluded.

In this study, we investigated the role of *SOX2* in human ESCs by manipulating the level of *SOX2* expression using feeder cell-free culture conditions. We show that depletion or over-expression of *SOX2* in human ESCs induced trophectoderm differentiation. In addition, our result suggests that *SOX2* may have an important role in trophoblast development.

## Results

### Depletion of *SOX2* in human ESCs using small interfering RNA

To investigate the role of *SOX2* in human ESCs, we used small interfering RNA (siRNA) to knockdown gene expression. We first examined the effect of *SOX2* expression in the KhES1 cell line using several siRNAs. Although three siRNAs (listed in the experimental procedures) showed similar effects on human ESCs (data not shown), we used the most effective siRNA (Dharmacon ID# 106971) in further studies. This siRNA reduced *SOX2* expression to 25% of the control level (Fig. 1A). We furthermore confirmed SOX2 down-regulation as a 34-kDa signal by Western blotting analysis (Fig. 1B). This signal was ascertained by *OCT4* siRNA transfection because it has been previously shown that depletion of *OCT4* in human ESCs decreased the SOX2 expression (Babaie *et al.* 2007; our unpublished data). The very earliest changes in cell morphology resulting from *SOX2* knockdown were observed 48 h post transfection with the appearance of enlarged nuclei and flattened cells after 72 h. These morphological changes were accompanied by a reduction in the SOX2 protein level, as determined by immunohistochemistry (Fig. 1C). The cells also down-regulated ESC-specific surface markers, such as stage-specific embryonic antigen (SSEA)-4, and the tumor rejection antigens (TRA)-1-60 and TRA-1-81, while up-regulating the differentiation marker SSEA-1 (Fig. 1D and data not shown). These results indicate that *SOX2* expression may be required to maintain human ESCs in a pluripotent state.

Three key pluripotency genes, *OCT4*, *SOX2*, and *NANOG*, are thought to form an interconnected autoregulatory loop in human ESCs (Boyer *et al.* 2005).Thus, we investigated changes in the expression of *OCT4* and *NANOG* and found that the *OCT4* transcript level was slightly decreased, whereas the *NANOG* level decreased to 60% of the control (Fig. 1A). When we examined another human ESC line, HES-3, siRNA reduced *SOX2* expression to 28% of the control. In this cell line, the *OCT4* and *NANOG* transcript levels decreased to 60% and 55% of the controls, respectively. These results indicate that *SOX2* may play a role in regulating the expression of *NANOG* and, perhaps, *OCT4*.

### Suppression of *SOX2* induced trophectodermal and, in part, endodermal differentiation

To determine the differentiation status of the cells, we assessed the expression of markers that represent lineage-specific ESC differentiation at 72 h after siRNA transfection by quantitative real-time PCR (Q-PCR). As expected, *SOX2* knockdown in human ESCs resulted in the up-regulation of trophectodermal


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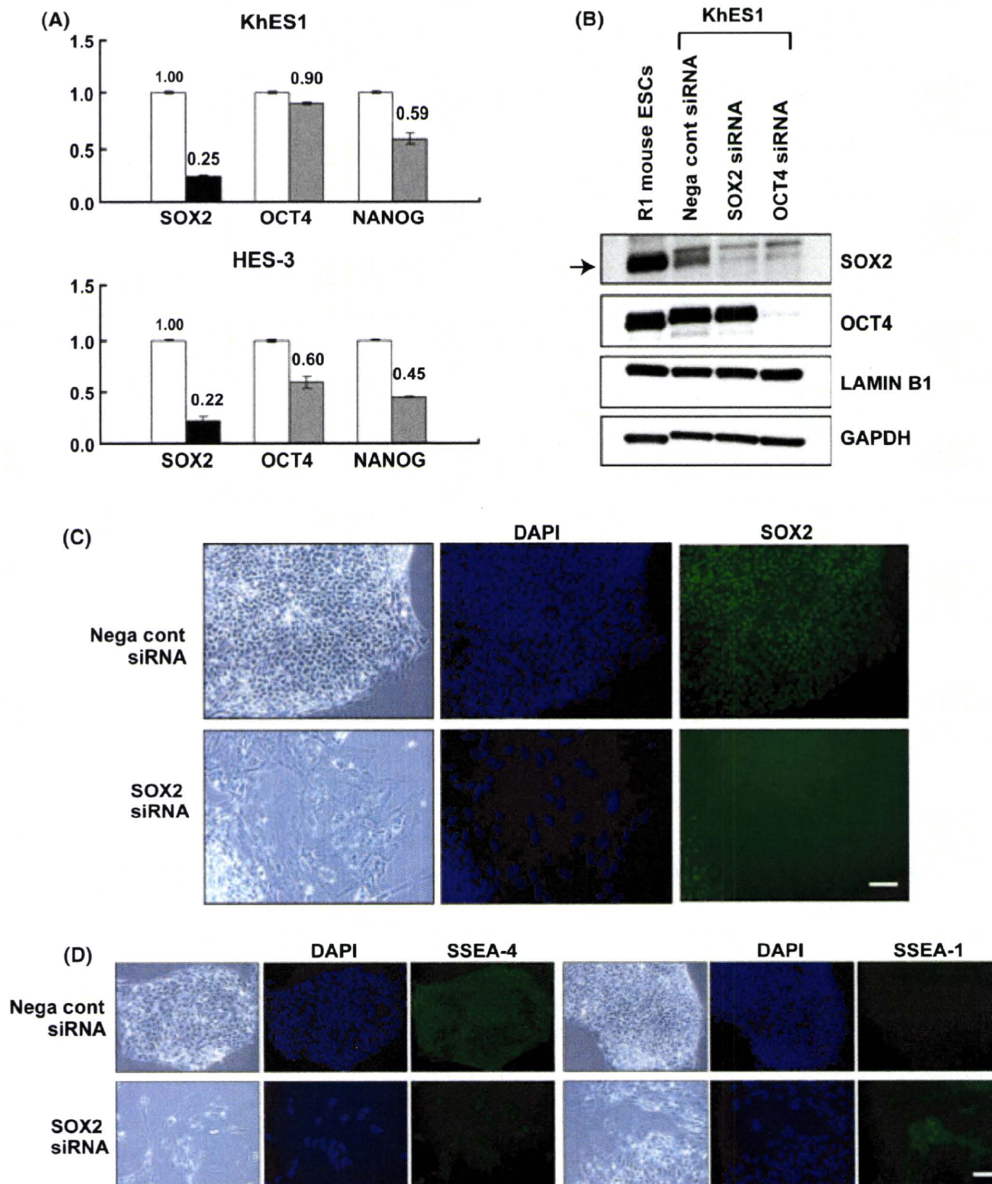


**Figure 1** Depletion of *SOX2* in human embryonic stem cells (ESCs) induced trophectodermal and endodermal differentiation. (A) *SOX2* siRNA reduced *SOX2* mRNA levels to approximately 25% of the negative control (Nega Control) siRNA. At the same time, the mRNA levels of *OCT4* and *NANOG* were decreased. (B) Western blotting analysis of SOX2 protein levels at 72 h after siRNA transfection. Both mouse and human SOX2 protein was detected as 34 kDa (indicated by an arrow). Decreasing level of SOX2 protein in human ESCs was detected by SOX2 or OCT4 siRNAs. Lamin B1 and GAPDH was used as loading controls. (C) Morphology of KhES1 ESCs 72 h after transfection with either Nega Control siRNA or *SOX2* siRNA. The level of SOX2 protein was confirmed by immunohistochemistry. (D) *SOX2* knockdown cells showed down-regulation of the ESC-specific surface marker stage-specific embryonic antigen (SSEA)-4 and up-regulation of the differentiation marker SSEA-1. (E, F) *SOX2* knockdown in human ESCs resulted in up-regulation of trophectodermal markers *EOMES*, *BMP4*, and *HAND1* (E, F) and endodermal markers *GATA6* and *FOXA2* (E). (G) The trophectodermal marker cytokeratin 8 (98.5%, $n$ = 200) and endodermal marker GATA6 (0.8%, $n$ = 1000) were detected in *SOX2*-down-regulated cells by immunohistochemistry. The Q-PCR data (A, D) represent the means (white bar for Nega control siRNA, and black or gray bar for SOX2 siRNA) ± SEM relative to the negative control (=1.0). The assays were normalized to *GAPDH*. The transfection experiments were carried out in triplicate three times ($n$ = 9) for KhES1 (A, E) and twice ($n$ = 6) for HES-3 cells (A). Statistical significance of the results was assessed using the Student's $t$-test. Scale bars = 100 μm (C, D, G). All analyses were performed at 48 h (F) or 72 h (others) after transfection. Abbreviation: *GSC*, goosecoid homeobox.

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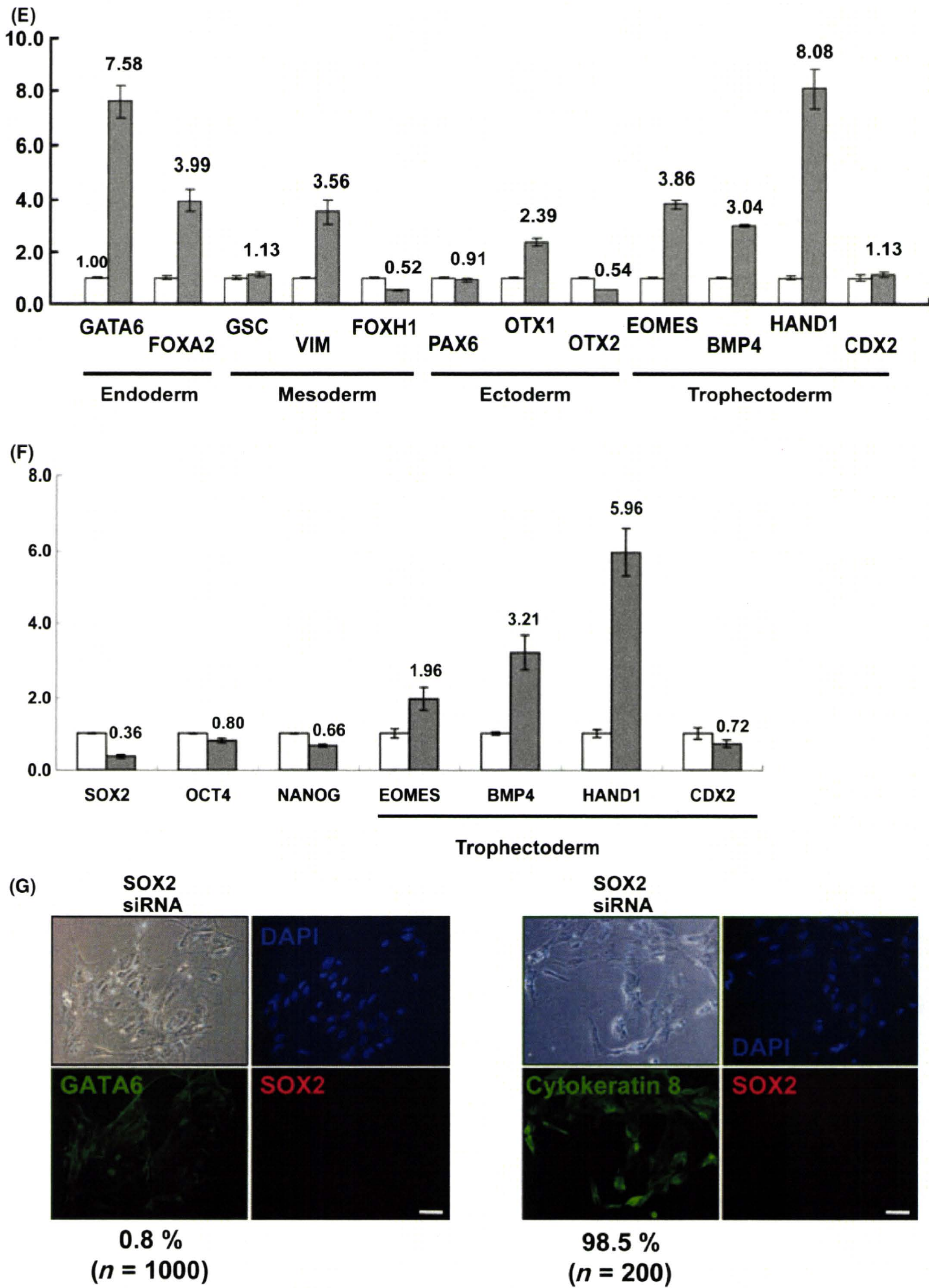


**Figure 1** (*Continued*).

markers *EOMES* (fourfold), *BMP4* (threefold), and *HAND1* (eightfold) (Fig. 1E). Interestingly, *CDX2* was not significantly up-regulated (Fig. 1E). We furthermore confirmed, at 48 h after the transfection, increasing of trophectodermal markers *EOMES* (two-hold), *BMP4* (threefold), and *HAND1* (sixfold), with reduced *SOX2* expression to 36% of the control level (Fig. 1F). In this time point, *CDX2* was not



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significantly up-regulated. Together, we concluded that SOX2 reduction in human ESCs induced up-regulation of trophectodermal markers except with *CDX2*. The trophectodermal marker cytokeratin 8 was detected in the majority (98.5%, $n$ = 200) of SOX2 down-regulated cells by immunohistochemical analysis (Fig. 1G). We also found up-regulation of the endodermal markers *GATA6* (7.5-fold) and *FOXA2* (fourfold) in the cells (Fig. 1E). Interestingly, the immunohistochemical analysis showed a small population of SOX2 down-regulated cells expressing the GATA6 protein (0.8%, $n$ = 1000) (Fig. 1G). Furthermore, the expression of ectodermal and mesodermal markers in *SOX2* siRNA-transfected cells did not significantly change relative to the controls. However, we observed that the ectodermal marker *OTX1*, or the mesodermal marker *VIM*, was up-regulated. In addition, another human ESC line, HES-3, showed similar results (data not shown). These results provide further support that *SOX2* expression is required to maintain ESC pluripotency by suppressing trophectodermal and, in part, endodermal differentiation.

### Over-expression of *SOX2* in human ESCs induces trophectodermal gene expression

Over-expression of *NANOG* in human ESCs enables feeder-free growth in the absence of conditioned medium (Darr *et al.* 2006), whereas over-expression of *OCT4* in human ESCs induces activation of endoderm-associated genes (Rodriguez *et al.* 2007). Thus, we assessed the effect of *SOX2* over-expression in human ESCs. We first examined transient *SOX2* over-expression in human ESCs using the lipofection method followed by semi-quantitative RT-PCR analysis up to 72 h after transfection. *SOX2* expression levels peaked 24 h post transfection, and thereafter, the levels decreased (Fig. 2A). Under our immunohistochemical staining, SOX2 expression in human ES cells is homogeneous in the colonies with an undifferentiated state (Fig. 1B, data not shown). Approximately 20% of human ESCs were confirmed over-expression of SOX2 by immunohistochemistry (Fig. 2B). When compared with wild-type or mock-transfected cells, *SOX2*-overexpressing cells showed significant up-regulation of the early trophectodermal markers *EOMES* or *CDX2*, but not later stage trophectodermal markers, such as *BMP4* or alpha polypeptide, a glycoprotein hormone (*CGα*) (Fig. 2C). In contrast, no significant changes in the expression patterns were observed for the endodermal markers *GATA6* and alpha-fetoprotein (*AFP*), the mesodermal markers goosecoid homeobox and brachyury homologue (*T*), or the ectodermal markers *OTX1* and *OTX2*. PAX6 expression was slightly up-regulated only 24 h after transfection. Interestingly, we could not detect significant down-regulation of the pluripotency markers *OCT4* and *NANOG*. Taken together, these results suggest that over-expression of *SOX2* in human ESCs may induce increased expression of early trophectodermal genes and that the cells may be re-specified to enter the trophectoderm lineage.

### Increasing *SOX2* expression in human ESCs induced trophectodermal differentiation

Transient inducible *SOX2* gene expression may only be effective for a few days in human ESCs. Although we observed up-regulation of the trophectoderm-associated genes *EOMES* and *CDX2*, we could not detect morphological changes caused by transient SOX2 over-expression in human ESCs. To furthermore investigate the effect of *SOX2* over-expression, we established a Tet-on system in human ESC lines, which contained the regulator plasmid pCAG-rtTA$^{On}$-hyg and response plasmid pTRE-SOX2-neo (Fig. 3A), as described in the experimental procedures. We initially isolated two independent cell lines (#14 and #27) that allowed doxycycline (Dox)-induced *SOX2* expression in a dose-dependent manner. High levels of Dox (more than 2 μg/mL) caused some detrimental effects to the survival of wild-type human ESCs (data not shown), thus we used 1 μg/mL Dox in the following experiments. Exposure to Dox resulted in increased SOX2 expression, as shown by Western blotting (Fig. 3B) and immunohistochemistry (Fig. 3C). Q-PCR results indicated that a three- to four-fold increase in *SOX2* expression in the cells at day 3 and a two-fold increase at day 5 (Fig. 3E). In contrast, rtTA expression was the same in the presence or absence of Dox-induced SOX2 expression (Fig. 3C).

The double-transfected ES clones (#14 and #27) were morphologically indistinguishable from the parental KhES1 ESCs when cultured under standard human ESC culture conditions. The addition of Dox (i.e. SOX2 over-expression) caused a flattened morphology in the middle of the colonies after day 3 (Fig. 3D). We found that 30%–50% of human ESC colonies showed such morphological changes at day 3. Similar to our previous experiments (Fig. 2C), *SOX2* over-expression in human ESCs induced up-regulation of the trophectodermal markers

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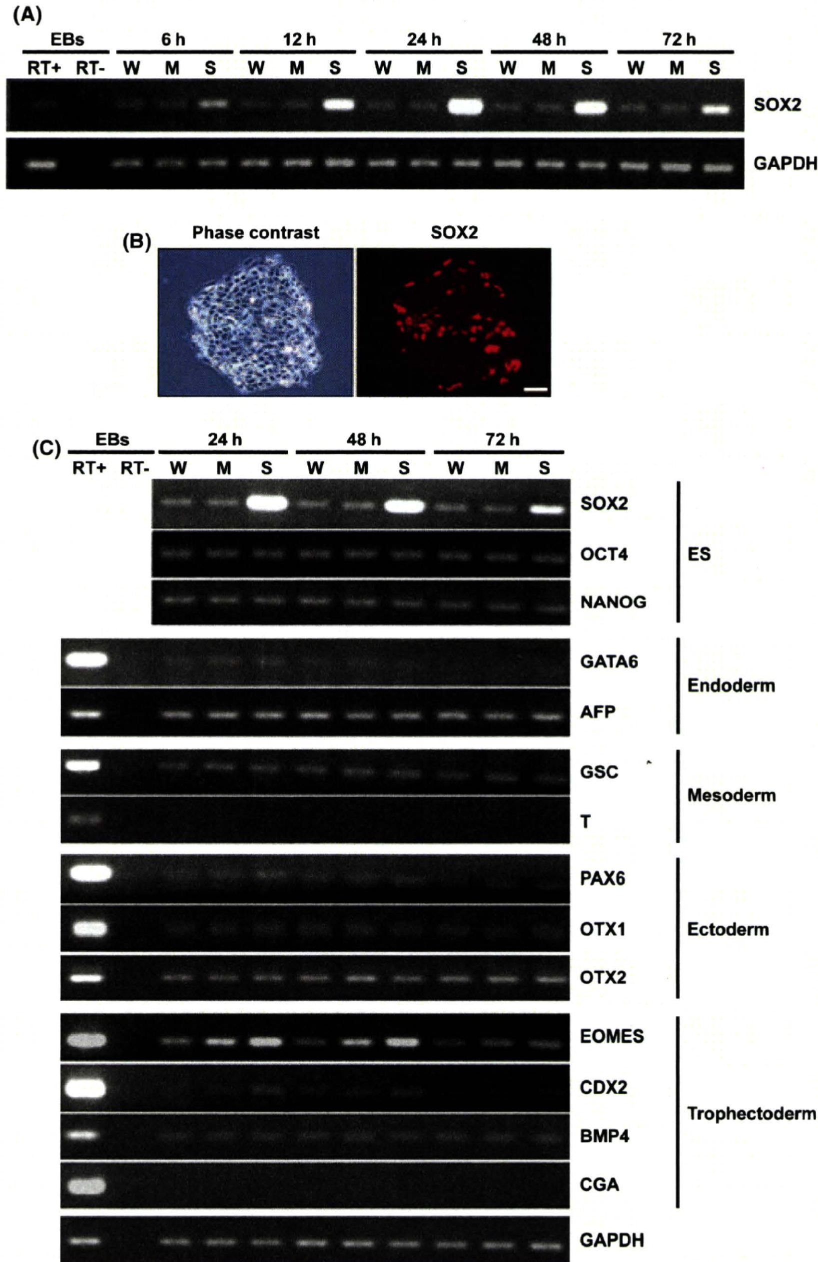


**Figure 2** Expression of embryonic stem cell (ESC)-specific and differentiation lineage marker genes in human ESCs transiently overexpressing *SOX2*. (A) The expression level of *SOX2* was examined from 6 to 72 h after transfection. *SOX2* expression levels peaked at 24 h. (B) Approximately 20% of human ESCs showed SOX2 over-expression by immunohistochemistry. (C) ESC-specific and differentiation marker genes were examined by semi-quantitative RT-PCR. Increased *SOX2* expression in human ESCs promoted trophectodermal marker genes, *EOMES* and *CDX2*. *SOX2*, *OCT4,* and *NANOG* were used as ESC-specific marker genes. Differentiation marker genes used were *GATA6* and *AFP* for endoderm, *GSC* and *T* for mesoderm, *PAX6*, *OTX2,* and *OTX1* for ectoderm, and *EOMES*, *CDX2*, *BMP4*, *CGA* for trophectoderm. *GAPDH* was used as an internal control. RT+ and RT− indicated the presence or absence of reverse transcriptase in the first-strand cDNA reaction, respectively. RT+ EBs were used as positive controls for differentiation marker genes. Scale bars = 100 μm (B). Abbreviations: W, wild type; M, mock transfected; S, SOX2 cDNA transfected; EBs, embryoid bodies; *GSC*, goosecoid homeobox; *T*, brachyury homologue; *CGA*, glycoprotein hormones, α polypeptide.


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*EOMES* and *CDX2*, but no up-regulation of other lineage markers as determined by semi-quantitative RT-PCR analysis (data not shown). To enable a better understanding of this differentiation event, we used Q-PCR to screen the expression of trophectodermal markers. As expected, *CDX2* was up-regulated approximately two- to three-fold at day 3 and three- to five-fold at day 5 (Fig. 3E). *EOMES* was also up-regulated approximately three- to five-fold at day 3 and two-fold at day 5 (Fig. 3E).

To furthermore determine the differentiation status of the cells, we examined *SOX2*-overexpressing cells by immunohistochemistry at day 5. Exposure to Dox increased *SOX2* expression (Fig. 4A). Next, we examined the levels of OCT4 and NANOG, and found, to our surprise, that the expression of both was decreased in these cells (Fig. 4B,C). We furthermore confirmed over-expression of *SOX2* in human ESCs decreased the level of OCT4 and NANOG using double immunohistochemical staining (Fig. S1 in Supporting Information). *OCT4* and *NANOG* expression is required for the maintenance of human ESC pluripotency, suggesting that *SOX2* over-expression may induce differentiation of human ESCs. Furthermore, SOX2 over-expression in human ESCs up-regulated the expression of a differentiation cell surface marker SSEA-1 (Fig. 4D). This result furthermore supports the notion that *SOX2* over-expression induces differentiation in human ESCs. Earlier, we had showed that there was up-regulation of the trophectodermal-associated gene *EOMES* and *CDX2* (Fig. 3D), so to confirm the status of trophectoderm differentiation we additionally examined trophectoderm markers by immunohistochemistry. We found that there was a substantial increase in the expression of a trophectodermal marker, cytokeratin 8 (Fig. 4E), whereas no significant increase in the endodermal marker GATA6, the neuronal marker PAX6 or the mesodermal marker brachyury (data not shown) was evident. Finally, we found that CDX2 expression could be detected in the morphologically trophectodermal cells consistent with our Q-PCR results (Fig. 4F). Following a further 2 days induction (day 7), we found a more than 10-fold increase in the number and level of CGα-expressing cells (Fig. 4G). Interestingly, we did not detect an increase in other trophectodermal, placental lactogen positive cells by immunohistochemistry in *SOX2*-overexpressing cells (data not shown).

Taken together, our observations indicate that the over-expression of *SOX2* in human ESCs decreases OCT4 and NANOG expression and induces trophectodermal differentiation accompanied by increased *CDX2* expression.

## Discussion

Human ESCs have the potential to provide material for clinical applications such as regenerative medicine and drug discovery in the future. Understanding the mechanisms governing human ESC self-renewal, differentiation and proliferation is important for advancing these studies. At present, it is thought that the three transcription factors *OCT4*, *SOX2*, and *NANOG* have essential roles in maintaining human ESC self-renewal (Boyer *et al.* 2005). The function of *OCT4* and *NANOG* in human ESCs are well characterized, whereas that of *SOX2* is still poorly understood. Here, we show that regulated *SOX2* expression is essential for maintaining the pluripotency of human ESCs. Either decreasing or increasing the level of *SOX2* resulted in differentiation of human ESCs into the trophoblast lineage.

### Repression of SOX2 in human ESCs induces trophectoderm and endoderm differentiation

Recent studies have suggested that a reduction in *SOX2* expression in human ESCs resulted in the loss of the undifferentiated state with increased expression of trophectoderm lineage markers (Fong *et al.* 2008). However, in their study, human ESCs were cultured with mouse MEFs as feeders, meaning that the undifferentiated or differentiated state of the cells might be influenced by the feeders. In this study, using a feeder-free culture system, we addressed directly the effect of *SOX2* reduction in human ESCs. Here, we showed that *SOX2* repression in human ESCs caused changes to cell morphology, loss of ESC-specific markers such as SSEA-4, and increased expression of a differentiation marker, SSEA-1. We also found decreased *NANOG* transcription and, possibly decreasing *OCT4* levels. These results indicated that *SOX2* expression is required to maintain human ESCs in an undifferentiated state. Furthermore, Q-PCR combined with immunohistochemical analysis showed that repression of SOX2 in human ESCs induced trophectodermal and endodermal differentiation. Thus, our study is the first to show that a reduction of SOX2 in human ESCs specifically causes differentiation to endoderm as well as trophectoderm. Indeed, a lack of *SOX2* induces differentiation into trophectoderm or extraembryonic endoderm cells from the ICM/epiblast lineage in mouse embryos

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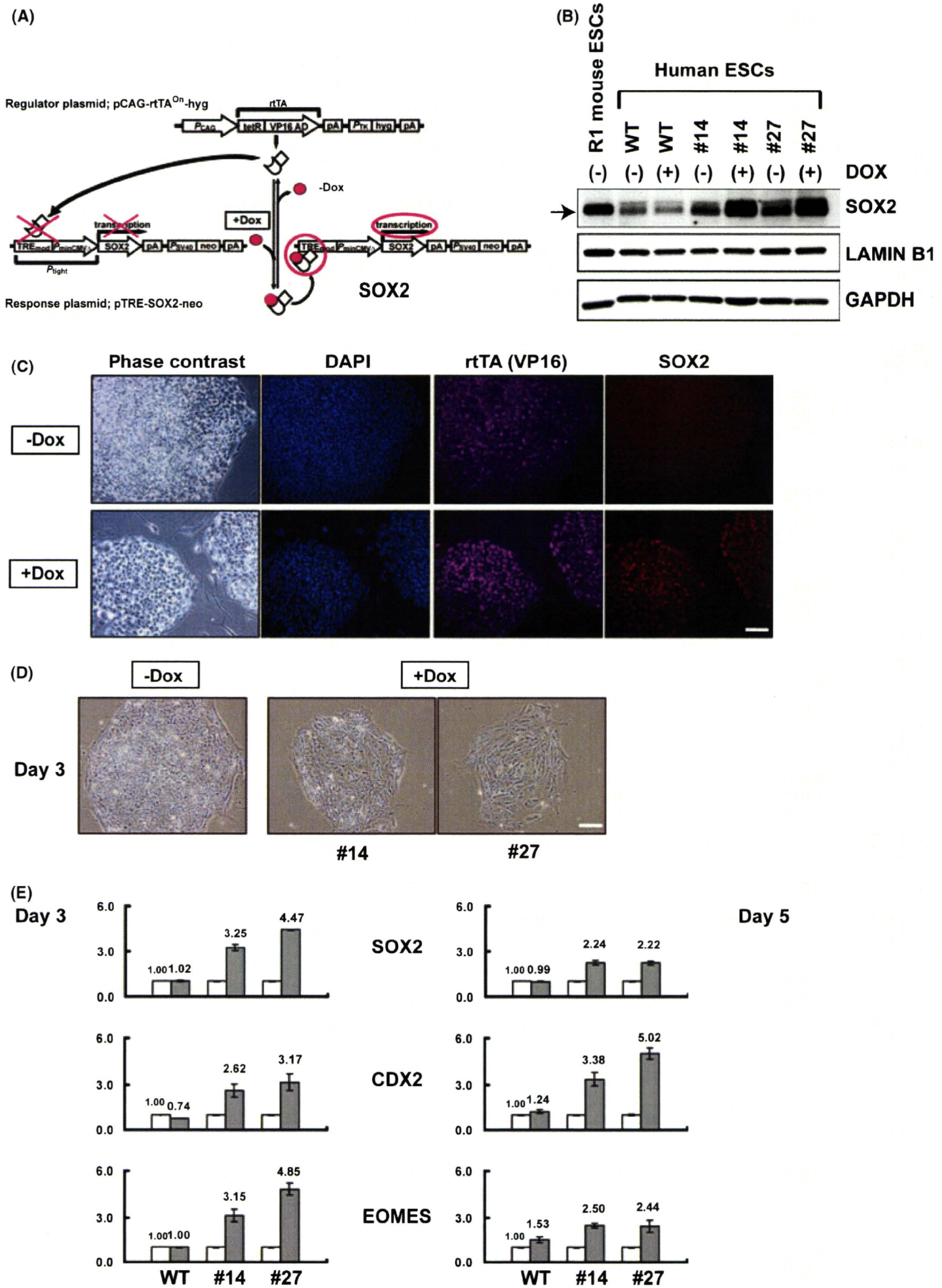




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(Avilion *et al.* 2003), consistent with our results in human ESCs. Additionally, trophectoderm differentiation in SOX2-repressing human ESCs is different from previous studies as we could not detect up-regulation of *CDX2* in this study. Interestingly, *CDX2* is not up-regulated even in ES cells from the Sox2-null mouse (Masui *et al.* 2007). Thus, we believe that feeder cells may bias against the intrinsic lineage differentiation in human ESCs. Here, our studies using a feeder cell-free culture system clarified new roles for *SOX2* in human ESCs.

### Over-expression of *SOX2* in human ESCs induces trophectoderm differentiation

Transient over-expression of *SOX2* induced up-regulation of the early trophectodermal markers *EOMES* or *CDX2*. In contrast, other lineage markers were not significantly changed. We found slight up-regulation of *PAX6* expression only 24 h after transfection, but after that, the level of *PAX6* was reduced to control levels. Expression of other ectodermal marker genes was not significantly changed. Thus, we believe that the increased expression of early trophectodermal genes is a primary effect of *SOX2* over-expression in human ESCs. Compared with transient over-expression, the Tet-on gene inducible system can permit longer induction of *SOX2* over-expression in human ESCs. *SOX2* over-expression in human ESCs caused changes to cell morphology, and increasing SSEA-1 differentiation marker expression. *OCT4* and *NANOG* expression is essential for maintaining human ESCs in an undifferentiated state, but we found that their expression was reduced. Thus, over-expression of *SOX2* in human ESCs resulted in differentiation. Increased expression of trophectoderm markers and no significantly increased expression of other lineage markers, led us to conclude that over-expression of *SOX2* in human ESCs causes trophectoderm differentiation.

### Model of *SOX2* function in human ESCs

Here, we show that regulated *SOX2* expression plays an important role in the maintenance of pluripotency in human ESCs. Double staining with SOX2 and differentiation markers by immunohistochemistry indicated that the majority of ESCs depleted of *SOX2* by RNA interference differentiated into trophectoderm. We speculate that the induction of *EOMES* by *SOX2* repression may be key for trophectoderm differentiation (Fig. 5 (1)). First, over-expression of either *CDX2* or *EOMES* induces differentiation of mouse ESCs into the trophectoderm lineage (Niwa *et al.* 2005). Second, in our study *EOMES* was significantly up-regulated upon *SOX2* repression in human ESCs, whereas *CDX2* was not. Furthermore, *OCT4* and *SOX2* are known to interact physically and co-occupy many sites in ES cells, including sites for the *EOMES* gene (Boyer *et al.* 2005), supporting the idea that *SOX2* directly represses *EOMES* expression.

We also showed that over-expression of *SOX2* in human ESCs induced activation of trophectodermal genes, including *CDX2*. We speculate that *CDX2* may be key for the activation of trophectodermal genes in this case (Fig. 5 (2)). In mouse ESCs, *CDX2* up-regulation increases *EOMES* expression (Niwa *et al.* 2005). Although *SOX2* repressed *EOMES* expression in

**Figure 3** Generation of the *SOX2* gene in human embryonic stem cells (ESCs) by the Tet-on inducible system. (A) The regulator and response plasmid constructions for the Tet-on system. The pCAG-rtTA$^{On}$-hyg regulator plasmid expresses reverse tetracycline-controlled transactivator (rtTA) under the control of the CAG promoter and has the hygromycin (hyg)-resistance gene. The pTRE-SOX2-neo response plasmid expresses *SOX2* under the control of the Tetracycline-Response Element (TRE) and has the neomycin (neo)-resistance gene. The $P_{tight}$ contains a modified TRE ($TRE_{mod}$) upstream of the altered minimal cytomegalovirus promoter ($P_{minCMV\Delta}$). Only after rtTA binds to $TRE_{mod}$ in the presence of Dox can transcription of *SOX2* be activated. (B) Dox increased *SOX2* expression in the double-transfected ES clone by Western blotting analysis at Day 4. Lamin B1 and GAPDH was used as loading controls. (C) Characteristics of the clone transfected with both the regulator and response plasmids. On day 3, Dox increased *SOX2* expression in the double-transfected ES clone, whereas rtTA was expressed in the nuclei regardless of Dox induction, as determined by immunohistochemical staining. Nuclei were counterstained with DAPI. (D) Morphological changes in human ESCs by *SOX2* over-expression. The double-transfected ESC clones (line #14 and #27) overexpressing *SOX2* exhibited a flattened morphology in the middle of the colonies after day 3. (E) *SOX2* over-expression in human ESCs resulted in up-regulation of the trophectodermal markers *EOMES* and *CDX2*. The data represent the means ± SEM relative to the non-Dox treatment as a control (=1.0). The assays were normalized to *GAPDH*. The induction experiments were carried out in triplicate three times ($n$ = 9). Statistical significance of the results was assessed using the Student's $t$-test. Scale bars = 100 μm. Abbreviations: Dox, doxycycline; −Dox, without Dox in the medium; +Dox, with Dox in the medium; pA, polyadenylation signal.

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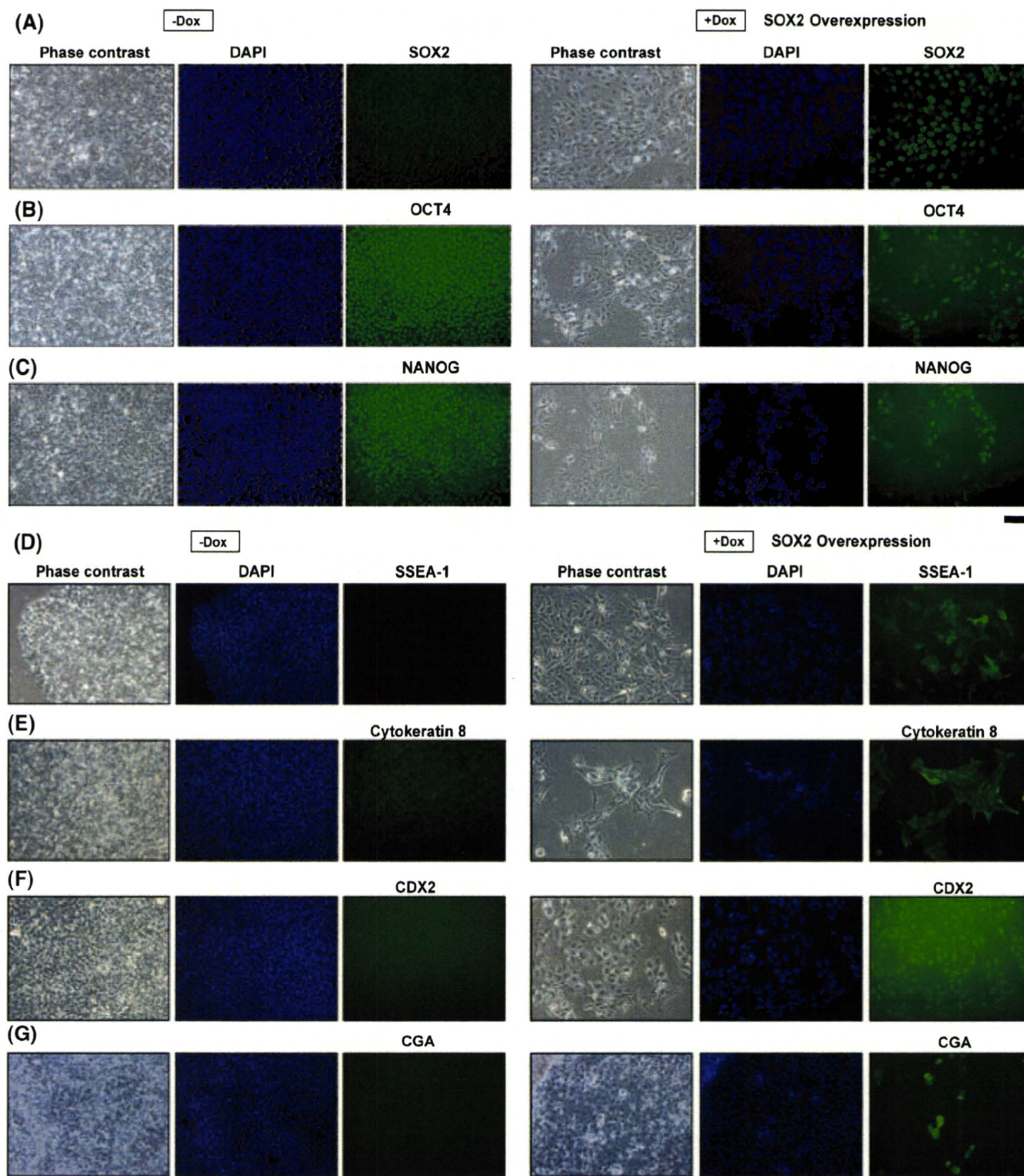


**Figure 4** Over-expression of *SOX2* in human embryonic stem cell (ESCs) induced trophectodermal differentiation. Immunohistochemical analysis was carried out at day 5 (A–F) or day 7 (G) after the induction of *SOX2* over-expression. (A) Increasing SOX2 expression by DOX induction compared with control human ESCs. (B, C) Over-expression of SOX2 repressed expression of the core transcription factors OCT4 (B) and NANOG (C) in human ESCs. (D) SOX2 over-expression in human ESCs resulted in up-regulation of the differentiation marker stage-specific embryonic antigen-1. (E–G) Immunohistochemistry of trophectoderm markers cytokeratin 8, CDX2, and CGA. (E) Increased cytokeratin 8 expression induced by SOX2 over-expression compared with control cells. (F) CDX2 was only detected in human ESCs overexpressing *SOX2*. (G) A few CGA-positive cells were present in control samples, but greater than 10-fold more positive cells with stronger fluorescence were present with *SOX2* over-expression. Scale bars = 100 μm. Abbreviations: Dox, doxycycline; −Dox, without Dox in the medium; +Dox, with Dox in the medium; *CGA*, glycoprotein hormones, α polypeptide.

human ESCs, *EOMES* was ultimately induced by *CDX2* expression. However, we cannot exclude the possibility that up-regulation of EOMES may be independent of *CDX2* expression. In that case, some other gene may induce *EOMES* expression instead of *CDX2*. *CDX2*, *EOMES*, or both then furthermore


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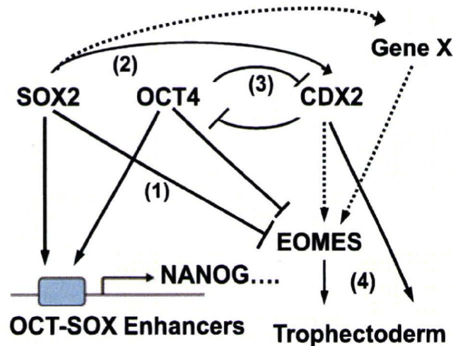


**Figure 5** A model of *SOX2* function in human embryonic stem cells (ESCs). The appropriate level of *SOX2* expression contributes to the maintenance of pluripotency in human ESCs. (1) *SOX2* suppresses *EOMES* expression to inhibit differentiation of the trophectoderm lineage. Repression of *SOX2* permits *EOMES* expression, resulting in the differentiation of human ESCs into trophectoderm. (2) *SOX2* can induce *CDX2* expression, whereas (3) *OCT4* suppresses *CDX2* expression in human ESCs. (4) Over-expression of *SOX2* caused up-regulation of *CDX2* and *EOMES* expression, resulting in trophectoderm differentiation. Increasing EOMES expression is indirectly induced by SOX2 through CDX2 or some other gene (X).

induced trophectodermal-associated genes, resulting in human ESC differentiation into the trophectoderm lineage (Fig. 5B (4)). Thus, *SOX2* potentially activates trophectoderm differentiation via *CDX2* expression, suggesting that some genes required for *CDX2* suppression are important for maintaining human ESC self-renewal (because CDX2 is not normally expressed in human ESCs). One strong candidate is *OCT4* (Fig. 5 (3)). Indeed, *OCT4* repression induced *CDX2* expression in human ESCs (Babaie *et al.* 2007) (our unpublished data), supporting this concept. In addition, our study showed that over-expression of SOX2 resulted in decreased OCT4 expression in human ESCs.

Alternatively, over-expression of *SOX2* may induce OCT4 down-regulation as a primary effect. In this case, CDX2 up-regulation may be induced by OCT4 down-regulation. To elucidate which is the primary effect of SOX2 over-expression, *CDX2* up-regulation or *OCT4* down-regulation, we performed time course analysis of gene expression. However, both *CDX2* up-regulation and *OCT4* down-regulation were detected from day 2 (data not shown). Thus, further studies are needed to determine to clarify the primary effect of *SOX2* over-expression in human ESCs.

*OCT4* and *SOX2* are thought to globally maintain the undifferentiated state of ES cells cooperatively, whereas over-expression of *OCT4* and *SOX2* induce differentiation of human ESCs (Rodriguez *et al.* 2007) (data from this study). In part, both may work antagonistically, and balanced expression of both genes may be essential for the maintenance of ESCs, as previously proposed for mouse ESCs (Avilion *et al.* 2003).

It was previously shown that BMP4 induced human ESC differentiation into trophectoderm (Xu *et al.* 2002). In this study, over-expression of *SOX2* in human ESCs did not significantly increase the expression of *BMP4*, suggesting *BMP4* independence. In contrast, depletion of *SOX2* in human ESCs may be different. Later trophectoderm marker genes such as *HAND1* and *BMP4* were up-regulated by *SOX2* depletion. We believe that in this case, the observed increase in *BMP4* expression represented a late trophectoderm gene rather than a trophectoderm differentiation inducer. However, further studies need to be carried out to exclude the possibility that depletion of *SOX2* induced trophectoderm differentiation via BMP4.

## Potential gene regulatory network between *OCT4*, *SOX2*, and *NANOG* in human ESCs

*OCT4*, *SOX2*, and *NANOG* are thought be regulated by autoregulatory loops (Boyer *et al.* 2005). Previously, it was shown that down-regulation of *OCT4* in human ESCs resulted in *SOX2* and *NANOG* down-regulation (Zaehres *et al.* 2005; Babaie *et al.* 2007) (our unpublished data). In addition, *NANOG* down-regulation in human ESCs causes *OCT4* down-regulation, but not down-regulation of *SOX2* (Hyslop *et al.* 2005). In this study, we showed that *SOX2* down-regulation caused significant *NANOG* down-regulation. *OCT4* expression was slightly reduced in the KhES1 cell line and significantly reduced in the HES-3 cell line. RNA interference cannot completely remove *SOX2* expression in human ESCs, thus further studies using *SOX2*-deficient human ESCs are necessary to determine if *SOX2* directly regulates the expression of *OCT4* in human ESCs.

In this study, we have shown that over-expression of *SOX2* caused decreasing *OCT4* and *NANOG* expression. We propose that this negative regulation is derived from an indirect rather than direct pathway. For example, over-expression of *SOX2* induces increased expression of *CDX2*, and induced *CDX2* expression in human ESCs may cause depression of *OCT4* expression. Finally, down-regulation of *OCT4* may cause decreased *NANOG* expression in the cells by an autoregulatory loop between *OCT4* and *NANOG*.

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### Comparison of the role of SOX2 between mouse and human ESCs

In the mouse, *SOX2*-deficient ESCs differentiate into trophectoderm cells, and <5% of these cells are positive for other lineage markers by immunohistochemistry (Masui *et al.* 2007). In this study, 98.5% of the *SOX2*-repressed human ESCs were positive for trophectoderm markers by immunohistochemistry, whereas 0.8% of the cells were positive for endoderm makers. Furthermore, during trophectoderm differentiation, *CDX2* was not up-regulated in either mouse or human ESCs. Thus, the role of *SOX2* in human ESCs was postulated to be similar to its repressive role in mouse ESCs.

In contrast, over-expression of *SOX2* in mouse and human ESCs seems to cause different results. Elevating the level of *SOX2* in mouse ESCs triggers the differentiation of a wide range of cell types, neuronal differentiation, or massive cell death (Mitsui *et al.* 2003; Zhao *et al.* 2004; Kopp *et al.* 2008), but is not specific for trophectoderm differentiation. In this study, we show that the primary effect of elevating the level of *SOX2* was differentiation of human ESCs into the trophectoderm lineage. *SOX2* is one of the key transcriptional factors required for ESC self-renewal. Thus, these different lineage specifications of ESCs caused by *SOX2* over-expression may reflect a substantial difference between mouse and human ESCs.

### Potential roles of *SOX2* during human trophoblast development

*SOX2* over-expression in human ESCs caused an increase in chorionic (CGα positive) cells, but not trophoblastic giant (placental lactogen positive) cells. Trophectoderm gives rise to two cell populations: polar trophectoderm and mural trophectoderm. Chorionic cells are derived from polar trophectoderm via extraembryonic ectoderm, whereas trophoblastic giant cells are derived from both mural and polar trophectoderm (Rossant & Cross 2001; Simmons & Cross 2005). This may suggest that *SOX2* promotes proliferation of polar trophectoderm or its descendants including extraembryonic ectoderm or chorionic cells. Indeed, *SOX2* mutant mice rescued with wild-type ES cells showed that *SOX2* plays a critical role in the chorion 7.5 days postcoitum (Avilion *et al.* 2003). Thus, *SOX2* may have an important role in human trophoblast development.

In this study, we showed that over-expression of *SOX2* induced trophectoderm differentiation accompanied by an increase in *CDX2* expression. *CDX2* is essential for the maintenance of trophoblast stem (TS) cells in the mouse (Niwa *et al.* 2005). Thus, we speculated whether over-expression of *SOX2* in human ESCs could induce TS-like cells and/or expansion of trophoblast cells under mouse TS cell culture conditions. Our preliminary studies showed that human morphologically TS-like cells were observed, but further improvements are needed to establish human TS cell lines.

Recently, it was shown that constitutive expression of *SOX7* or *SOX17* in human ESCs produces extra-embryonic endoderm and definitive endoderm, respectively (Seguin *et al.* 2008). It would be interesting to determine how over-expression of individual *SOX* genes, such as *SOX2*, *SOX7*, and *SOX17*, cause different lineage restriction in human ES cells.

Inducible gene expression systems, either regulating over-expression or repression of a transgene, are excellent tools for understanding basic science and in gene therapy for regenerative medicine. Here, we developed a Tet-on system in human ESCs and addressed how *SOX2* regulates pluripotency of human ESCs.

### Conclusion

In summary, regulated *SOX2* expression is essential for maintaining the pluripotent state of human ESCs. Both *SOX2* down-regulation and up-regulation caused trophectodermal differentiation. We also showed that *SOX2* may play an important role in the maintenance of trophoblast development.

## Experimental procedures

### Culture of human ESCs

The human ESC lines KhES1 and HES-3 were established and cultured as previously described (Pera *et al.* 2004; Suemori *et al.* 2006). Briefly, ES cells were cultured as colonies on mitomycin C-treated MEF feeder cells and subcultured every 3–5 days by enzymatic dissociation. For feeder-free cultures, human ESCs were dissociated into small clumps and cultured on plates coated with Matrigel (BD Biosciences) in MEF conditioned medium with 5 ng/mL basic fibroblast growth factor (bFGF, human recombinant, Millipore). Dox was added to the culture media after human ESCs had attached to Matrigel-coated dishes.

### Construction of expression plasmid

Full-length human *SOX2* cDNA was cloned by RT-PCR using the following primers: forward, 5′-ACCATGTACAA-CATGATGGAG-3′; and reverse, 5′-GAATTCCTCACA-


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TGTGTGAGA-3′. The PCR product was subcloned into the pGEM-T easy vector (Promega), and the sequence was confirmed by nucleotide sequence analysis. The *SOX2* overexpression vector with a CAG promoter was created by inserting the *SOX2* ORF into the pCAG/PGK neo vector (Adachi *et al.* 2006).

To create the regulator plasmid pCAG-rtTA$^{On}$-hyg, we inserted a CAG promoter and the reverse tetracycline-controlled transactivator (rtTA) sequence from the pTet-On vector (Clontech) into the pTK-Hyg vector (Clontech). To create the response plasmid pTRE-SOX2-neo, we inserted the SOX2 sequence into the multiple cloning site of the $P_{tight}$ sequence from the pTRE-Tight vector (Clontech) and also inserted a neomycin (neo)-resistant gene driven by an SV40 promoter.

### Transfection

For transient transfections, $2 \times 10^5$ human ESCs on Matrigel-coated 35-mm dishes were transfected with 2 μg of *SOX2* expression plasmid, or alternatively, with 1.7 μg of mock plasmid in 5 μL of FuGENE HD (Roche Diagnostics) transfection reagent. One day after transfection, the medium was replaced, and samples were collected after 72 h.

Human ESC Tet-on lines expressing the *SOX2* gene were established using a protocol described previously (Adachi *et al.* 2006). Briefly, KhES1 human ESCs plated on a hygromycin-resistant SL10 (sub-line of STO) cell line were transfected with linearized pCAG-rtTA$^{On}$-hyg (10 μg/60-mm tissue culture dish) in Opti-MEM (Invitrogen) using Lipofectamine™ 2000 (Invitrogen) and selected in the presence of 50 μg/mL hygromycin B (Invitrogen). After selection, hygromycin-resistant colonies were picked and transferred to new MEF feeder cells. The regulator-transfected ES cells plated on neomycin-resistant MEF feeder cells were furthermore transfected with linearized pTRE-*SOX2*-neo and selected in the presence of 100 μg/mL G418 (Sigma). After selection, neomycin-resistant colonies were picked and transferred to new MEF feeder cells.

### RNA interference

RNA interference (RNAi) experiments were performed with siGENOME SMARTpool (M-011778-00), siGENOME Duplex (D-011778-01) (Dharmacon) or Silencer Pre-designed siRNA (ID#106971) (Ambion) reagents against human *SOX2*. The Silencer Cy3-Labeled Negative Control #1 (Ambion) was used as a negative control. Human ESCs were transfected for 24 h with 50 nM of the siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions in Matrigel-coated 12-well plates (for immunohistochemical analysis) or 6-well plates (for RNA expression analysis) at a density of $1 \times 10^5$ cells or $2.5 \times 10^5$ cells per well, respectively. A second transfection was performed on adherent cells 24 h after the first transfection, and analyses were performed on samples at day 3.

### Immunohistochemical analyses

For immunohistochemical analyses, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, and then incubated with 5% bovine serum albumin (BSA), 5% normal donkey serum, or 5% normal goat serum in PBS. The cells were incubated with primary antibodies in PBS containing 2.5% BSA overnight at 4 °C, followed by incubation with Alexa Fluor 488- or 546-conjugated secondary antibodies (1 : 250–500; Molecular Probes) in PBS containing 2.5% BSA for 30 min at room temperature. Antibodies against the following proteins were used: SSEA-1 (1 : 50–100; Developmental Studies Hybridoma Bank), SSEA-4 (1 : 100; R&D systems), TRA-1-60 (1 : 50; Chemicon), TRA-1-81 (1 : 50, Chemicon), SOX2 (clone 245610, 1 : 200; R&D systems) or (Y-17, 1 : 250–500; Santa Cruz Biotechnology), herpes simplex virus VP16 (clone 1–21, 1 : 250; Santa Cruz Biotechnology) or (1 : 500–1000; Clontech), GATA6 (H-92, 1 : 100; Santa Cruz Biotechnology), cytokeratin 8 (clone Ks 8.7, 1 : 100; PROGEN), OCT4 (C-10, 1 : 1000, Santa Cruz Biotechnology), NANOG (1 : 100, ReproCell, Tokyo, Japan), CDX2 (clone AMT28, 1 : 10; Abcam) or (1 : 50, Cell Signaling Technology), CGα (1 : 1500, Abcam), placental lactogen (1 : 200, Abcam), brachyury (N-19, 1:100; Santa Cruz Biotechnology), and PAX6 (1 : 500, Chemicon). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and analyzed by fluorescence microscopy.

### Western blotting

Protein lysates from human and mouse R1 ESCs were electrophoresed on 10% SDS-PAGE gel (5 μg/lane), transferred onto polyvinylidene difluoride (PVDF)-membrane, and probed with the following primary antibodies anti-SOX2 (1 : 2000; R&D Systems), anti-OCT4 (1 : 5000, Santa Cruz Biotechnology), anti-Lamin B1 (1 : 20000, abcam), and anti-GAPDH (6C5, 1 : 10000. Santa Cruz Biotechnology). After incubation with horseradish-peroxidase (HRP)-conjugated secondary antibodies, proteins were detected using SuperSignal West Dura (Pierce). Lamin B1 and GAPDH was used as loading controls.

### RNA extraction, RT-PCR and quantitative RT-PCR analyses

Total RNA was extracted from feeder-free ESCs or 15-day-old induced embryoid bodies using the RNeasy Micro Kit (Qiagen) before cDNA was synthesized from 1–2 μg total RNA using Omniscript™ Reverse Transcriptase (Qiagen). PCRs were optimized to facilitate semi-quantitative comparison of samples within the log phase of amplification. Gene-specific primers were designed based on published sequences (Table S1 in Supporting Information). PCR products were separated on a 2% agarose gel and visualized by ethidium-bromide staining. Quantitative PCR reactions were carried out with the Power SYBER Green PCR Master Mix (Applied Biosystems) and the Applied Biosystems 7500 Real-Time

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PCR System. The expression levels of the genes of interest were normalized to that of *GAPDH*.

### Statistical analysis

Comparisons between groups were performed using the Student's *t*-test with a *P*-value of <0.05 considered as a significant change.

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## Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

**Figure S1** Over-expression of *SOX2* in human ESCs induced decreasing level of OCT4 (A) and NANOG (B).

**Table S1** List of primers used for RT-PCR or Q-PCR

Additional Supporting Information may be found in the online version of this article.

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# Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells

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**Abstract** There are many reports of defined culture systems for the propagation of human embryonic stem cells in the absence of feeder cell support, but no previous study has undertaken a multi-laboratory comparison of these diverse methodologies. In this study, five separate laboratories, each with experience in human embryonic stem cell culture, used a panel of ten embryonic stem cell lines (including WA09 as an index cell line common to all

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