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Molecular Biomarkers of Embryonic Stem Cells

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1. Introduction

Embryonic stem cells (ESCs) are pluripotent cells capable of both limitless selfrenewal and differentiation into all embryonic lineages, and thus ESCs can give rise to any adult cell type. When ESCs are stably maintained in culture and their pluripotency is strictly enforced, they can serve as an unlimited source for tissue replacement in regenerative medicine for degenerative diseases such as neural disorders, heart disease, and type I diabetes. They also offer enormous potential for drug discovery and toxicology, human developmental biology, and cancer research. Studies of human ESCs (hESCs) biology have developed rapidly since the first reports of their derivation in 1998 (Thomson *et al.*, 1998). Many studies have tried to manipulate the growth and differentiation conditions of hESCs with variable success (Biswas and Hutchins, 2007; Hoffman and Carpenter, 2005). hESCs have been differentiated into the derivatives of all three germ layers: ectoderm, mesoderm, and endoderm. Specifically, these derivatives include cardiomyocytes, neural cells, hepatocyte-like cells, endothelial cells, pancreatic hormone expressing endocrine cells, and hematopoietic progenitor cells (Barberi *et al.*, 2007; Carpenter *et al.*, 2003; D'Amour *et al.*, 2006; Levenberg *et al.*, 2007; Lu *et al.*, 2007; Roy *et al.*, 2006; Wang *et al.*, 2007), and thus hESCs have great potential for use in regenerative medicine to restore heart disease, neuronal functions, hepatic disease, blood vessels, and type I diabetes. In addition, mouse ESCs (mESCs) can generate hepatocytes (Gouon-Evans *et al.*, 2006; Soto-Gutierrez *et al.*, 2007), insulin-producing cells (Schroeder *et al.*, 2006), cerebellar neurons (Salero and Hatten, 2007), and even germ cells (West *et al.*, 2006) *in vitro*, suggesting that hESC can be applied much more widely to regenerative medicine in the future. On October 2010, Geron corporation in United States announced plans to initiate the phase I clinical trial of hESC-derived oligodendrocyte progenitor cells. However, the clinical application of hESCs is restricted thus far for alleged ethical and scientific reasons. First, hESC research often faces opposition from those who object to the destruction of human embryos. Second, ESC therapy potentially poses the risk of tumorigenesis. ESCs frequently form teratocarcinomas when transplanted into mice. Moreover, the ability of ESCs to provide differentiated cells for regenerative medicine will require continual maintenance of the undifferentiated stem cells for long periods in culture. However, chromosomal stability during extended cell passage cannot be guaranteed, and recent cytogenetic studies of ESCs have revealed karyotypic aberrations (Baker *et al.*, 2007). Third, cell replacement therapies have been limited by the availability of sufficient quantities of cells for transplantation. Although there are many reports describing a method to maintain ESC properties in culture, the large-scale culture of

ESC lines is still problematic and susceptible to substantial challenges at present (Thomson, 2007). Fourth, the potential for immunorejection should be a concern in its therapeutic use, and thus histocompatible ESCs will be required. Genetically, matched pluripotent ESCs generated *via* somatic cell nuclear transfer or parthenogenesis are a potential source of patient-derived histocompatible cells and tissues for transplantation (Kim *et al.*, 2007; Menendez *et al.*, 2005; Yang *et al.*, 2007). Selected hESCs can serve as a source of histocompatible tissues for transplantation (Kim *et al.*, 2007). The largest impact on recent ESC biology is the generation of ESC-like cells termed "induced pluripotent stem cells (iPSCs)" from fibroblasts that are created by introducing four genes, Oct4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka, 2006). Although the first report described the generation of mouse iPSCs, human iPSCs have also been generated by introducing the same four genes as the mouse iPSCs derived from adult human dermal fibroblasts (Takahashi *et al.*, 2007) or the other distinct genes, Oct4, Sox2, Nanog, and LIN28, from human fetal fibroblasts (Yu *et al.*, 2007). These cells could differentiate into cell types of the three germ layers *in vitro* and produce teratomas, suggesting that iPSCs have the potential to generate patient- and disease-specific stem cells. Despite the importance of our knowledge of ESCs both in cell biology and clinical medicine, the molecular mechanism underlying the cell biological characteristics of ESCs such as the mechanism that maintains pluripotency and that regulates ESC differentiation, remains largely unknown.

Recent we showed the function of CD9, which is highly expressed in undifferentiated state in ESCs, as well as in embryos using the conventional gene targeting strategy to reveal whether CD9 can serve as a molecular marker to detect, classify, and isolate a particular subpopulation of ESCs and to monitor their state of differentiation (Akutsu *et al.*, 2009). This chapter also reviews other ESC molecular markers (Oct4, Sox2, Nanog, Klf4 and Rex1) in addition to CD9. The accumulation of these ES molecular marker studies will be provided a more detailed view of ESCs and facilitated our understanding of early embryonic development and cell-based therapies.

2. The membrane protein CD9

CD9 is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. Most of these members are cell-surface proteins. CD9 is expressed on the cell surface of mouse and rat male germline stem cells and of neural stem cells. Therefore, CD9 may be involved in the common machinery in stem cells of many self-renewing tissues. In addition, CD9 is also involved in cell development, growth, motility, cell differentiation, and egg-sperm fusion (Hadjiargyrou and Patterson, 1995; Kanatsu-Shinohara *et al.*, 2004b; Kaprielian *et al.*, 1995; Miyado *et al.*, 2000; Miyado *et al.*, 2008). The expression of CD9 in embryonic as well as adult stem cells populations may indicate a role of CD9 in stem cell self-renewal. Oka et al. have been reported that *CD9* is highly expressed in undifferentiated ESCs but rapidly down-regulated after cells differentiation (Oka *et al.*, 2002). Upon application of an antibody against CD9, mouse ESCs can not form compact ES-like colonies. Moreover, ESCs are dead in the presence of the anti-CD9 antibody. Therefore, CD9 may play a role in maintenance of undifferentiated mouse ESCs (Oka *et al.*, 2002). Despite high potential role of CD9 in mouse ESCs, however, *CD9* null mice are born healthy and grew normally. Therefore, the question whether CD9 has a role in pluripotent cells of the inner cell mass has not been addressed. Based on these findings, we recently reported that CD9 is dispensable for maintenance of an undifferentiated state and pluripotency (Figure 1)
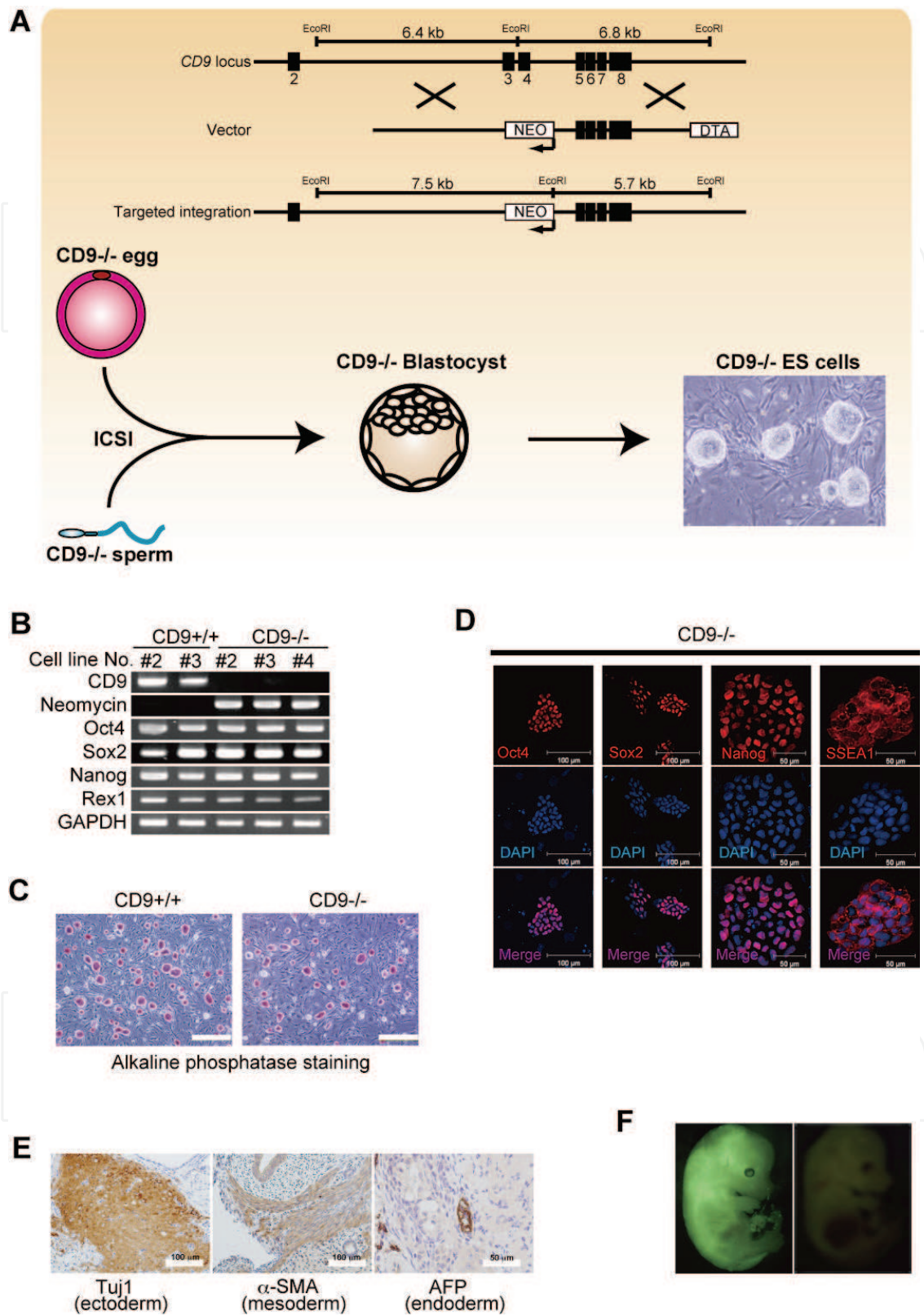
Fig. 1. **Generation of CD9 knockout ESCs**.
A. Strategy for generation of CD9-KO ES cells. The schematic maps of the CD9 allele (top), the KO vector carrying the neomycin cassette (middle), and the KO allele generated by homologous recombination (bottom) were shown in scale. *CD9-/-* fertilized eggs can not be

obtained by a cross of *CD9-/-* female and male mice. To address this issue, we used ICSI to insert *CD9-/-* sperm directly into the cytoplasm of *CD9-/-* egg and bypass the fusion step. As a result, *CD9-/-* ES cells were successfully isolated from blastocyst of the *CD9-/-* fertilized egg. **B**. RT-PCR analysis of ES cell-marker genes. Transcripts of *Oct4*, *Sox2*, *Nanog*, and *Rex1* were detected in both *CD9-/-* and *CD9+/+* ESCs without feeder cells. A neomycin-resistance gene was targeted to delete a part of the third exon and all of the fourth exon of *CD9*. Therefore, neomycin gene was detected in *CD9-/-* ES cell lines. **C**. Alkaline phosphatase staining shows undifferentiated *CD9-/-* ESCs as well as *CD9+/+* ESCs. Bar = 500 μm. **D**. *CD9-/-* and *CD9+/+* ESCs were fixed and stained with antibodies against Oct4, Nanog and Sox2. Nuclei were counterstained with DAPI. Bar = 50 μm. **E**. Teratomas of *CD9* knockout ESCs containing all three germ layers. **F**. Chimeric embryos derived from *CD9* knockout ESCs. When EGFP-positive *CD9-/-* ESCs, which were homozygotes for the partially deleted *CD9* allele and marked by the constitutively-active *EGFP* transgene, were injected into blastocysts, the embryos developed to chimeras at E13.5 in which widespread contributions of GFP-positive cells were observed in fluorescent stereomicroscopic observation (left panel). Right panel is the control embryo, showing an absence of fluorescence.

(Akutsu *et al.*, 2009). In this report, we established mouse ESCs lacking *CD9* by gene targeting. These *CD9-/-* ESCs exhibited the morphology and growth properties of ESCs, which express the ES marker factors Oct4, Sox2 and Nanog and have the ability to give rise to teratomas composed of tissues from all three germ layers. *CD9-/-* ESCs also generated mouse chimeras, contributing to various tissues. However, it has been reported that CD9 strongly expresses in mouse and human ES cells, suggesting that CD9 may be one marker of pluripotent stem cells (Nash *et al.*, 2007; Oka *et al.*, 2002). Therefore, our *CD9* knockout ESCs may explain the role of CD9 as a hallmark trait of stem cells-self-renewal and differentiation capacity. Thus, we should consider that CD9 might be one of markers for identification of pluripotent stem cells without functional significance like Oct4.

3. The transcription factor OCT4

Oct4 (octamer-binding transcription factor 4) also known as POU5F1 (POU domain, class 5, transcription factor 1) is a protein that is expressed by all pluripotent cells during mouse embryogenesis, and is also abundantly expressed by undifferentiated mouse ESCs and ECC cell lines (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Scholer *et al.*, 1989a; Scholer *et al.*, 1989b), as well as in EGC cell lines (Donovan and de Miguel, 2003). So far, however, experiments show that *Oct4* expression is generally weaker in germline stem cells (GSCs) (Kanatsu-Shinohara *et al.*, 2004a). Oct4 has also been established as a marker for human pluripotent ESCs. Therefore, downregulation of *Oct4* is required for the differentiation of somatic lineages. *Oct4*-deficient mouse embryos only develop to a stage that looks like a blastocyst, and although cells are allocated to the interior, these blastocysts are actually only composed of trophectoderm cells. As these structures lack a genuine ICM, they cannot be used to produce ESC cell lines (Nichols *et al.*, 1998). Oct4 has therefore been viewed as being involved in preventing trophectoderm and perhaps somatic-cell differentiation from the ICM, as well as being crucial for maintaining the pluripotent state during embryonic development. Recently, it has been reported that Oct4 only is sufficient to reprogram human neural stem cells to pluripotency (Kim *et al.*, 2009). Therefore, Oct4 is a master gene for

pluripotency. In mouse ESCs, the manipulation of *Oct4* expression through inducible or repressible *Oct4* transgenes indicates that the relative amount of Oct4 protein ultimately determines cell fate (Niwa *et al.*, 2000). However, the target genes that are actually responsible for implementing Oct4 decisions are only partly known (Du *et al.*, 2001; Saijoh *et al.*, 1996). Similarly, the potential interactions of Oct4 with other (co)factors, except for Sox2 (SRYrelated high-mobility group (HMG)-box protein-2; (Pevny and Lovell-Badge, 1997)), remain unclear.

4. The transcription factor SOX2

SRY (sex determining region Y)-box 2, also known as Sox2, is a transcription factor. Sox2 is a member of the HMG-domain DNA-binding-protein family that is implicated in the regulation of transcription and chromatin architecture (Pevny and Lovell-Badge, 1997). Sox2 forms a ternary complex with either Oct4 or the ubiquitous Oct1 protein on the enhancer DNA sequences of *Fgf4* (Yuan *et al.*, 1995). This allows Sox2 to participate in the regulation of the ICM and its progeny or derivative cells. Consistent with this role, *Sox2* is expressed in ESCs, but it is also expressed in neural stem cells. Therefore, Sox2 is essential to maintain self-renewal of undifferentiated embryonic stem cells. When gene targeting was used to inactivate *Sox2*, the primitive ectoderm was defective, but it could be rescued (albeit only to survive longer) by the injection of wild-type ESCs into the *Sox2*-/- blastocysts (Avilion *et al.*, 2003). Reduction of *Sox2* expression induces mouse ESCs to differentiate cells into the trophoectoderm lineage, indicating that *Sox2* function is essential for maintaining pluripotency. These results also are suggested by *Sox2* ablation *in vivo*. Interestingly, the forced expression of *Oct4* rescues the pluripotency of *Sox2*-null ESCs (Masui *et al.*, 2007). These findings indicate that Sox2 has a unique function in maintaining the pluripotency of ESCs that is related to the transcriptional activation of *Oct4*.

5. The transcription factor NANOG

Nanog is a highly divergent homeodomain-containing protein commonly accorded a central position in the transcriptional network of pluripotency (Boyer *et al.*, 2005; Cole *et al.*, 2008; Loh *et al.*, 2006; Wang *et al.*, 2006). It is essential for early embryonic development (Mitsui *et al.*, 2003). Undifferentiated, wild-type ESCs normally express Nanog. However, the physiological levels of Nanog in ESCs do not prevent their differentiation after LIF withdrawal. So, under physiological conditions, Nanog seems to be one of several factors that are expressed in pluripotent cells and are downregulated at the onset of differentiation. *Nanog*-/- mouse ESCs differentiate slowly into extra-embryonic endoderm lineages, which is consistent with the absence of a primitive ectoderm in *Nanog*-/- embryos that were analysed at E5.5 *in vivo* (Mitsui *et al.*, 2003). So Nanog expression is responsible for the maintenance of a primitive ectoderm in the embryo. Unlike wild-type ESCs and those forced to express *Oct4*, mouse ESCs that are overexpressing *Nanog* are resistant, but not completely refractory, to the spontaneous differentiation that occurs after LIF withdrawal or by chemical induction (for example, after treatment with 3-methoxybenzamide or all-*trans* retinoic acid). The persistence of Nanog therefore seems to delay, rather than block, the differentiation of ESCs; that is, the threshold of differentiation is increased rather than abolished. In contrast to Nanog overexpression, the reduced expression seen in *Nanog*+/- ESCs results in labile pluripotency whereby spontaneous differentiation is more likely to

occur after longer times spent in culture ('passages') (Hatano *et al.*, 2005). So, the amount of Nanog per cell is crucial for stably maintaining an undifferentiated state even in the presence of LIF. In addition, Nanog is not one of the Yamanaka 4 factors employed to reprogram mouse fibroblasts (Maherali *et al.*, 2007; Okita *et al.*, 2007; Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006; Wernig *et al.*, 2007). Moreover, addition of Nanog to these 4 factors has not been reported to increase efficiencies. However, Nanog is expressed weakly or not at all in incompletely reprogrammed cells that fail to activate properly the endogenous pluripotent transcriptional circuitry (Silva and Smith, 2008; Sridharan *et al.*, 2009; Takahashi and Yamanaka, 2006). Selection or screening for activation of endogenous Nanog expression facilitates isolation of fully reprogrammed iPSCs that can contribute to adult chimeras and give germline transmission (Okita *et al.*, 2007). Furthermore, in human cells Nanog does facilitate molecular reprogramming (Yu *et al.*, 2007). It has also been shown that Nanog promotes the transfer of pluripotency after ES cell fusion (Silva *et al.*, 2006). However, conditional gene deletion in ESCs revealed that Nanog is not essential for propagation of pluripotency *ex vivo* (Chambers *et al.*, 2007). *Nanog* null ESCs are more prone to differentiate but can be maintained indefinitely. Moreover, they contribute extensively to somatic chimeras, similar to *CD9* null ESCs.

6. Other transcriptional factors KLF4 and REX1

The mechanism by which Klf4 regulates ES cell self-renewal was first revealed by its identification as a highly up-regulated target gene of LIF signaling in ES cells (Li *et al.*, 2005). ES cells overexpressing Klf4 had a great propensity for self-renewal based on secondary embryoid body (EB) formation. *Klf4*-transduced EBs expressed higher levels of Oct4, consistent with the notion that Klf4 regulates ES cell self-renewal (Li *et al.*, 2005). The role of Klf4 in regulating pluripotency of ES cells is further revealed by global analysis of promoter occupancy by Yamanaka 4 factors (Kim *et al.*, 2008a). The results identified a transcriptional hierarchy within the four reprogramming factors with both auto-regulatory and feed-forward regulation. In addition, the study indicated that Klf4 is an upstream regulator of a large feed-forward loop that contains Oct4, Sox2, and c-Myc, as well as other common downstream factors including Nanog (Kim *et al.*, 2008a). Combining the results of these studies, it appears that Klf4 exerts a crucial role in somatic cell reprogramming and maintenance of ES cell self-renewal. On the other hand, Klf4 also exhibits both cytostatic and anti-apoptotic effect that is context-dependent. The ability of Klf4 in maintaining immortality of iPSCs maybe explained in part by the requirement of c-Myc as a member of reprogramming factor. Thus, in a manner similar to the cooperation between Klf4 and Ras to affect transformation (Rowland and Peeper, 2006), Klf4 and c-Myc cooperate to affect iPS cell self-renewal. Thus, Klf4 may suppress apoptosis induced by c-Myc and c-Myc neutralizes Klf4's cytostatic effect by suppressing p21WAF1/CIP1 (Yamanaka, 2007). In this manner, the balance between Klf4 and c-Myc might play a critical role in the establishment of an immortalized state of iPSCs. In addition, re-expression of Klf4 in an appropriate environment can regenerate the naïve ground state from mouse epiblast stem cells (EpiSCs), which are derived from columnar epithelial epiblast of the early post-implantation embryo (Brook and Gardner, 1997; Hanna *et al.*, 2010; Hanna *et al.*, 2009; Tesar *et al.*, 2007). Therefore, the essential requirement of Klf4 for reprogramming of somatic cells has subsequently been substantiated (Di Stefano *et al.*, 2009; Shi *et al.*, 2008). On the other hand, *Klf4+/-* mice were phenotypically and histologically normal (Katz *et al.*, 2005; Segre *et al.*, 1999). *Klf4-/-* mice

were born at the expected Mendelian ration. Therefore, Klf4 is also dispensable for maintenance of self-renewal and pluripotency of ESCs.

In addition to Oct4, Sox2, Nanog and Klf4, other putative transcription factors expressing pluripotent stem cells in stem-cell-specific manner have been also identified by several investigators. For example, Rex1 (for reduced expression-1, also known as *Zfp42*) was first identified a gene that expresses in F9 embryonal carcinoma (EC) cells and is down-regulated after retinoic acid (RA) treatment to induce differentiation (Hosler *et al.*, 1989). This gene encodes a C2H2 zinc-finger protein that is closely similar to Yy1, an evolutionally-conserved component of polycomb- related complex 2 (Gordon *et al.*, 2006). Its highly-specific expression in pluripotent stem cells has been confirmed in mouse and human ESCs (Eiges *et al.*, 2001; Rogers *et al.*, 1991), making it one of the most famous markers of pluripotency tested in various stem cells such as multipotent adult progenitor cells (Jiang *et al.*, 2002) and amniotic fluid cells (Karlmark *et al.*, 2005). Moreover, Rex1 is also known as a marker of the naïve ground state (Nichols and Smith, 2009). This has been argued that the blastocyst origin of human ESCs is evidenced by their expression of Rex1. However, its function in ESCs has not yet been characterized well although it has been reported that a targeted deletion of *Rex1* results in loss of the ability to differentiate into visceral endoderm induced by RA in F9 EC cells (Thompson and Gudas, 2002), and that a gene silencing by RNA interference for Rex1 results in loss of capacity to self-renew in ESCs (Zhang *et al.*, 2006). In addition, it has been recently reported that over-expression of Rex1 in ESCs neither induces differentiation in the presence of LIF nor maintains self-renewal in the absence of LIF. *Rex1-/-* ESCs can be established and contribute whole embryos after blastocyst injection, indicating that they possess proper pluripotency. Moreover, *Rex1-/-* mice were produced by the intercross of heterozygotes, and both male and female homozygotes were normal and fertile (Masui *et al.*, 2008). These findings support that Rex1 is also dispensable for maintenance of pluripotency in ESCs,

7. Concluding remarks

ESCs can bring unique application to medical and pharmaceutical research. Of note, recent advances in ESC biology have led to the successful generation of iPSCs, which could solve many scientific and ethical problems associated with regenerative medicine and cell-based therapies for degenerative human diseases. Thus, the understanding molecular biomarkers for ESCs is becoming increasingly important for the detection, classification, and isolation of a particular population of ES/iPS cells, and for monitoring the state of differentiation. This chapter discusses that Oct4 only is functionally essential for maintenance of pluripotency in ESCs (Table 1). This is consistent with evidence that Oct4 alone is able to reprogram mouse and human neural stem cells (Kim *et al.*, 2009; Kim *et al.*, 2008b). Therefore, other molecular biomarker highly expressed in ESCs might be markers for identification of pluripotent stem cells without functional significance like Oct4.

Although we have mainly focused here on studies using mouse ESCs, it will be important to understand how these findings relate to human ES cell studies. Studies on human ESCs may be best compared with studies on pluripotent mouse EpiSC lines, which have been established from post-implantation embryos (Brons *et al.*, 2007; Tesar *et al.*, 2007). ESCs and EpiSCs differ from one another in their factor requirements in vitro and in their capacity to incorporate into developing chimaeras. The recent demonstration of revertibility of primed EpiSC state to naïve ESC state is reported in mouse and human (Hanna *et al.*, 2010; Hanna *et al.*, 2009). In the near future, naïve human ESCs will need to be generated from blastocyst

Disrupted genes	Knockout ESCs	Passed pluripotency test	References
Oct4	not established[a]	N/D	Okamoto et al., 1990
Sox2	established[b]	chimera	Masui et al., 2007
Nanog	established	chimera	Chambers et al., 2007
Klf4	established	chimera	Nakatake et al., 2006
Rex1	established	chimera	Masui et al., 2008
CD9	established	chimera	Akutsu et al., 2009

N/D: not done
[a]Oct4 deficient-blastocysts lack ICM.
[b]Sox2 null ESCs are maintenanced by the forced expression of Oct4 .

Table 1. Function of the best-characterized ES cell markers

embryos. Because the naïve human pluripotent stem cells will provide a critical tool to model the earliest steps in human embryonic development. Understanding how pluripotent molecular biomarker assemblies change as cells move from one pluripotent compartment to another will allow us to view how the dynamic alterations in cell phenotype that underlie developmental transitions are dictated, which will be surely enhanced our knowledge of ESCs and of early embryonic development and cell-based therapies.

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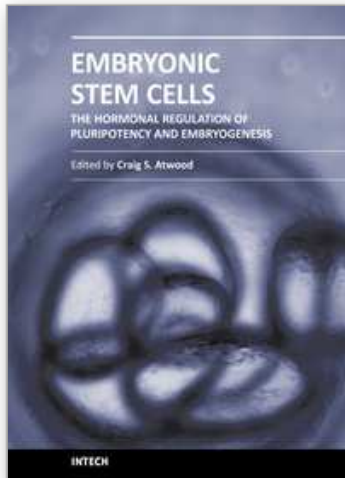
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Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem cell differentiation into tissue types derived from the ectoderm, mesoderm and endoderm.

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