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The Role of *SOX2* in Maintaining Pluripotency and Differentiation of Human Embryonic Stem Cells

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1. Introduction

Embryonic stem cells (ESCs) are pluripotent and self-renewing cells that are derived from the inner cell mass (ICM) of the developing blastocysts (Evans and Kaufman, 1981; Martin, 1981). ESCs have the ability to maintain self-renewal and to differentiate into all types of cells. With respect to primates, ESCs were first established from the rhesus monkey (*Macaca mulatta*) (Thomson et al., 1995) and subsequently from humans (Thomson et al., 1998). Human ESCs are of particular interest because of their potential application to regenerative medicine and drug discovery. In addition, human ESCs may provide insights into human embryo development in culture. Therefore, identification of the molecular mechanisms that govern human ESC self-renewal, differentiation and proliferation is of considerable interest.

A small number of genes, the so-called "core transcription factors", is thought to have a central role in the control of the stem cell state in concert with other genes including other transcription factors. The ESC state is largely governed by three core transcription factors, *OCT4*, *SOX2*, and *NANOG*. In the mouse, Oct4 and Nanog have a distinctive expression pattern in ESCs and during embryonic development, and genetic and molecular analyses have shown that both genes have essential roles for maintaining the stable pluripotent state (Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998; Niwa et al., 2000). By contrast, Sox2 is widely expressed during embryonic development and occurs not only in the inner cell mass and epiblast but also in neural tissues, extra embryonic ectoderm, gut endoderm, esophagus and trachea (Avilion et al., 2003; Williamson et al., 2006; Wood and Episkopou, 1999). Indeed, *SOX2* is required for development during the peri-implantation period, and is required for trophoblast formation and neural development (Avilion et al., 2003; Kelberman et al., 2006; Taranova et al., 2006). Oct4 acts as a heterodimer with Sox2 in mouse

ESCs; Sox2 assigns a position for key regulators for the maintenance of mouse ESCs with undifferentiated state (Ambrosetti et al., 2000; Avilion et al., 2003; Masui et al., 2007).

Mouse ESCs have often been used as a model system for human ESCs. However, various differences are known to exist between the ESCs of the two species, including the molecular mechanisms for 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 grown in serum-free medium, and acts by inhibiting neural differentiation (Ying et al., 2003). However, the addition of BMP4 to human ESC culture promotes primitive endoderm or trophectoderm differentiation (Pera et al., 2004; Xu et al., 2002). These inter-species differences have become a highly contentious issue following the establishment of a new type of pluripotent cell line called EpiSCs (Brons et al., 2007; Tesar et al., 2007). EpiSCs were first established by explanting late epiblast from mouse embryos; these explanted cells expressed the core transcription factors Oct4, Sox2 and Nanog. EpiSCs can differentiate into three germ layers but they are inefficient in generating chimeras, suggesting that they have a more limited developmental potential than ESCs. Interestingly, mouse EpiSCs and human ESCs have similar growth requirements and gene expression patterns, and both types of cells are distinguishable from mouse ESCs. It is unclear whether human ESCs are really a counterpart of mouse EpiSCs, since EpiSCs have not been extensively studied to date.

Disregarding the issue of EpiSCs, mouse and human ESCs are believed to have similar molecular mechanisms for maintenance of the undifferentiated state. Chromatin immunoprecipitation (ChIP) assays combined with genome-wide location methodologies showed that in both human and mouse ESCs, *OCT4*, *SOX2* and *NANOG* have common target sites within the regulatory regions of many genes. This is true of active, highly expressed genes, such as *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). Downregulation of *OCT4* in mouse and human ESCs induces trophectoderm differentiation (Babaie et al., 2007; Hay et al., 2004; Matin et al., 2004; Niwa et al., 2000; Zaehres et al., 2005). By contrast, overexpression of *OCT4* induces ESCs to differentiate into endoderm or mesoderm cells (Niwa et al., 2000; Rodriguez et al., 2007). Reduction in expression of *NANOG* in mouse ESCs induces endoderm differentiation (Chambers et al., 2003; Mitsui et al., 2003), while in human ESCs, it induces both endoderm and trophectoderm differentiation (Hyslop et al., 2005; Zaehres et al., 2005). Overexpression of *NANOG* promotes stabilization of an undifferentiated state in ESCs. Mouse ESCs can maintain an undifferentiated state in the absence of LIF (Chambers et al., 2003; Chambers et al., 2007; Mitsui et al., 2003). The human ESCs allows feeder-free growth or growth without conditioned medium from feeder cells (Darr et al., 2006). In mouse ESCs, repression of *Sox2* expression induces trophectoderm differentiation (Masui et al., 2007), whereas overexpression induces non-specific lineage differentiation, neuronal differentiation or massive cell death (Kopp et al., 2008; Mitsui et al., 2003; Zhao et al., 2004). Furthermore, *Sox2*-deficient mice are defective in the maintenance of 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.

Reprogramming of somatic cells into induced pluripotent stem (iPS) cells provides another approach to investigating the nature of the undifferentiated state in ESCs. Surprisingly, the overexpression of only four transcription factors, Oct4, Sox2, Klf4 and c-Myc, can convert

somatic fibroblasts to pluripotent cells that can contribute to the germline in chimeric mice, similarly to ESCs (Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). Reprogramming can be induced not only by Oct4, Sox2, Klf4 and c-Myc but also by use of combinations including Nanog, Lin28 and other genes. Generally, overexpression of Sox2 is required unless the somatic cells already have endogenous Sox2 expression (Hanna et al., 2010; Stadtfeld and Hochedlinger, 2010). Moreover, the level of *SOX2* expression has a direct effect on the rate of direct reprogramming of somatic cells to iPSCs (Yamaguchi et al., 2011). These results provide further support for the assumption that SOX2 has a definitive role in the maintenance of ESCs.

In this study, we investigated the role of *SOX2* in human ESCs by manipulating the level of *SOX2* expression. We show that depletion or overexpression of *SOX2* in human ESCs induced trophectoderm differentiation. We further showed that overexpression of *SOX2* during human ESC differentiation promoted neural and glandular epithelium development.

2. The role of *SOX2* in maintaining pluripotency and differentiation of human ESCs

2.1 The role of SOX2 in maintaining pluripotency of human ESCs

2.1.1 Depletion of *SOX2* in human ESCs induces trophectodermal and some endodermal differentiation

To investigate the role of *SOX2* in human ESCs, we used a small interfering RNA (siRNA) to knockdown gene expression. We first optimized the protocol by examining the effect of a previously described siRNA on OCT4 expression in the KhES1 cell line (Dharmacon ID D-019591-05) (Babaie et al., 2007) (a detailed protocol is described in Adachi et al., 2010). This siRNA reduced OCT4 expression in our protocol as determined by quantitative real-time PCR (Q-PCR) (Figure 1A) and Western blotting (40-, 44-kDa signal, Figure 1B), indicating that this protocol was appropriate for the study. Next, we analyzed the levels of *SOX2* expression in KhES1 cells transfected with a range of siRNAs, since selection of appropriate siRNA sequences was crucial to the study. Overall, we found that D-011778-01 siRNA (Dharmacon) was the most effective of the tested siRNAs. A Q-PCR analysis indicated that D-011778-01 siRNA reduced *SOX2* mRNA expression to 25% of the control level (Fig. 1A). Western blotting confirmed a reduction in the level of SOX2 protein in the cells (34-kDa signal, Figure 1B). SOX2 repression in human ESCs caused changes to cell morphology from 48 hours post-transfection. Cells with enlarged nuclei and a flattened morphology were present after 72 hours. These morphological changes were accompanied by a reduction in the SOX2 protein level as determined by immunohistochemistry (Fig. 1C). In addition, the cells showed downregulated expression of ESC-specific surface markers, anti-stage specific embryonic antigen (SSEA)-4, anti-tumor rejection antigens (TRA)-1-60 and TRA-1-81, and also upregulated expression of the differentiation marker SSEA-1 (Fig. 1D, and data not shown). These results indicate that *SOX2* expression may be required for maintenance of human ESCs in a pluripotent state.

As mentioned earlier, three core transcription genes, *OCT4*, *SOX2* and *NANOG*, are thought to form an interconnected autoregulatory loop in human ESCs (Boyer et al., 2005; Loh et al., 2006). In agreement with previous reports, *OCT4* knockdown in human ESCs caused a decrease in both *NANOG* and *SOX2* levels to 10-30% of the control level, indicating that *OCT4* may regulate expression of these core transcription factors. In the KhES1 cells with knockdown of SOX2, the *OCT4* transcript level was slightly decreased, while the *NANOG* level decreased to 60% of the control (Fig. 1A). When we examined another human ESC line,

HES-3, we found that D-011778-01 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 control, respectively. These results indicate that *SOX2* may play a role in regulating expression of *NANOG* and, perhaps, *OCT4*. Future studies using *SOX2*-deficient human ESCs should provide conclusive evidence on whether *SOX2* regulates the expression of *OCT4* and *NANOG*.

Our data indicate that *SOX2* expression is required to maintain human ESCs in an undifferentiated state, although the exact differentiation status of the cells was uncertain. Immunohistochemical analysis showed that the majority of SOX2 downregulated cells (98.5%, n = 200) were positive for the trophectodermal marker cytokeratin 8, and a small population of cells with downregulation of SOX2 were positive for the endodermal marker GATA6 (0.8%, n = 1,000) (Fig. 1E). We could not detect any increase in other linage markers including the ectodermal marker PAX6 and the mesodermal marker brachyury (T) homolog (K.A. and E.K., unpublished observation). Q-PCR showed that *SOX2* knockdown in human ESCs resulted in upregulation of the trophectodermal markers *EOMES*, *BMP4* and *HAND1* at 72 hours after transfection. Interestingly, *CDX2* was not significantly upregulated (Fig. 1F). Upregulation of trophectodermal markers, except CDX2, was also present at 48 hours after transfection. At this time point, GATA3 was upregulated (Fig 1G). GATA3 is a gene expressed in the trophectoderm; *Gata3* directly regulates *Cdx2* transcription via a conserved GATA motif at the intron 1 region of the *Cdx2* locus in the mouse (Home et al., 2009). Thus, *CDX2* shows an exceptional expression pattern compared to other early trophectodermal markers, possibly indicating that in these cells some other gene may be involved in its regulation. We also found that expression of the endodermal markers *GATA6* and *FOXA2* was upregulated in cells with downregulation of SOX2 (Fig. 1F). Furthermore, expression of ectodermal and mesodermal markers in *SOX2* siRNA transfected cells was not significantly altered relative to controls. However, we observed that the ectodermal marker *OTX1* and the mesodermal marker *VIM* were upregulated. Thus, the results from assays of transcripts were consistent with the immunohistological observations. Taken together with similar results from the HES-3 cell line, we conclude that *SOX2* expression maintains ESC pluripotency by suppressing trophectodermal and, to a lesser extent, endodermal differentiation.

It was previously shown that reduction in *SOX2* expression in human ESCs resulted in the loss of the undifferentiated state with an associated increase in the expression of trophectoderm lineage markers including CDX2 (Fong et al., 2008). However, in this study, human ESCs were cultured with mouse MEF feeder cells; consequently, it was not possible to exclude the possibility that the feeder cells also influenced the undifferentiated or differentiated state of the ESCs. In this study, we used a feeder-free culture system and were thus able to address directly the effect of *SOX2* reduction on human ESCs. Our results without feeder cells showed that *CDX2* was not upregulated in cells with suppressed expression of SOX2; this finding is consistent with previous results from ES cells of the Sox2 null mouse (Masui et al., 2007). Furthermore, our results were the first to show that a reduction in SOX2 expression in human ESCs specifically caused differentiation of endoderm as well as trophectoderm. A lack of *SOX2* has been found to induce differentiation into trophectoderm or extraembryonic endoderm cells from the ICM/epiblast lineage in mouse embryos (Avilion et al., 2003), consistent with our results in human ESCs. Thus, we believe that feeder cells may bias against the intrinsic lineage differentiation in human ESCs. Our analysis of human ESCs in a feeder cell-free culture

system identified new roles for *SOX2*. Clearly, this system could be also be used to investigate the role of other genes in human ESCs.

Figure 1A-C

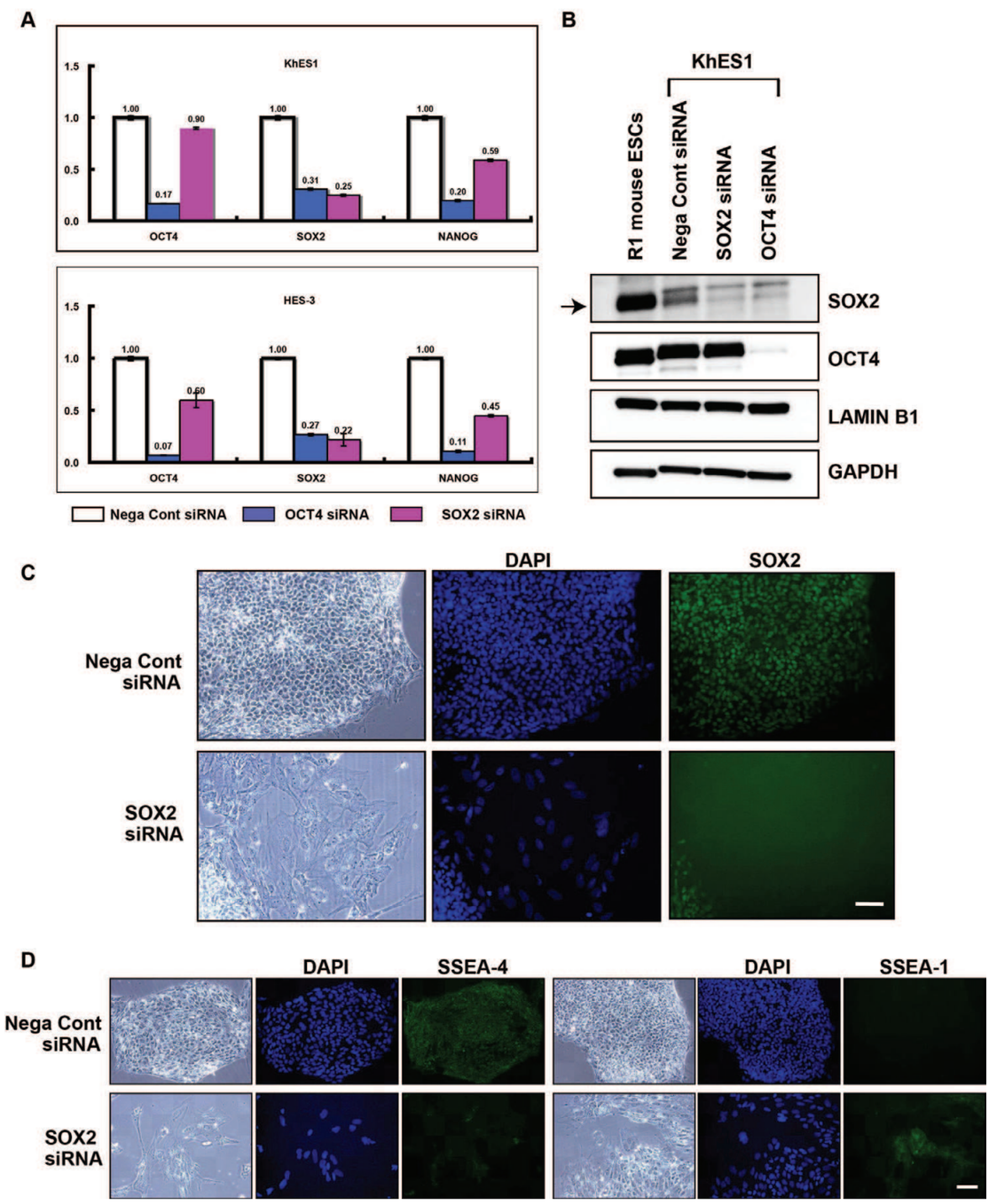


Figure 1E-G

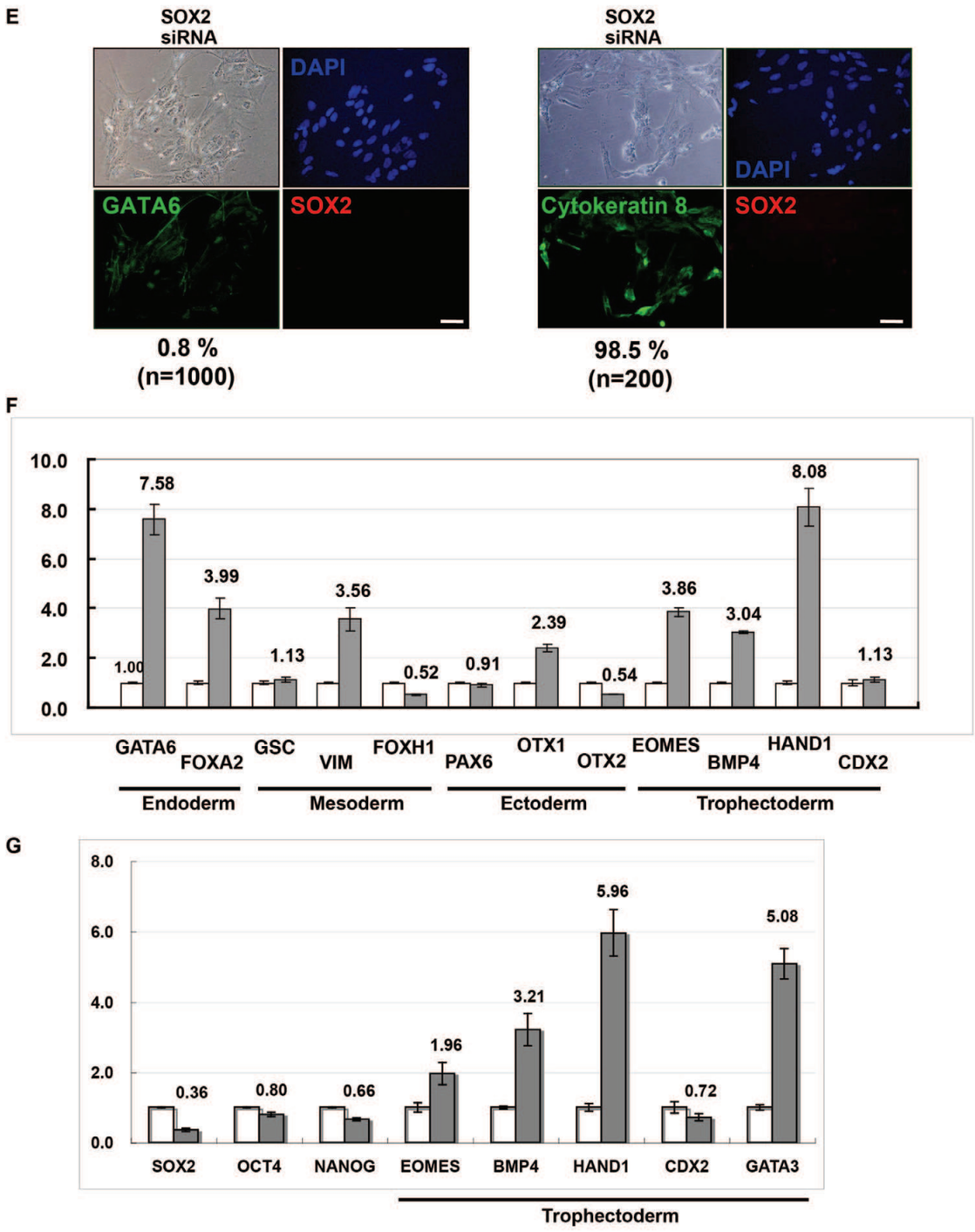


Fig. 1. Depletion of *SOX2* in human ESCs induces trophectodermal and some endodermal differentiation.

Transfection with *SOX2* siRNA reduced *SOX2* mRNA levels to approximately 25% of the negative control (Nega Control siRNA). *OCT4* and *NANOG* mRNA levels also decreased. (B) Western blotting analysis of SOX2 protein levels at 72 hours after siRNA transfection. Both mouse and human SOX2 protein was detected as a 34 kDa band (indicated by arrow). Decreased levels of SOX2 protein was present in human ESCs after transfection with SOX2 or OCT4 siRNAs. Lamin B1 and GAPDH was used as loading controls. (C) Morphology of KhES1 cells 72 hours after transfection with either Nega Control siRNA or *SOX2* siRNA. The level of SOX2 protein was confirmed by immunohistochemistry. (D) *SOX2* knockdown cells showed downregulation of the ESC-specific surface marker SSEA-4 and upregulation of the differentiation marker SSEA-1. (E) Immunohistochemical detection of the trophectodermal marker cytokeratin 8 (98.5%, n = 200) and endodermal marker GATA6 (0.8%, n = 1,000) in *SOX2*-downregulated cells. (F, G) *SOX2* knockdown in human ESCs resulted in upregulation of the trophectodermal markers *EOMES*, *BMP4* and *HAND1* (F, G) and endodermal markers *GATA6* and *FOXA2* (F). The Q-PCR data (A, D) represent the means (white bar for Nega control siRNA, and 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 three times in triplicate (n = 9) for KhES1 cells (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 mm (C, D, E). Analyses were performed at 48 hours (G) or 72 hours (others) after transfection. Abbreviations: *GSC*, goosecoid homeobox. This figure is modified from (Adachi et al., 2010).

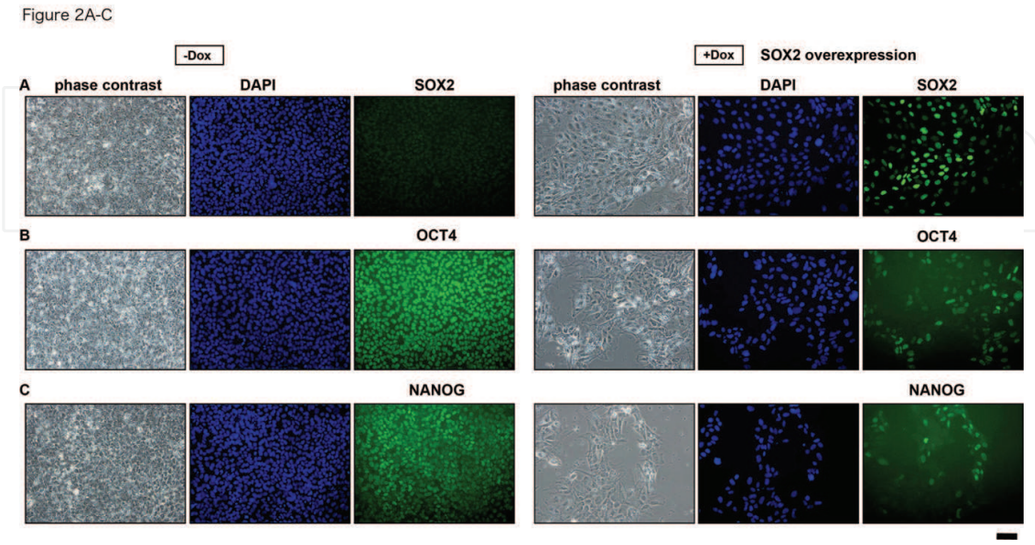
2.1.2 Overexpression of *SOX2* in human ESCs induces trophectodermal differentiation

We used a lipofection method to induce transient *SOX2* overexpression in human ESCs and analyzed the effects with an RT-PCR analysis (Adachi et al., 2010). We monitored *SOX2* expression for 72 hours after transfection. *SOX2* expression levels peaked at 24 hours post-transfection and, thereafter, the levels decreased. An immunohistochemical analysis indicated that about 20% of the human ESCs overexpressed the *SOX2* protein. When compared with wild type or mock transfected cells, *SOX2* overexpressing cells showed significant upregulation of the early trophectodermal markers *EOMES* or *CDX2*, but not of later trophectodermal markers, such as *BMP4* or the glycoprotein hormones alpha chain. By contrast, the expression patterns were not changed in the endodermal markers *GATA6* and alpha-fetoprotein (*AFP*), the mesodermal markers goosecoid homeobox (*GSC*) and brachyury (*T*) homolog or the ectodermal markers *PAX6*, *OTX1* and *OTX2*. Interestingly, we did not detect significant downregulation of the pluripotency markers *OCT4* and *NANOG*. Thus, transient overexpression of *SOX2* in human ESCs may induce expression of early trophectodermal genes, but this was not sufficient for re-specification of the cells into the trophectoderm lineage.

In order to achieve constitutive overexpression of SOX2 in human ESCs, we used the Tet-On/Off system of monkey ESCs and developed this for human ESCs (Adachi et al., 2006; Adachi et al., 2010). We isolated two independent cell lines (#14 and #27) that showed doxycycline (Dox)-induced *SOX2* expression in a dose-dependent manner. Human ESCs, in general, are sensitive to chemicals and we found that high levels of Dox (more than 2 mg/ml) had some detrimental effects on the survival of wild-type human ESCs (data not shown). Therefore, we used 1 mg/ml Dox in our experiments. Human ES clones #14 and #27 were morphologically indistinguishable from parental KhES1 ESCs when cultured

under standard human ESC culture conditions. The addition of 1 mg/ml Dox to the medium resulted in a two- to four-fold increase in *SOX2* expression (as determined by Q-PCR) on day 5. From day 3 of culture, 30-50% of the human ESC colonies displayed a flattened morphology in their middle. Moreover, *SOX2* overexpression in human ESCs induced upregulation of the trophectodermal markers *EOMES* and *CDX2*, but not of other lineage markers as determined by semi-quantitative RT-PCR and Q-PCR analysis (data not shown). After day 5, immunohistochemical staining showed that *SOX2* overexpression in human ESCs resulted in trophectoderm differentiation. Exposure to Dox increased *SOX2* expression (Figure 2A); however, the levels of expression of OCT4 and NANOG, which are essential for maintenance of the undifferentiated state, decreased in these cells (Figure 2B-C). Furthermore, SOX2 overexpression in human ESCs upregulated the expression of a differentiation cell surface marker SSEA-1 (Figure 2D). This result further supports the notion that *SOX2* overexpression induces differentiation in human ESCs. We found that there was a substantial increase in the expression of the trophectoderm marker cytokeratin 8 (Figure 2E), but there were no significant increases in expression of the endoderm marker GATA6, the ectoderm marker PAX6 or the mesoderm marker brachyury (data not shown). Finally, we found that CDX2 expression could be detected in cells with the morphological appearance of trophectoderm, consistent with our Q-PCR results. Following a further 2 days induction (day 7), we found that the number of cells expressing CGα increase more than 10-fold (Fig. 2G). Interestingly, immunohistochemical staining did not detect increases in other trophectodermal, placental lactogen positive cells among *SOX2*-overexpressing cells (data not shown).

Taken together, our observations indicate that overexpression of *SOX2* in human ESCs resulted in trophectodermal differentiation accompanied by increased *CDX2* expression. On the basis of the decrease in expression of OCT4 and NANOG, the increase in expression of trophectoderm markers, and the absence of a significant increase in expression of other lineage markers, we concluded that overexpression of *SOX2* in human ESCs causes trophectoderm differentiation.



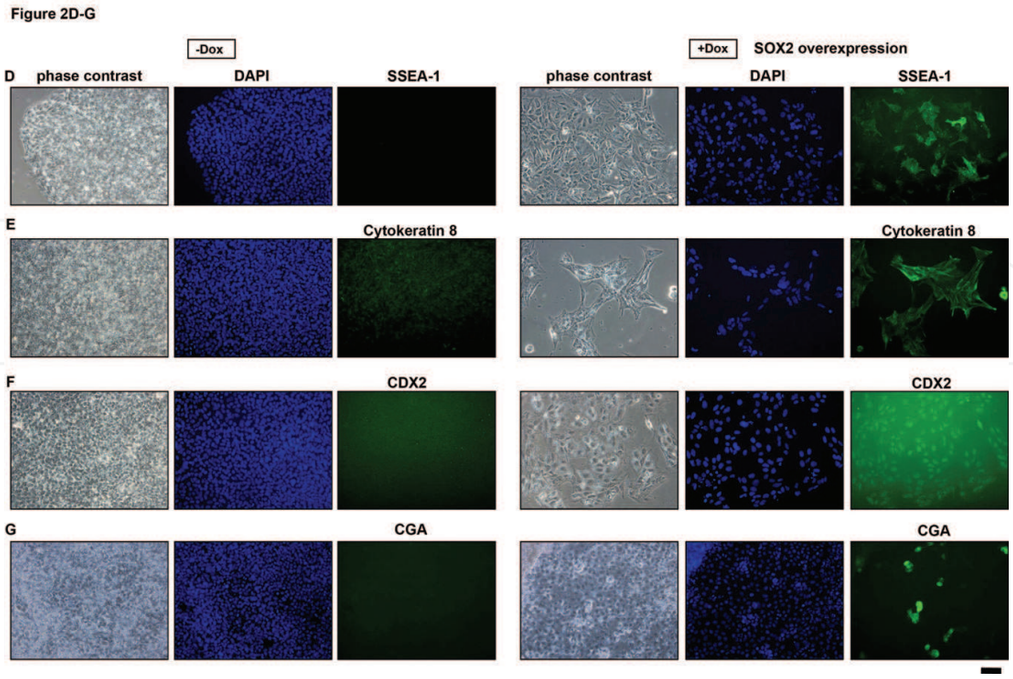


Fig. 2. Overexpression of *SOX2* in human ESCs induces trophectodermal differentiation. Immunohistochemical staining at day 5 (A-F) or day 7 (G) after induction of *SOX2* overexpression. (A) DOX induces increased SOX2 expression compared to control human ESCs. (B, C) Overexpression of SOX2 repressed expression of the core transcription factors OCT4 (B) and NANOG (C) in human ESCs. (D) SOX2 overexpression in human ESCs resulted in upregulation of the differentiation marker SSEA-1. (E-G) Immunohistochemical staining of trophectoderm for cytokeratin 8, CDX2 and CGA. (E) Increased cytokeratin 8 expression was induced by SOX2 overexpression compared to control cells. (F) CDX2 was only detected in human ESCs overexpressing *SOX2*. (G) A few CGA positive cells were present in control samples, but a more than 10 fold increase in positive cells with stronger fluorescence was present after *SOX2* overexpression. Scale bars = 100 mm. Abbreviations: Dox, doxycycline; -Dox, without Dox in the medium; +Dox, with Dox in the medium; *CGA*, glycoprotein hormones alpha chain. This figure is adapted from (Adachi et al., 2010).

2.1.3 Expansion of CDX2 positive cells from *SOX2*-overexpressing human ESCs

Our investigation demonstrated that overexpression of *SOX2* induced trophectoderm differentiation, which was 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). Therefore, we speculated whether overexpression of *SOX2* in human ESCs could induce TS-like cells and/or expansion of trophoblast cells under mouse TS cell culture conditions. At day 4, human ESCs that had been induced by Dox to overexpress *SOX2* started to change morphologically, to resemble trophoblast-like cells prior to flattening. The cells continued to proliferate, while retaining immunohistochemically positive staining for CDX2 (Figure 3A, B). On rare occasions, CDX2 positive cells were found in wild type or non-Dox induced human ESCs (Figure 3B).

CDX2 positive cells were detected for 3 to 4 weeks after Dox induction. Most of the CDX2 positive cells formed a glandular epithelium-like structure on the feeder cells (Figure 3C I).

Cells that were morphologically similar to mouse TS-like cells surrounded these structures, but CDX2 expression was weak or undetectable by immunohistochemistry in the majority of the cells (data not shown). A minority of the cells was CDX2 positive, and may represent candidate human TS cells (Figure 3C II). The numbers of other trophoblast cells that were positive for CGα immunohistochemical staining (data not shown) also greatly increased. Thus, under mouse TS cell culture conditions, overexpression of *SOX2* in human ESCs resulted in an increase in the rate of differentiation of trophoblast cells. However, the majority of the trophoblast cells lost CDX2 expression, thereby preventing derivation of human TS cell lines. SOX2 overexpression was therefore not sufficient for maintenance of CDX2 expression in the TS-like cells. Instead, these culture conditions induced an increase in

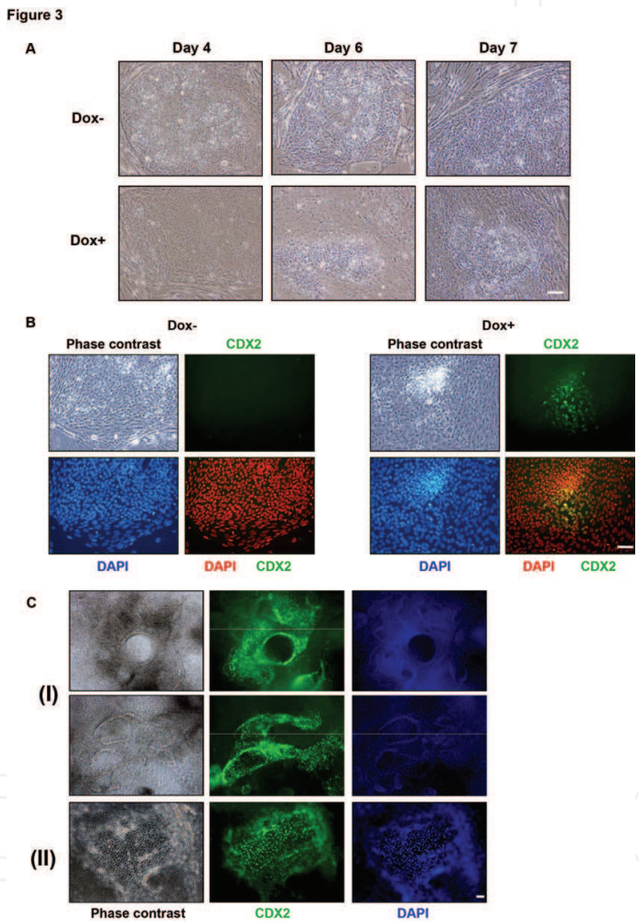


Fig. 3. Expansion of trophoblast and glandular epithelium cells from human ESCs by *SOX2* overexpression using the TS cell culture medium.
(A) Initial morphological changes in trophoblast-like cells differentiating from human ESCs. Trophoblast-like cells were detectable at day 4 after induction of *SOX2* overexpression. (B,C) Immunohistochemical staining for the trophectoderm marker *CDX2*. (B) *CDX2* was only detected in human ESCs induced to overexpress *SOX2*. No *CDX2* positive cells were present in parental cell lines or double transfected cell lines without Dox treatment. (C) *CDX2* positive cells were detected one month after Dox induction. (I) Glandular epithelium-like cells, (II) putative TS-like cells. Scale bars = 100 mm.

the number of CDX2 positive putative glandular epithelium-like cells, which showed more rapid proliferation than putative TS cells. Glandular differentiation did not occur only in response to *SOX2* overexpression in human ESCs, suggesting that initiation of glandular epithelium differentiation is not related to SOX2 overexpression.

2.2 Increased *SOX2* expression in human ESCs induces differentiation of neural and glandular epithelium

The teratoma formation assay has often been used to investigate the developmental potential of ESCs. We, therefore, used this assay to determine whether *SOX2* overexpression in human ESCs enabled formation of trophoblastic tumors or caused them to develop into other tissues. To examine the effect of *SOX2* overexpression during human ESC differentiation, we injected the double-transfected ES clones (lines #14 and #27) subcutaneously into the backs of SCID mice and monitored their differentiation with or without Dox treatment. Under this system, SOX2 should be an effective inducer of differentiation not only for human ESCs but also their differentiated descendants (Figure 4A) Parental human ESC lines formed teratomas comprised of all three germ layers in the presence or absence of Dox treatment (Suemori et al., 2006; data not shown). Similarly, double-transfected ES clones formed teratomas comprised of all three germ layers in the absence of Dox treatment (Figure 4B, data not shown). However, after Dox treatment (i.e. *SOX2* overexpression conditions), the double-transfected ES clones did not form teratomas like those observed in the absence of Dox. In this case, the teratomas were mainly comprised of neural epithelium and glandular epithelium (Figure 4C). We were unable to investigate how glandular epithelium differentiated from human ESCs. Glandular epithelium can differentiate from extraembryonic trophoblastic tissue and also the embryo proper, such as from gut or lung tissues, where SOX2 is expressed during development. Our results from use of the mouse TS cell culture protocol indicated that glandular epithelium could differentiate from human ESCs with or without SOX2 overexpression, and may suggest that SOX2 increases expansion of cell numbers rather than inducing initial differentiation.

By contrast to culture of mouse ESCs, we had to remove differentiated colonies to enable continual culture of human ESCs. As described earlier, we did not detect any cells at day 5 that were positive for the neuronal marker PAX6 among SOX2-overexpressing human ESCs. Two days later, PAX6-positive cells could be detected but only in the piled-up, embryoid body-like differentiated colonies formed by *SOX2*-overexpressing cells (K.A. and E.K., unpublished observation). We believe that SOX2 overexpression promoted expansion of PAX6-positive neural cells or neural progenitors rather than neuronal differentiation from human ESCs. In combination with our cell culture data, these observations indicated that overexpression of *SOX2* enhanced specific-lineage development of neural and glandular epithelium in differentiating human ESCs.

2.3 A putative model of SOX2 function in human ESCs

We have shown that the regulated expression of *SOX2* plays an important role in the maintenance of pluripotency in human ESCs. Decrease or increase of the level of *SOX2* expression resulted in human ESCs differentiating into the trophectodermal lineage. However, our results also suggest that trophectodermal differentiation may occur through different pathways. Although trophectoderm genes were upregulated in human ESCs with either repression or overexpression of SOX2, as determined by mRNA and protein studies, *CDX2* expression was exceptional and seemed to alter in concert with the level of expression

of SOX2. Overexpression of either *CDX2* or *EOMES* induces differentiation of mouse ESCs into the trophectoderm lineage (Niwa et al., 2005). *OCT4* and *SOX2* are known to interact physically and co-occupy many sites in ES cells, including those on the EOMES gene (Boyer et al., 2005). In our study, *EOMES* was significantly upregulated upon *SOX2* repression in human ESCs, whereas *CDX2* was not, suggesting that induction of EOMES by *SOX2* repression in human ESCs may be a key step in trophectodermal differentiation. In contrast, overexpression of *SOX2* in human ESCs and activation of *CDX2* expression may be key for trophectodermal differentiation. In this study, we found that SOX2 overexpression in human ESCs resulted in decreased expression of OCT4 and NANOG, leading to activation of trophectodermal genes. Indeed, OCT4 repression induces CDX2 and EOMES expression in human ESCs (Babaie et al., 2007; K.A. and E.K., unpublished data). It will be interesting to determine how SOX2 overexpression reduces stem cell related genes while, at the same

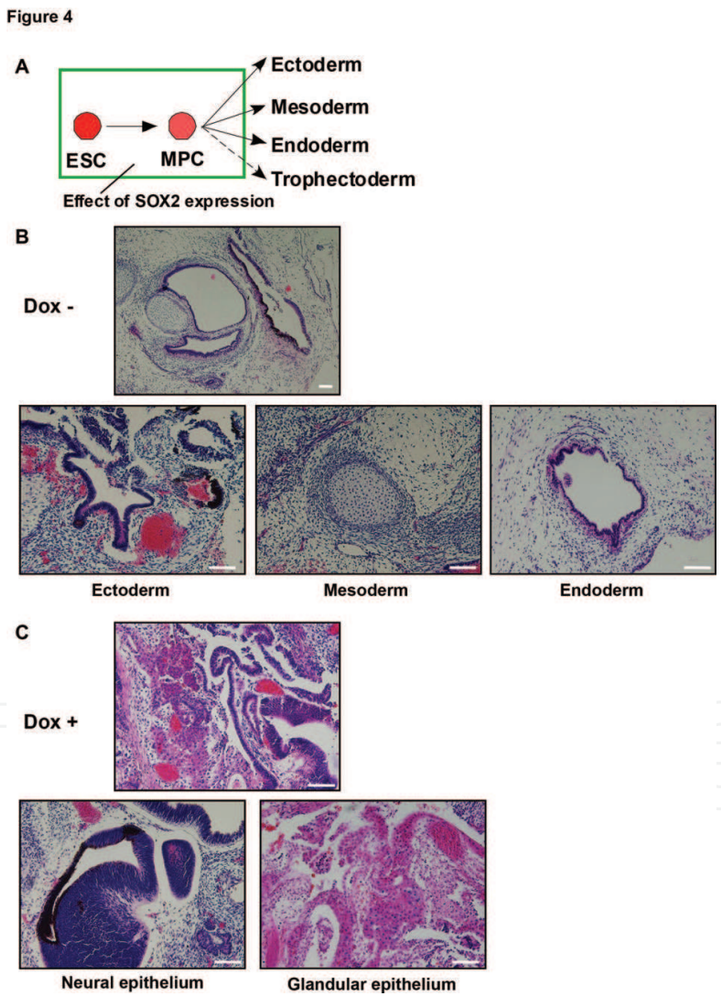


Fig. 4. Induction of teratomas by transplantation of human ESCs overexpressing *SOX2*. (A) SOX2 overexpression was effective for both human ES cells and their descendants. (B) In the absence of Dox, human ESCs generated teratomas composed of all three germ layers. (C) *SOX2* overexpressing ESCs induced by Dox in drinking water produced teratomas composed mainly of neuronal and glandular epithelium-like tissue. Scale bars = 100 mm.

time, increasing trophectodermal lineage genes. Elucidation of this process may provide novel insights into the gene regulatory networks for human ESC maintenance and differentiation.

3. Conclusion

In summary, the regulation of *SOX2* expression is essential for maintaining the pluripotent state of human ESCs. Both *SOX2* downregulation and upregulation caused trophectodermal differentiation. *SOX2* overexpression also promoted neural and glandular epithelium tissues during human ESC differentiation as a late or secondary effect. Our results demonstrate that in human ESCs, *SOX2* plays an important role in the maintenance of pluripotency and, possibly, trophoblast, neural and glandular epithelium development.

4. Acknowledgments

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5. References

Adachi, K., Kawase, E., Yasuchika, K., Sumi, T., Nakatsuji, N., and Suemori, H. (2006). Establishment of the gene-inducible system in primate embryonic stem cell lines. *Stem Cells*, Vol.24, No.11, (November 2006), pp. 2566-2572, ISSN 1066-5099.

Adachi, K., Suemori, H., Yasuda, S.Y., Nakatsuji, N., and Kawase, E. (2010). Role of SOX2 in maintaining pluripotency of human embryonic stem cells. *Genes Cells*, Vol.15, No.5, (May 2010) pp. 455-470, ISSN 1365-2443.

Ambrosetti, D.C., Scholer, H.R., Dailey, L., and Basilico, C. (2000). Modulation of the activity of multiple transcriptional activation domains by the DNA binding domains mediates the synergistic action of Sox2 and Oct-3 on the fibroblast growth factor-4 enhancer. *J. Biol. Chem.*, Vol.275, No.30, (July 2000), pp. 23387-23397, ISSN 0021-9258.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev., Vol.17, No.1, (January 2003), pp. 126-140, ISSN 0890-9369.

Babaie, Y., Herwig, R., Greber, B., Brink, T.C., Wruck, W., Groth, D., Lehrach, H., Burdon, T., and Adjaye, J. (2007). Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. *Stem Cells*, Vol.25, No.2, (February 2007), pp. 500-510, ISSN 1066-5099.

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., *et al.* (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell*, Vol.122, No.6, (September 2005), pp. 947-956, ISSN 0092-8674.

Brons, I.G., Smithers, L.E., Trotter, M.W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A., *et al.* (2007).

Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature,* Vol.448, No. 7150, (July 2007), pp. 191-195, ISSN 1476-4687.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*, Vol.113, No.5, (May 2003), pp. 643-655, ISSN 0092-8674.

Chambers, I., Silva, J., Colby, D., Nichols, J., Nijmeijer, B., Robertson, M., Vrana, J., Jones, K., Grotewold, L., and Smith, A. (2007). Nanog safeguards pluripotency and mediates germline development. *Nature*, Vol.450, No.7173, (December 2007), pp. 1230-1234, ISSN 1476-4687.

Daheron, L., Opitz, S.L., Zaehres, H., Lensch, W.M., Andrews, P.W., Itskovitz-Eldor, J., and Daley, G.Q. (2004). LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells*, Vo.l22, No.5, (May 2004), pp. 770-778, ISSN 1066-5099.

Darr, H., Mayshar, Y., and Benvenisty, N. (2006). Overexpression of NANOG in human ES cells enables feeder-free growth while inducing primitive ectoderm features. *Development,Vol.* 133, No. 6, (March 2006), pp. 1193-1201, ISSN 0950-1991.

Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature*, Vol.292, No.5819, (July 1981), pp. 154-156, ISSN 0028-0836.

Fong, H., Hohenstein, K.A., and Donovan, P.J. (2008). Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. *Stem Cells*, Vol.26, No.8 (August 2008), pp. 1931-1938, ISSN 1066-5099.

Hanna, J.H., Saha, K., and Jaenisch, R. (2010). Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. *Cell*, *Vol.143*, No.4 (November 2010), pp. 508-525, ISSN 0092-8674.

Hay, D.C., Sutherland, L., Clark, J., and Burdon, T. (2004). Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells. *Stem Cells*, Vol.22, No.2 (February 2004), pp. 225-235, ISSN 1066-5099..

Home, P., Ray, S., Dutta, D., Bronshteyn, I., Larson, M., and Paul, S. (2009). GATA3 is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. *J. Biol. Chem.* Vol.284, No. 42, (October 2009), pp. 28729-28737, ISSN 0021-9258.

Humphrey, R.K., Beattie, G.M., Lopez, A.D., Bucay, N., King, C.C., Firpo, M.T., Rose-John, S., and Hayek, A. (2004). Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells*, Vol.22 (July 2004), pp. 522-530, ISSN 1066-5099, .

Hyslop, L., Stojkovic, M., Armstrong, L., Walter, T., Stojkovic, P., Przyborski, S., Herbert, M., Murdoch, A., Strachan, T., and Lako, M. (2005). Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. Stem Cells, Vol.23, No. 8 (September 2005), pp. 1035-1043, ISSN 1066-5099.

Kelberman, D., Rizzoti, K., Avilion, A., Bitner-Glindzicz, M., Cianfarani, S., Collins, J., Chong, W.K., Kirk, J.M., Achermann, J.C., Ross, R., *et al.* (2006). Mutations within Sox2/SOX2 are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans. *J Clin. Invest.* , Vol.116, No.9 (September 2006), pp. 2442-2455, ISSN 0021-9738.

Kopp, J.L., Ormsbee, B.D., Desler, M., and Rizzino, A. (2008). Small increases in the level of sox2 trigger the differentiation of mouse embryonic stem cells. *Stem Cells,* Vol.26, No.4 (April 2008), pp. 903-911, ISSN 1066-5099.

Loh, Y.H., Wu, Q., Chew, J.L., Vega, V.B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., *et al.* (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet.* Vol.38, No.4, (April 2006), pp. 431-440, ISSN 1061-4036.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.*, Vol.78, No.12, (December 1981), pp. 7634-7638, ISSN 0027-8424.

Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A.A., *et al.* (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat. Cell. Biol.*, Vol.9, No.6, (June 2007), pp. 625-635, ISSN 1465-7392.

Matin, M.M., Walsh, J.R., Gokhale, P.J., Draper, J.S., Bahrami, A.R., Morton, I., Moore, H.D., and Andrews, P.W. (2004). Specific knockdown of Oct4 and beta2-microglobulin expression by RNA interference in human embryonic stem cells and embryonic carcinoma cells. *Stem Cells*, Vol.22, No. 5, (September 2004), pp. 659-668, ISSN 1066-5099.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*, Vol.113, No. (5), (May 2003), pp. 631-642, ISSN 0092-8674.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*, Vol.95, No. 3, (July 1998), pp. 379-391, ISSN 0092-8674.

Niwa, H., Burdon, T., Chambers, I., and Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev.*, Vol.12, No. 13, (July 1998), pp. 2048-2060, ISSN 0890-9369.

Niwa, H., Miyazaki, J., and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet. Vol.24, No.4 (April 2000), pp. 372-376, ISSN 12061-4036.

Niwa, H., Toyooka, Y., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, R., and Rossant, J. (2005). Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. *Cell*, Vol.123, No.5, (December 2005), pp. 917-929, ISSN 0092-8674.

Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature*, Vol.448, No. 7151, (June 2007), pp. 313-317, ISSN 0028-0836.

Pera, M.F., Andrade, J., Houssami, S., Reubinoff, B., Trounson, A., Stanley, E.G., Ward-van Oostwaard, D., and Mummery, C. (2004). Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. *J. Cell Sci.* , Vol.117, No.7, (March 2004), pp.1269-1280, ISSN 0021-9533.

Rodriguez, R.T., Velkey, J.M., Lutzko, C., Seerke, R., Kohn, D.B., O'Shea, K.S., and Firpo, M.T. (2007). Manipulation of OCT4 levels in human embryonic stem cells results in induction of differential cell types. *Exp. Biol. Med. (Maywood)*, Vol.232, No.10, (November 2007), pp. 1368-1380, ISSN 1535-3702.

Stadtfeld, M., and Hochedlinger, K. (2010). Induced pluripotency: history, mechanisms, and applications. *Genes Dev.*, Vol. *24*, No. 20, (October 2010), pp. 2239-2263, ISSN 0890-9369.

Suemori, H., Yasuchika, K., Hasegawa, K., Fujioka, T., Tsuneyoshi, N., and Nakatsuji, N. (2006). Efficient establishment of human embryonic stem cell lines and long-term

maintenance with stable karyotype by enzymatic bulk passage. *Biochem. Biophys. Res. Commun.*, Vol.345, No.3, (July 2006), pp. 926-932, ISSN 0006-291X.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, Vol.126, No.4, (August 2006), pp. 663-676, ISSN 0092-8674.

Taranova, O.V., Magness, S.T., Fagan, B.M., Wu, Y., Surzenko, N., Hutton, S.R., and Pevny, L.H. (2006). SOX2 is a dose-dependent regulator of retinal neural progenitor competence. *Genes Dev.*, Vol.20, No.9, (May 2006), pp. 1187-1202, ISSN 0890-9369 .

Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L., and McKay, R.D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature*, Vol.448, No.7150, (July 2007), pp. 196-199, ISSN 1476-4687.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science*, Vol.282, No.5391 (November 1998), pp. 1145-1147, ISSN 0036-8075.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., and Hearn, J.P. (1995). Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. U.S.A.*, Vol.92, No.17, (August 1995), pp. 7844-7848, ISSN 0027-8424.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E., and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature*, Vol.*448*, No.7151, (July 2007), pp. 318-324, ISSN 0028-0836.

Williamson, K.A., Hever, A.M., Rainger, J., Rogers, R.C., Magee, A., Fiedler, Z., Keng, W.T., Sharkey, F.H., McGill, N., Hill, C.J., *et al.* (2006). Mutations in SOX2 cause anophthalmia-esophageal-genital (AEG) syndrome. *Hum. Mol. Genet.,* Vol.15, No.9, (May 2006), pp. 1413-1422, ISSN 0963-6906.

Wood, H.B., and Episkopou, V. (1999). Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. *Mech. Dev.*, Vol.86, No.1-2, (August 1999), pp. 197-201, ISSN 0925-4773.

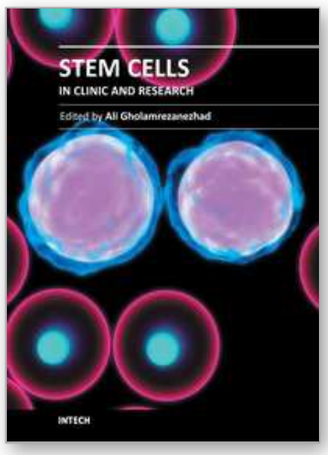
Xu, R.H., Chen, X., Li, D.S., Li, R., Addicks, G.C., Glennon, C., Zwaka, T.P., and Thomson, J.A. (2002). BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat. Biotechnol.*, Vol.20, No.12, (December 2002), pp. 1261-1264, ISSN 1087-0156.

Yamaguchi, S., Hirano, K., Nagata, S., and Tada, T. (2011). Sox2 expression effects on direct reprogramming efficiency as determined by alternative somatic cell fate. *Stem Cell Res.*, Vol.*6*, No.2, (March 2011), pp. 177-186, ISSN 1876-7753.

Ying, Q.L., Nichols, J., Chambers, I., and Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell, Vol.115, No.3, (October 2003pp. 281-292, ISSN 0092-8674.

Zaehres, H., Lensch, M.W., Daheron, L., Stewart, S.A., Itskovitz-Eldor, J., and Daley, G.Q. (2005). High-efficiency RNA interference in human embryonic stem cells. *Stem Cells,* Vol.*23*, No.3, pp. 299-305, ISSN 1066-5099.

Zhao, S., Nichols, J., Smith, A.G., and Li, M. (2004). SoxB transcription factors specify neuroectodermal lineage choice in ES cells. Mol. Cell Neurosci., Vol.27, No.3, (November 2004), pp. 332-342, ISSN 1044-7431.



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Based on our current understanding of cell biology and strong supporting evidence from previous experiences, different types of human stem cell populations are capable of undergoing differentiation or trans-differentiation into functionally and biologically active cells for use in therapeutic purposes. So far, progress regarding the use of both in vitro and in vivo regenerative medicine models already offers hope for the application of different types of stem cells as a powerful new therapeutic option to treat different diseases that were previously considered to be untreatable. Remarkable achievements in cell biology resulting in the isolation and characterization of various stem cells and progenitor cells has increased the expectation for the development of a new approach to the treatment of genetic and developmental human diseases. Due to the fact that currently stem cells and umbilical cord banks are so strictly defined and available, it seems that this mission is investigationally more practical than in the past. On the other hand, studies performed on stem cells, targeting their conversion into functionally mature tissue, are not necessarily seeking to result in the clinical application of the differentiated cells; In fact, still one of the important goals of these studies is to get acquainted with the natural process of development of mature cells from their immature progenitors during the embryonic period onwards, which can produce valuable results as knowledge of the developmental processes during embryogenesis. For example, the cellular and molecular mechanisms leading to mature and adult cells developmental abnormalities are relatively unknown. This lack of understanding stems from the lack of a good model system to study cell development and differentiation. Hence, the knowledge reached through these studies can prove to be a breakthrough in preventing developmental disorders. Meanwhile, many researchers conduct these studies to understand the molecular and cellular basis of cancer development. The fact that cancer is one of the leading causes of death throughout the world, highlights the importance of these researches in the fields of biology and medicine.

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