MOLECULAR CHARACTERIZATION OF OCT4-EXPRESSING YOLK SAC ENDODERM STEM CELL LINES

A Dissertation

by

BISRAT GODEFAY DEBEB

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Biomedical Sciences

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Approved by:

Chair of Committee,	Bert Binas
Committee Members,	Ellen W. Collisson
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	Charles R. Long
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ABSTRACT

Molecular Characterization of Oct4-Expressing Yolk Sac Endoderm Stem Cell Lines.

(May 2008)

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Chair of Advisory Committee: Dr. Bert Binas

The extraembryonic endoderm (XEN) defines the yolk sac, a set of membranes that provide essential support for mammalian embryos. Recently, the committed XEN-precursor was identified in the embryonic Inner Cell Mass (ICM) as a group of cells that intermingles with the closely related, anatomically indistinguishable epiblast (EPI)-precursor that gives rise to the fetus. In vitro, the EPI-precursor is represented by the well-known embryonic stem (ES) cell lines, but cell lines representing the XEN-precursor are not known. Furthermore, since the XEN-precursor cells were discovered only very recently, the unexpected fact that they express the key pluripotency marker Oct4 has not been explored. Recently, however, our laboratory has isolated rat XEN cell lines that express Oct4, leading to the following two questions: (i) Do these new XEN cell lines represent XEN-precursor cells? (ii) Is their Oct4 expression regulated similarly as previously known from ES cells? These two questions are addressed here by lineage marker and reporter gene analyses.

Whole culture analyses showed that rat XEN cell lines expressed markers of all XEN stages including XEN-precursor, primitive endoderm (PrE) and/or visceral endoderm (VE), and parietal endoderm (PE) but trophoectoderm and EPI-precursor markers were missing. In line with this, immunocytochemistry demonstrated heterogeneity and directly visualized the XEN-precursor, PrE/VE, and PE subpopulations. Low-density plating and time-dependent immunocytochemistry on resulting colonies strongly suggested that XEN-precursor cells generate the other XEN stages. Moreover, by analyzing single-cell derived clones, it was shown that culture heterogeneity results from the self-renewal and differentiation of a single cell. Reporter gene analyses using the 5' regulatory region of the mouse Oct4 gene revealed that a DNA fragment containing the previously described distal enhancer drove reporter gene expression only in ES cells whereas inclusion of an upstream fragment led to high expression in both mouse ES and rat XEN cells.

In conclusion, our rat XEN cell lines contain XEN-precursor cells that differentiate extensively, providing for the first time an in vitro model that mimics the natural process of early XEN differentiation. In addition, they regulate Oct4 gene transcription differently than ES cells suggesting heterogeneous Oct4 regulation within the mammalian ICM.

DEDICATION

To my wife, Makida, for her love, patience and support and to my son, Nolawi, for providing me his lovely smiles while I was writing my dissertation.

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I take special pride in expressing my deep gratitude to my parents and brothers for their unconditional love and continued support. I am also indebted to my sisters-in-law for their encouragement and support.

Thanks also go to my Habesha friends for making my time at Texas A&M University a great experience with lots of memories.

Finally, I thank the Department of Veterinary Pathobiology for financial support that helped me complete my study.

NOMENCLATURE

dpc	days post coitum
EC	Embryonic carcinoma cells
EG	Embryonic germ cells
EPI	Epiblast
EPI-P	Epiblast-precursor
ES	Embryonic stem cells
ICM	Inner cell mass
LIF	Leukemia inhibitory factor
PE	Parietal endoderm
PrE	Primitive endoderm
PGCs	Primordial germ cells
PYS	Parietal yolk sac
RA	Retinoic acid
TE	Trophectoderm
TS	Trophoblast stem cells
VE	Visceral endoderm
VYS	Visceral yolk sac
XEN	Extraembryonic endoderm
XEN-P	Extraembryonic endoderm-precursor

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Lineage specification in the blastocyst

It is important to understand the morphological changes that lead to formation of the blastocyst in order to know the cellular origins of the first three lineages. Following fertilization of the oocyte, the zygote cleaves several times leading to the formation of the morula. During the morula stage, cells compact against each other by forming intercellular junctions. The outer layer of the late morula are epithelized and become trophoectoderm (TE) while the inner cells generate the pluripotent inner cell mass (ICM). During late blastocyst stages the ICM becomes organized into the epiblast (EPI) and primitive endoderm (PrE) cells (Fig. 1**;** Rossant, 2007). Thus, by the late blastocyst stage, the mammalian embryo is composed of three distinct cell types: the TE, EPI and PrE, which are committed to their different later lineages and are no longer totipotent. The TE is exclusively restricted to form the fetal portion of the placenta and the trophoblast giant cells. The EPI gives rise to the entire fetus as well as extraembryonic mesoderm cells that line the visceral yolk sac and placental villi. The PrE subsequently differentiates into visceral endoderm (VE) and parietal endoderm (PE).

The VE cells cover the epiblast and extraembryonic ectoderm (trophoblast) at the

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This dissertation follows the style of *Development*.

egg cylinder stage of development. VE cells in contact with the trophoblast are more columnar and cuboidal, while VE cells overlying the epiblast are flatter and more epithelial in shape (Bielinska et al., 1999; Rossant, 2007). The VE cells are morphologically and functionally similar to gut endoderm. They have microvilli and phagocytic and pinocytic vesicles that allow efficient absorption and digestion of maternal nutrients. VE cells also synthesize and secrete proteins involved in nutrient transport, such as transferrin and apolipoproteins (Bielinska et al., 1999). While PE cells are terminally differentiated, VE cells retain the ability to differentiate into PE (Gardner, 1983; Ninomiya et al., 2005) and can even trans-differentiate into somatic cells (Sobis et al., 1993).

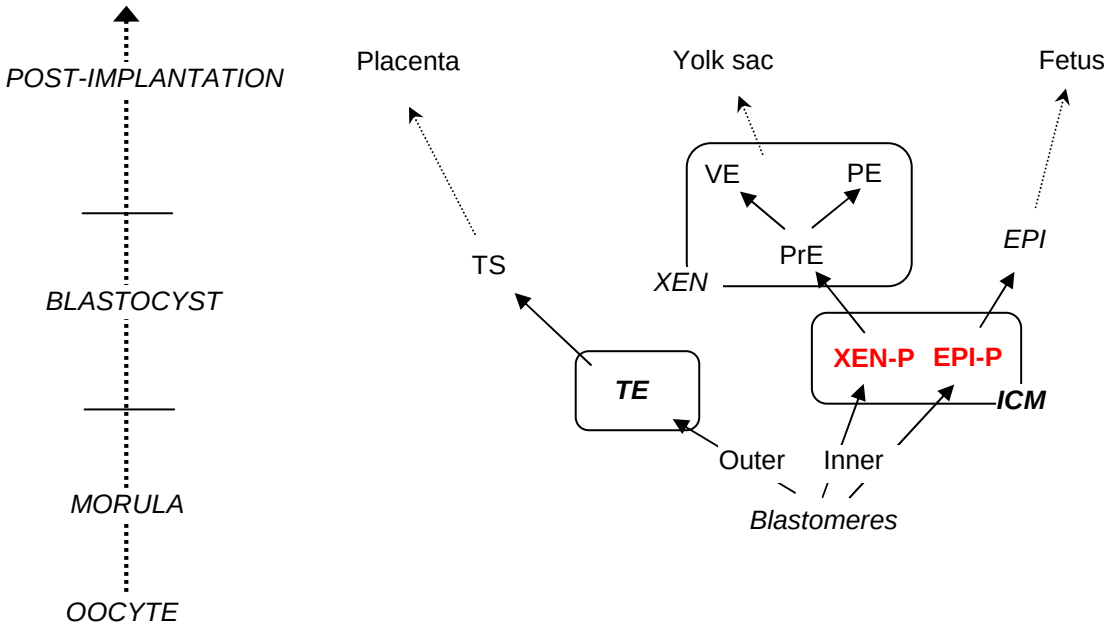

Fig. 1. Stages of early embryonic development and the cell lineages present in vivo. The three fundamental cell lineages in a mammalian embryo are the TE, EPI and XEN, which give rise to the placenta, fetus and yolk sac, respectively, postimplantation. EPI-P, EPI-precursor; XEN-P, XEN-precursor.

The PE, a single layer of cells that develops in association with TE, synthesizes copious amounts of basement membrane proteins (such as laminin, type IV collagen) to form Reichert's membrane in conjunction with the trophoblast giant cell layer (Hogan et al., 1980). The combination of PE cells, giant cells and the intervening thick basement membrane comprises the early functioning, transient parietal yolk sac.

The first lineage choice (TE vs. ICM)

Early differentiation of the mammalian embryo leads to the development of two distinct lineages, the TE and the ICM. These lineages can be distinguished by the expression patterns of several lineage-specific transcription factors.

An important gene that promotes early TE development is the Caudal-like transcription factor, Cdx2 which is expressed in the TE of the blastocyst (Beck et al., 1995; Strumpf et al., 2005). Cdx2 protein is first detectable in the nuclei of select cells at the eight-cell stage (Niwa et al., 2005) and becomes restricted to outer cells of the late morula (Niwa et al., 2005; Strumpf et al., 2005). Cdx2 mutant embryos form a normal-appearing blastocyst, although ICM markers such as Oct4 and Nanog fail to be repressed in the TE (Strumpf et al., 2005), indicating a defect in restriction of the ICM/TE lineage. Cdx2 mutant TE eventually collapses and undergoes apoptosis, leading to blastocyst lethality before implantation. In addition to Cdx2, transcription factor genes like Eomesodermin and Mash2 show a reciprocal pattern of expression to Oct4 in the early embryo, becoming restricted to the TE by the blastocyst stage (Ciruna and Rossant, 1999; Russ et al., 2000; Guillemot et al., 1994; Niwa et al., 2005). Targeted mutation of

these genes reveals that they are required for trophoblast lineage development. Eomesodermin mutants die shortly after implantation because the TE fails to proliferate and presumably fails to provide support for the embryonic lineages (Russ et al., 2000). Mash2 mutants develop to E10.5 but die because of placental defects caused by loss of spongiotrophoblast (Guillemot et al., 1994).

The POU (Pit-Oct-Unc) domain transcription factor Oct4 is essential for ICM development. Oct4 is initially detectable in nuclei of all cells of the early embryo, but its expression becomes restricted to the ICM upon blastocyst formation (Palmieri et al., 1994). Oct4 mutant mice die around the time of implantation, lack ICM derivatives such as epiblast and yolk sac, and only TE-like cells can be recovered (Nichols et al., 1998). In addition, no ES cells can be derived from Oct4 mutant blastocysts, and conditional knock-down of Oct4 in ES cells results in their transformation into trophoblast-like cells (Niwa et al., 2000). Thus, Oct4 is required for ICM specification and maintenance, and for suppressing trophoblast cell fate, and the antagonism between Oct4 and Cdx2 reinforces the ICM/TE lineage fates. Another gene, Sox2 (SRY box containing gene), is also believed to be important in ICM cell fate specification. Like Oct4, Sox2 is expressed in all blastomeres of the cleavage stage embryo and becomes restricted to the ICM and epiblast at the blastocyst stage (Wood and Episkopou, 1999). Mutation of Sox2 results in early postimplantation lethality with failure of epiblast growth (Avilion et al., 2003). Cultured Sox2−/− ICM cells show characteristics of differentiated trophoblast (Avilion et al., 2003), and thus Sox2 seems to share a role with Oct4 in maintaining ICM cell fate.

The second lineage choice (EPI vs. PrE)

At E4.5 in the mouse blastocyst, the second lineage decision leads to the establishment of two morphologically distinct populations from the ICM: the EPI, which is the embryonic lineage, and the PrE, which will give rise to an extraembryonic tissue, the yolk sac endoderm. The PrE forms as a monolayer on the surface of the ICM directly facing the blastocoele, while the EPI lies between the PrE and the TE.

The establishment and segregation of the two lineages within the ICM have been understood only recently. Traditionally, it has been thought that ICM cells of the early blastocyst are a homogenous population of bipotential cells each with the ability to become both EPI and PrE. But recent findings indicate that the early ICM consists of a heterogeneous population of cells (Fig. 2). Chazaud et al. (Chazaud et al., 2006) found that lineage-specific markers for the EPI and the PrE are expressed in non-overlapping manner in the ICM of the early blastocyst before morphological segregation of these two lineages. In this study, Oct4 was expressed at equivalent levels in all ICM cells, while Nanog and Gata6, the EPI and the PrE-specific transcription factors, were expressed in a mutually exclusive manner. Thus, ICM cells expressing Oct4 and Nanog are EPI-precursors while those expressing Oct4 and Gata6 are PrE-precursors (XEN-precursors). Moreover, individual labeled ICM cells from the early blastocyst contributed to either EPI or PrE lineages but rarely to both (Chazaud et al. 2006). The finding that the early ICM is not homogenous was supported by another study that used single-cell microarray analysis (Kurimoto et al., 2006) and showed that individual ICM cells of the early blastocyst have distinct EPI-like or PrE-like gene expression profiles. These findings and

the segregation and dispersed localization of Nanog and Gata6 expressing cells in the ICM suggests that allocation of cells to the EPI and PrE lineages is not influenced by any position-specific activity within the ICM, unlike previous thoughts that cell fate within the ICM is determined based on their position in the ICM cell cluster.

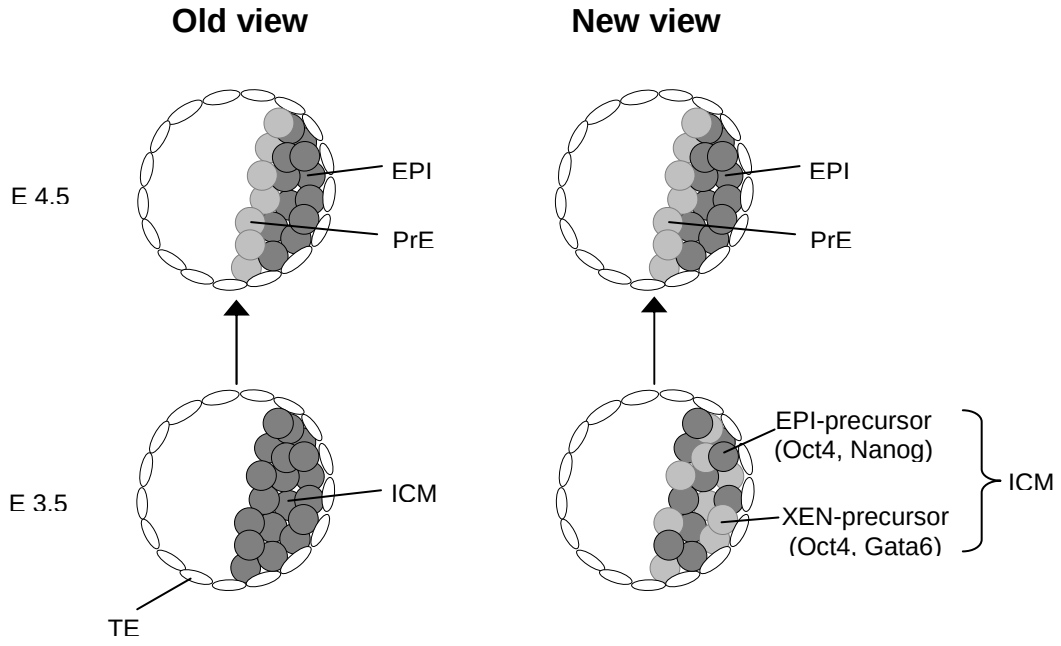

Fig. 2. The new understanding of early embryogenesis. During early blastocyst stage (E3.5), the ICM is a mosaic of EPI-precursor and XEN-precursor cells that both express Oct4 but later (E4.5), it segregates into the EPI and the PrE.

The identification of genes required for PrE formation has given insight into mechanisms regulating the initiation and maintenance of EPI/PrE lineage restriction. In the late blastocyst, the homeobox transcription factor Nanog is expressed in the EPI (Chambers et al., 2003; Mitsui et al., 2003), and the zinc finger transcription factor Gata6 is expressed in the PrE (Morrisey et al., 1998; Koutsourakis et al., 1999). The

knockout of Nanog in ES cells leads to differentiation to PrE-like cells and expression of the PrE marker Gata6 (Mitsui et al., 2003). Similarly, overexpression of Gata6 in ES cells leads to differentiation into a PrE-like cells and down-regulation of the EPI marker Oct4 (Fujikura et al., 2002; Shimosato et al., 2007). Likewise, Gata6 knockout mice are embryonically lethal at E5.5 – 6.5 due to defects in VE differentiation and subsequent XEN development (Morrisey et al., 1998; Koutsourakis et al., 1999) and Gata6-null ES cells fail to undergo VE differentiation in vivo and in vitro (Morrisey et al., 1998). This suggests that Gata6 is required for early XEN, including PrE, as well as for the development of both VE and PE. Thus, Nanog and Gata6 appear to be antagonistic, with Nanog promoting the EPI formation and blocking the PrE formation while Gata6 promoting the PrE lineage and blocking EPI formation. This antagonism may reinforce EPI and PrE cell fates to promote self-renewal and prevent switching among these lineages.

Similar to Gata6 mutations, the mutations in the transcription factors Hnf4 and Gata4 also lead to defective development of the VE (Chen et al., 1994; Watt et al., 2004). Likewise, loss of Grb2 prevents the formation of PrE and expression of Gata6 during the late blastocyst stage because Grb2, which encodes an adaptor protein for receptor tyrosine kinases, facilitates the sorting of the EPI/PrE lineages in the blastocyst (Cheng et al., 1998; Chazaud et al., 2006; Hamazaki et al., 2006).

Stem cells from mammalian blastocyst lineages

Stem cells from the blastocyst have provided a valuable tool for the genetic analysis of lineage-promoting factors and have confirmed the central importance of transcription factors in maintaining cell fates.

Permanent cell lines representing the EPI-precursor (called embryonic stem cells) and TE (called trophoblast stem cells) have been derived from the mouse blastocyst (Evans and Kaufmann, 1981; Martin, 1981; Tanaka et al., 1998). But so far, the third stem cell type that represents all the derivatives of the XEN lineage including the ICM-stage XEN (XEN-precursor) has not been published. In previously described XEN cell lines (Fowler et al., 1990; Wewer et al., 1982; Notarianni and Flechon, 2001; Kunath et al., 2005; Ouhibi et al., 1995) the key XEN-precursor marker Oct4 was not studied or not expressed. Moreover, insofar as studied, these cell lines contribute predominantly to the PE and not to the VE in vivo (Kunath et al., 2005). As will be described in detail in the forthcoming chapters, our laboratory is the first to isolate the XEN-precursor cell lines as evidenced by their molecular signature (including Oct4 expression), differentiation potential and repopulation of the PE and VE in chimeras.

The known mouse blastocyst-derived stem cell types use different signaling pathways to maintain stem cell proliferation, require different transcription factor expression for specifying the stem cell state, and contribute to their cognate lineage in chimeric embryos (Beddington and Robertson, 1989; Tanaka et al., 1998; Kunath et al., 2005). The properties of the existing stem cell types from the blastocyst will be described below.

Embryonic stem (ES) cells

ES cells can be derived from the epiblast lineage of the blastocyst (Evans and Kaufmann, 1981; Martin, 1981). They can be obtained in vitro by culture of ICM cells of the blastocyst in the presence of the cytokine, leukemia inhibitory factor (LIF) (Smith et al. 1988) and are often grown on a layer of mouse embryonic fibroblast 'feeder' cells. In LIF-supplemented medium, ES cells grow as smooth colonies of rounded cells and can grow indefinitely as undifferentiated cells that express markers like Oct4. Upon removal of LIF, however, they downregulate Oct4, rapidly lose self-renewal capacity and spontaneously differentiate into a variety of cell types of the body (Chambers and Smith, 2004). Generation of chimeras by aggregation of ES cells with eight-cell embryos or injection of ES cells into blastocysts revealed that ES cells are pluripotent but not totipotent. Although they contribute to all tissues of the fetus including the germline, they fail to contribute efficiently to the TE or XEN lineages (Beddington and Robertson, 1989).

Maintaining pluripotency of ES cells

Pluripotency refers to the ability of a cell to differentiate into various types of cells that belongs to all definitive tissues: ectoderm, mesoderm and endoderm. Understanding the molecular basis of pluripotency and the conditions required to maintain this state in the early embryo is vital for an understanding of the maintenance of pluripotent ES cells in culture (Smith, 2001; Niwa, 2007).

Maintaining cells in an undifferentiated state requires fine tuning of an intricate network of cellular factors that prevents the cell from entering any of the differentiation lineages. As will be discussed later in detail, Oct4 is a crucial determinant of pluripotency in ES cells as knockout of this gene leads to the formation of TE-like cells that are non-pluripotent (Nichols et al., 1998). Besides Oct4, the transcription factors Nanog and Sox2 have been identified to contribute to the pluripotency of ES cells (Niwa, 2007; Chambers et al., 2003; Mitsui et al., 2003; Avilion et al., 2003). The knockout of Nanog leads to conversion of all ICM cells to a PrE phenotype (Mitsui et al., 2003) and causes loss of pluripotency in ES cells (Chambers et al., 2003; Mitsui et al., 2003). Sox2 shows an expression pattern similar to Oct4 during early embryogenesis and its mutation results in early postimplantation lethality with failure of epiblast growth (Avilion et al., 2003).

Trophoblast stem (TS) cells

TS cells can be derived from blastocysts or early postimplantation trophoblast by culture in the presence of fibroblast growth factor 4 (Fgf4) plus heparin and primary embryo fibroblast-conditioned medium (Tanaka et al., 1998). Under these conditions, TS cells grow as epithelial colonies. It has also been shown that members of the transforming growth factor b (TGF-b) family Activin (Erlebacher et al., 2004) can replace the need for conditioned medium in maintaining the undifferentiated TS state. TS cells can be passaged for many generations without differentiation, but upon withdrawal of Fgf4, they will assume more differentiated morphologies and begin

expressing markers of the differentiated trophoblast (Tanaka et al., 1998). Like ES cells, TS cells also retain the capacity to contribute to normal tissues when reintroduced into the early embryo. However, TS cells only contribute to the trophoblast lineages of the placenta and not to the fetus itself (Tanaka et al., 1998).

Permanent cell lines from rat blastocysts (called “rat ES cell-like” cells) have been isolated, which like mouse ES cells show compact morphology, and express alkaline phosphatase and SSEA1 (Fandrich et al., 2002; Vassilieva et al., 2000). Although initially thought to be ES-like, further analysis of these cell lines by the Austin Smith group (Buehr et al., 2003) and in our laboratory (Debeb and Binas, unpublished results) confirmed that they express the TS cell markers Cdx2 and Eomesodermin but lack Oct4 and other ES cell markers. We now call these cell lines “TE-like” and we have used them as positive controls when analyzing TS cell genes by RT-PCR (Line B10 in Figs. 6B, 12B).

Extraembryonic endoderm (XEN) cells

XEN cells are the third stem cell type that can be derived from the blastocyst (Fowler et al., 1990; Wewer et al., 1982; Notarianni and Flechon, 2001; Kunath et al., 2005), but appear to represent a stage later than the PrE. XEN cells require feeders or feeder-conditioned medium for growth and exhibit round-epithelial interchangeable morphologies. Chimeras generated by injection of XEN cells into blastocysts showed exclusive contributions to the extraembryonic yolk sac, although predominantly

restricted to the parietal and not visceral yolk sac (Kunath et al., 2005; Shimosato et al., 2007).

Our laboratory has, for the first time, isolated XEN cell lines that have the features of all XEN derivatives including the XEN-precursor of the ICM. These cell lines are the basis of these projects and will be discussed in Chapters II and III.

The Oct4 gene

Oct4 (also termed Oct3, pou5f1) is a member of the POU transcription factor family that are able to activate the transcription of genes bearing cis-acting elements containing an octameric sequence (ATGCAAAT) called the octamer motif, within their promoter or enhancer region. The POU domain is a bipartite DNA binding domain containing the POU homeodomain and the POU specific domain (Herr and Cleary, 1995; Ryan and Rosenfeld, 1997). It encodes a protein of 352 amino acids.

Oct4 was first identified in the extracts of undifferentiated ES and embryonal carcinoma (EC) cells (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990a). This suggests an association of Oct4 with the early stages of mouse embryogenesis. Oct4 has also been identified in other mammalian species including human, bovine, and rat (van Eijk et al., 1999; Takeda et al., 1992; Hurt et al., 2004). There is a strong conservation in gene sequence and structure in the mammalian Oct4 genes. For example, sequence comparisons of the promoter/enhancer regions of human and cow Oct4 genes with that of the mouse ortholog revealed 87% and 82% overall protein sequence identity and alignment of upstream sequences of human, bovine and mouse promoters revealed

four conserved regions (CR1 through CR4) (Nordhoff et al., 2001). Moreover, the mammalian Oct4 gene is organized into five exons and is located in the region of the major histocompatibility complex (MHC). The Oct4 gene is mapped to chromosome 6 in humans (Takeda et al., 1992), to chromosome 17 in mouse (Scholer et al., 1990b; Yeom et al., 1991), to chromosome 20 in rat (Hurt et al., 2004) and to chromosome 23 in bovine (van Eijk et al., 1999).

Expression of Oct4

Oct4 is expressed in early mouse embryogenesis and in cells with a totipotent or pluripotent differentiation ability (Rosner et al., 1990; Scholer et al., 1990b; Yeom et al., 1996) (Fig. 3). Oct4 mRNA and protein are present in unfertilized oocytes and the protein is localized to the pronuclei following fertilization (Palmieri et al., 1994; Rosner et al., 1990). Embryonic expression of Oct4 gene is activated around the eight-cell stage, when levels of both mRNA and protein increase significantly in the nucleus (Palmieri et al., 1994; Yeom et al., 1991). There is a high and uniform expression of Oct4 mRNA and protein in all cells of the embryo through the morula stage (32-64 cells), but the levels decrease in the outer cells of the morula when they differentiate and form the TE. In contrast, Oct4 expression is maintained in the ICM of the blastocyst (Okamoto et al., 1990; Rosner et al., 1990). Following implantation, Oct4 expression is restricted to the epiblast (also called primitive ectoderm) although it is transiently expressed at high levels in cells of the primitive endoderm (also called hypoblast) (Palmieri et al., 1994). Oct4 expression is maintained in the epiblast but, as the primitive endoderm

differentiates into visceral and parietal endoderm, Oct4 protein levels transiently increase and then decrease to undetectable levels. During gastrulation, Oct4 expression becomes downregulated in the epiblast and by 7.5 dpc is confined exclusively to newly established primordial germ cells (PGCs), precursors of the gametes (Yeom et al., 1996). PGCs continue to express Oct4 as they proliferate and migrate to the forming genital ridges of the developing fetus.

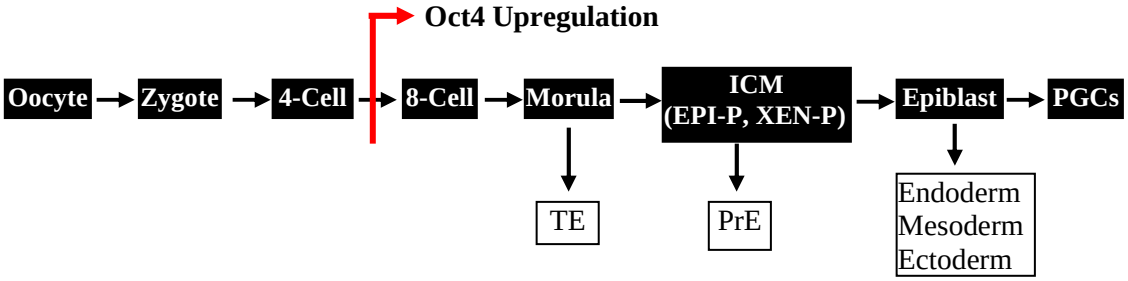

Fig. 3. Oct4 expression pattern in preimplantation and postimplantation development. Oct4 is expressed at low levels until the 8-cell stage, when the endogenous oct4 is turned on. Black boxes indicate stages that express Oct4 while white boxes show little or no Oct4. EPI-P, EPI-precursor; XEN-P, XEN-precursor; PGCs, Primordial germ cells; ICM, inner cell mass; TE, trophoectoderm; PrE, primitive endoderm.

Oct4 is also expressed in undifferentiated ES and EC cell lines that are derived from the ICM and epiblast, respectively. Differentiation of ES and EC cells with retinoic acid (RA) or other differentiating agents results in rapid Oct4 downregulation (Scholer et al. 1990a, 1990b; Minucci et al., 1996). Oct4 gene expression has also been detected at low or undefined levels in adult-derived somatic stem cells (Jiang et al., 2002; Matthai et al., 2006) and in tumors (Palumbo et al., 2002; Ezeh et al., 2005; Monk and Holding, 2001; Suo et al., 2005).

Role of Oct4 in pluripotency and development

The remarkable expression patterns of Oct4 during early embryonic development and in undifferentiated ES and EC cell lines suggest an important role of Oct4 in maintaining stem cell pluripotency. This importance has been demonstrated by gene targeting experiments where Oct4-deficient knock-out embryos developed to the blastocyst stage, but failed to make the ICM and were comprised solely of the trophoectoderm (Nichols et al., 1998). Moreover, the experimental reduction of Oct4 levels in ES cells causes the cells to lose pluripotency, and they differentiate into trophectoderm in culture (Hay et al., 2004; Niwa et al., 2000; Kim et al., 2002). The Oct4 gene controls the pluripotency of stem cells in a quantitative or dosage-dependent manner. This was elegantly demonstrated in ES cells by Niwa et al. (Niwa et al., 2000) using an inducible Oct4 expression coupled to homologous recombination of the endogenous Oct4 gene. In this study, a 50% decrease in expression of the endogenous Oct4 levels triggered trophoectodermal differentiation, whereas a 50% increase initiated upregulation of primitive endoderm and mesoderm markers. This confirms the findings of Palmieri et al. (Palmieri et al., 1994) who described Oct4 protein expression to appear higher in the newly forming primitive endoderm than that seen in the ICM population of murine blastocysts. In accord with this, a recent finding showed that a transient increase of Oct4 is involved in cardiac specification of the epiblast (Zeineddine et al., 2006).

The dose dependent effect of Oct4 was also observed to dictate the oncogenic potential of ES cells (Gidekel et al., 2003); high levels increase the malignant potential of ES cell-derived tumors while Oct4 inactivation induced regression of the malignant

component (Gidekel et al., 2003). This suggests that Oct4 plays a significant role in the malignant behavior of ES cells. Moreover, ectopic expression of Oct4 resulted in dysplastic growths in epithelial tissues by inhibiting cellular differentiation in a manner similar to that in ES cells (Hochedlinger et al., 2005).

In addition to its role in the maintenance of pluripotency of stem cells, Oct4 was also found to be important in keeping the viability of the mammalian germline (Kehler et al., 2004). Using conditional gene targeting, primordial germ cells lacking Oct4 expression have been shown to undergo apoptosis rather than differentiation to TE. Moreover, Oct4 was found to be crucial in inducing pluripotency in a recent outstanding study by Yamanaka and Takahashi (Yamanaka and Takahashi, 2006) who engineered ES-like cells from normal mouse skin cells. In this study, the researchers introduced four gene-transcription factors (Oct4, Sox2, Klf4 and c-myc) into fibroblast cells originating from mouse skin, and specifically selected those cells that, in response to these factors, expressed genes indicative of a pluripotent state. If this method can be translated to humans, patient-specific stem cells could be made without the use of donated eggs or embryos (Yamanaka and Takahashi, 2006; Yamanaka, 2007). This remarkable work was validated by other teams (Okita et al., 2007; Maherali et al., 2007; Wernig et al., 2007).

Role of Oct4 in transcription

Oct4 activates and represses transcription of different target genes. The genes encoding fibroblast growth factor 4 (Fgf4), the Osteopontin adhesion molecule (OPN), and the acidic zinc finger gene Rex1 provide models of Oct4-dependent positive

regulation while the repression of Cdx2 and HCG a and b genes are examples of a negative regulation.

Fgf4 is expressed in the ICM and is essential for the survival of postimplantation mouse embryos (Feldman et al., 1995). Fgf4 is a stem cell-specific growth factor, and has an enhancer element located within the 3'-untranslated region (UTR) of the gene, which is responsible for its stem cell-specific expression (Curatola and Basilico, 1990). This enhancer contains an octamer motif adjacent to a binding site to which Oct4 and Sox2 bind cooperatively to activate transcription synergistically (Yuan et al., 1995). In the absence of Sox2, Oct4 is not sufficient for Fgf4 enhancer activity. Targeting of the Oct4 gene demonstrated that Oct4-null embryos either do not express or have a reduced expression of Fgf4 (Nichols et al., 1998). Stimulation by Fgf4 increases the formation of diploid trophoblastic cells from outgrowths of Oct4-null embryos in culture. This result confirms the notion that Fgf4 acts as a paracrine factor, sustaining the proliferation of trophoblast cells (Tanaka et al., 1998). Addition of Fgf4 to the culture medium, however, does not rescue the lethality of the Oct4 deficient embryos suggesting the requirement for expression of other genes (like Sox2) transactivated by Oct4.

The OPN gene, which encodes an extracellular matrix component involved in cellular migration, is weakly expressed in the ICM and ES cells and is upregulated in primitive endodermal cells prior to their differentiation to parietal or visceral endodermal cells (Botquin et al., 1998; Palmieri et al., 1994). Botquin et al. (Botquin et al., 1998) found that retinoic acid treatment of F9 EC cells, which are usually used as a model for differentiation of the primitive endoderm, results in a transient increase of Oct4,

paralleled by an increased expression of OPN. A search for Oct4 potential binding sites within the cis-regulatory sequences of the OPN gene identified a novel palindromic sequence named palindromic-oct-regulatory-element (PORE) within the first intron of OPN. This sequence contains an ATGCAAAT octamer motif and an inverted half-site, CAAAT, separated by two nucleotides to which Oct4 binds in vivo as a monomer and a homodimer (Botquin et al., 1998).

The Rex1 gene is a developmentally regulated acidic zinc finger gene (Zfp-42) and a well recognized marker for the pluripotent state of both ES cells and EC cells (Rogers et al., 1991). Both EC and ES cells can be induced to differentiate into primitive endoderm-like cells by treatment with retinoic acid with the downregulation of pluripotency markers such as Rex1 (Rogers et al., 1991; Ben-Shushan et al., 1998). The Rex1 promoter contains an octamer motif (ATTTGCAT) at position -220 that is required for its activity in undifferentiated F9 cells and is involved in RA-mediated downregulation (Hosler et al., 1993; Rogers et al., 1991). This octamer motif is a binding site for octamer transcription factor members of the POU domain family of DNA binding proteins such as Oct4. Subsequent analysis has demonstrated that Oct4 can regulate the activity of Rex1 promoter through this octamer motif in a dose dependent manner (Ben-Shushan et al., 1998). In addition to the octamer motif, a positive regulatory element, which is located immediately 5’ of the octamer motif, was identified in the Rex1 promoter. Mutations in this element severely compromised the activity of the Rex1 promoter in F9 cells suggesting that this element plays a key role in the activation of Rex1 gene transcription (Ben-Shushan et al., 1998). Recently, Nanog (a

homeodomain protein known for its role in maintaining pluripotency) was found to be a transcription activator for the Rex1 promoter (Shi et al., 2006). The knockdown of Nanog in ES cells resulted in a reduction of Rex1 expression, whereas forced expression of Nanog in P19 EC cells stimulated Rex1 expression. Serial deletion studies mapped the Nanog-responsive element between -187 and -286 of the Rex1 promoter. Furthermore, Sox2, not Oct4, appeared to cooperate with Nanog in upregulating Rex1 although both Oct4 and Sox2 can tranactivate Rex1 promoter (Shi et al., 2006).

Oct4 also functions as a repressor of some specific target genes. For example, Oct4 represses the caudal-related homeobox transcription factor Cdx2, which is expressed in the TE lineage in the early stage embryos (Niwa et al., 2000; Strumpf et al., 2005). Cdx2 becomes expressed when Oct4 expression is suppressed (Niwa et al., 2000), and Oct4 expression becomes suppressed when Cdx2 is force-expressed (Niwa et al., 2005) in ES cells. In both cases, the result is a phenotypic change towards the TE lineage. Conversely, genetic lack of Cdx2 prevents downregulation of Oct4, and the TE lineage can not form (Strumpf et al., 2005; Niwa et al., 2005). Likewise, the murine Oct4 has been found to silence transcription of both human HCG a and b genes required for implantation and the maintenance of pregnancy (Liu and Roberts, 1996; Liu et al., 1997). Both HCG subunits are first expressed as trophectoderm begins to differentiate, when the Oct4 gene is normally downregulated. This suggests that their expression is normally repressed by Oct4, and become activated as Oct4 is downregulated.

Regulation of Oct4 gene expression

The regulatory control of the Oct4 expression has been studied extensively in stem cells and in developing embryos because the specific expression of Oct4 during early mouse development is required for the maintenance of pluripotent cells. Transgenic analysis of Oct4 expression in the developing mouse embryo was carried out using a LacZ reporter gene linked to various regions of Oct4 upstream sequences (Yeom et al., 1996). An 18-kb genomic fragment was found to correctly reproduce the endogenous pattern of Oct4 expression in both preimplantation and postimplantation embryos. Deletion analysis demonstrated that Oct4 gene expression is dependent on at least three upstream cis-regulatory regions: the minimal/proximal promoter, the proximal enhancer and the distal enhancer, based on their position with respect to the transcription initiation site (Fig. 4) (Yeom et al., 1996).

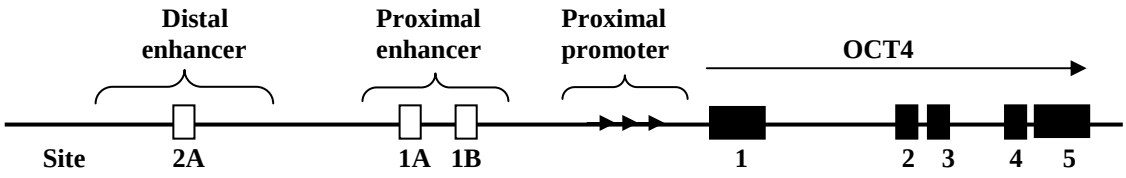

Fig. 4. Genomic structure of the Oct4 gene known regulatory elements. The gene is divided into five exons, depicted as black boxes. The identified upstream regulatory regions include the proximal promoter, proximal enhancer and distal enhancer.

The Oct4 proximal promoter, located within the first 250 bp of the transcription initiation sites, is a TATA-less promoter containing a cluster of overlapping binding sites recognized by specific DNA binding proteins. Among these is a GC box representing a high-affinity site for Sp1/Sp3 transcription factors that mediate Oct4 minimal promoter

activity (Sylvester and Scholer, 1994; Okazawa et al., 1991). Mutation of the GC box in the Oct4 promoter abolishes expression in both ES and EC cells (Minucci et al., 1996).

The proximal enhancer, located 1.2 kb upstream of the transcription start site, is known to mediate P19 EC cell-specific expression and RA-induced Oct4 downregulation (Okazawa et al., 1991). In the developing embryo, the activity of this enhancer is limited to the epiblast (Yeom et al., 1996). In undifferentiated cells of the preimplantation embryo, in ES cells and in F9 EC cells, the activity of the proximal enhancer is very low (Yeom et al., 1996; Minucci et al., 1996). The enhancer contains a cis-acting element which responds to RA repression, although it does not encompass a recognizable RA receptor binding site (Okazawa et al., 1991). This element can be subdivided into two sites (1A and 1B) (Fig. 4). Both sites bind distinct factors in vitro (Okazawa et al., 1991), but only site 1A is occupied in vivo in undifferentiated ES and EC cells (Minucci et al., 1996). This site is GC-rich and related to the consensus Sp1 binding site in the promoter (Minucci et al., 1996).

The distal enhancer, located ~2 kb upstream of transcription start sites, is active in undifferentiated cells of the preimplantation embryo and, later in development, is also responsible for the specific expression of Oct4 in PGCs (Yeom et al., 1996). ES and EG cells also require the distal enhancer for Oct4 expression. Thus, the activity of this enhancer is restricted to totipotent and pluripotent cell types. Within the distal enhancer is a site 2A, which is similar to, but in the opposite orientation of site 1A of the proximal enhancer (Fig. 4). In vivo, genomic foot-printing demonstrated that site 2A is also occupied in undifferentiated ES and EC cells (Minucci et al., 1996).

Rationale

By the late blastocyst stage, the mammalian conceptus consists of three fundamental tissue lineages: the placental trophoblast that is derived from the TE, the fetus proper that is derived from the EPI, and the yolk sac endoderm that is derived from the PrE. During the first embryonic lineage choice, the outer cells of the mammalian morula become committed towards the TE lineage. The remaining cells, called the ICM, give rise to the EPI and PrE cells (the second embryonic lineage choice) by the late blastocyst stage. It has previously been thought that the ICM cells of the early blastocyst are homogenous population of bipotential cells each with the ability to become both EPI and PrE. But, recently it was discovered that the ICM is heterogeneous and consists of two closely related, anatomically indistinguishable groups of cells: the committed EPI-precursor and the committed XEN-precursor cells (Chazaud et al., 2006; Kurimoto et al., 2006). The committed EPI-precursor gives rise to the embryo proper while the committed XEN-precursor gives rise to the yolk sac endoderm, a tissue that plays a major role in the nutritive support of the embryo and is required for several inductive events, such as anterior patterning and blood island formation (Jollie, 1990; Baron, 2003; Rossant, 2007).

Cell lines from early embryonic lineage precursors are powerful tools for the study and manipulation of development and differentiation (Rossant, 2007). These resulting insights, as well as the stem cell lines themselves, play increasingly important roles in regenerative medicine, reproduction, and cancer research. Permanent cell lines that represent the EPI-precursor (called ES cells) and the TE lineage (called TS cells)

have been established and characterized (Evans and Kaufman, 1981; Martin, 1981; Tanaka et al., 1998). However, cell lines from the third stem cell type that represents the ICM-stage XEN (XEN-precursor) lineage has not been described. Mouse XEN cell lines published recently (Kunath et al., 2005) do not express Oct4 and are biased towards the PE in vivo, and thus do not represent the XEN-precursor. Our laboratory has isolated cell lines from rat blastocysts that have XEN morphology, and preliminary results showed these lines stably express Oct4, a key ICM marker.

The hypothesis of our investigation was that these rat XEN cells represent the XEN-precursor of the ICM. In the first chapter, we offer compelling evidence that our rat XEN cells stably and reproducibly maintain the molecular signature of the ICM-stage XEN-precursor (Oct4+ Gata6+ SSEA1+Nanog- Cdx2- Sox2-), incorporate into the ICM and show the full XEN-precursor differentiation potential in vitro and in vivo. In the second chapter, we investigated the regulation of Oct4 gene expression in the rat XEN cells and compared it to the regulation in ES cells. We present data demonstrating that a novel enhancer drives Oct4 gene expression in rat XEN cells and that there is differential regulation in these cells and mouse ES cells.

CHAPTER II

LINEAGE MARKER EXPRESSION IN RAT YOLK SAC ENDODERM STEM CELL LINES

Overview

The ICM of mammalian embryos consists of two intermingled, short lived cell types: epiblast (EPI)-precursor and extraembryonic endoderm (XEN)-precursor. In vitro the EPI-precursor cells are represented by the well-studied ES cells but cell lines representing the XEN-precursor stage remain elusive. Our laboratory has recently isolated rat XEN cell lines that we hypothesize represent the XEN-precursor stage since they express Oct4. The data presented here provide strong evidence for our hypothesis as our rat XEN cell lines exhibited the molecular signature (Oct4+Gata6+Nanog-Cdx2-) and differentiation potential (primitive/visceral and parietal XEN) expected from the XEN-precursor cells. These findings establish the XEN-precursor as a self-renewable entity and provide a model of early XEN differentiation.

Introduction

Cell lines isolated from mammalian preimplantation-stage embryos play key roles in the study of embryogenesis, differentiation, and cellular reprogramming, work that is considered important for regenerative medicine, reproduction, cancer research and species conservation. Known blastocyst-derived stem cell lines include ES cells (Evans

and Kaufmann, 1981; Martin, 1981), TS cells (Tanaka et al., 1998), XEN stem cells (Kunath et al., 2005), and some less-defined rat blastocyst stem cells that seem to be extraembryonic (Buehr et al., 2003; Fandrich et al., 2002). Transplantation and expression profiling experiments revealed that the ICM of early mouse embryos is not homogeneous as previously thought, but consists of two closely related, anatomically indistinguishable groups of cells (Chazaud et al., 2006; Kurimoto et al., 2006): (i) the committed epiblast precursor (EPI-precursor) that gives rise to the extraembryonic mesoderm and the embryo proper; (ii) the committed extraembryonic endoderm precursor (XEN-precursor) that gives rise to the yolk sac endoderm. The yolk sac provides essential roles in nutrition, axial patterning, and hematopoiesis (Jollie, 1990; Baron, 2003; Rossant, 2007).

The well-known mouse ES cell lines (Evans and Kauffman, 1981; Martin, 1981) are now thought to be the in vitro correlate of the EPI-precursor (Chazaud et al., 2006). By contrast, cell lines analogously representing the XEN-precursor have not yet been described, and therefore the nature of the XEN-precursor and its relationship to EPI-P/ES cells are poorly understood. The presently known XEN cell lines (Kunath et al., 2005) cannot efficiently contribute to the visceral yolk sac lineage and seem to exhibit none of the features that collectively define the ICM stage (i.e. both EPI-precursor and XEN-precursor), most noticeably expression of the pluripotency marker Oct4 (Chazaud et al., 2006; Kurimoto et al., 2006). It has, however, been observed that mouse ES cells carrying a null mutation of the Nanog gene become committed to the XEN lineage while maintaining the ability to proliferate and express the gene encoding Oct4 (Mitsui et al.,

2003), a key transcription factor whose mRNA and protein is expressed in all cells of the ICM (Chazaud et al., 2006; Kurimoto et al., 2006). This suggests that just like their EPI-precursor siblings, XEN-precursor cells can exist as a distinct, self-renewing entity in vitro. Here, we present wild-type rat blastocyst-derived cell lines that show the lineage marker profile and differentiation potential expected from the XEN-precursor cells.

Materials and methods

Derivation and maintenance of XEN cell lines

Derivation of cell lines was performed by Dr. B.Binas (except for CX5- that was isolated by J.Epple-Farmer). Primary mouse and rat embryo fibroblasts (PMEFs and PREFs) were derived by standard methods (Nagy et al., 2002). D3 mouse ES cells (Doetschman et al., 1985) were maintained on mitomycin-treated PMEFs in the presence of 1000 u/ml LIF. For the derivation of rat XEN cell lines, 4.5 days-post-coitum (dpc) blastocysts were plated into Nunc 4-well dishes onto mitomycin (10 μg/ml)-treated embryo feeders in DMEM (with glutamine and sodium pyruvate) containing 15% fetal calf serum (ES-qualified) and 1,000 u/ml LIF at 37^0C and 5% CO_2. Lines CX1 (rat strain WKY), CX2 (strain BDIX), and CX5 (WKY) were derived, respectively, on PREF, PMEF, and Li1, a permanent rat fibroblast feeder cell line that was obtained by spontaneous immortalization of PREFs from a day-11 rat embryo (strain SD). In about 2 weeks after plating, the blastocyst outgrowths had completely converted into XEN, which was mechanically transferred into a new well. The rat XEN cell lines were maintained in the same medium used for derivation, and transferred every 2-3 days by

trypsinization (0.25% trypsin-EDTA) onto mitomycin-treated Li1 cells (~50,000 Li1 cells/cm^2). After derivation, the cells have been growing permanently without slowing down for >50 passages and the experiments described here were mostly performed between passages 30 and 40. For the derivation of mouse XEN cell lines, 3.5 dpc blastocysts from NMRI strain (CX3, CX4) and strain BalbcxB6 F1 (CX6) were plated onto mitomycin-treated PMEFs in DMEM containing 15% serum. The mouse XEN cell lines were then routinely maintained on PMEFs by trypsinization. In all experiments mouse ES cells (line D3) were used for comparison because rat ES cells have not yet been derived.

RNA extraction, reverse transcription and RT-PCR

Total RNA was extracted from whole culture cells using TRIZOL (Invitrogen) following the manufacturer's instructions and the concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA was then subjected to DNaseI treatment using a DNA-free kit (Ambion, Austin, TX) at 37°C for 30 minutes to remove any contaminating genomic DNA. 2 µg of total RNA was used for reverse transcription (RT) reaction. To extract RNA from microsamples, the RNeasy microkit (Qiagen, Valencia, CA) that included DNase treatment was used. From these samples, half of the eluted RNA (~6µl) was used for the RT. The RT reaction was performed with random hexamers using the first strand cDNA synthesis Superscript II kit (Invitrogen) in a total volume of 20 µl according to the procedure provided by the manufacturer. After the RT reaction, the enzyme was denatured at 70°C

for 15 minutes. "No- RT" control (RNA) was also made from each sample to confirm the absence of contaminating genomic DNA.

Equal amounts of the cDNA samples were subjected to PCR amplification in a 25 µl reaction volume using 2.5 units of Taq DNA polymerase, 10x PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.2 µM gene-specific forward, and 0.2 µM reverse PCR primers. Primers were designed using the Primer3 software of the Whitehead Institute (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). In order to amplify the genes with equal efficiency from the rat and mouse cell lines, we aligned the sequences for each gene and designed dual-specific primers from the homologous regions. Hprt was used as an internal control. By using species-specific Hprt primers that distinguish mouse and rat, we excluded cross contaminations between the rat and mouse lines. The sequences of each specific primer set, including their product size and accession numbers are listed in Table 1. Cycle conditions for the PCR were as follows: 94°C for 1 minute; 25–35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute; 72°C for 5 minutes. The PCR products were visualized on a 1.5% agarose gel containing ethidium bromide.

Real-time PCR was performed in duplicate in a volume of 25 µl (containing 5 µl of 10x-diluted cDNA template, 1 µl (10 µm) of each primer, 0.5 µl Rox dye and 12.5 µl of SYBR Green PCR supermix (Invitrogen). The products were detected with the ABI Prism 7700 sequence detection system (Applied Biosystems). Fluorescence was quantified as a Ct value with Oct4 gene expression levels calibrated using Hprt as an internal control. The differences between the mean Ct values of the gene of interest (Oct4) and the housekeeping gene (Hprt) were denoted (delta-Ct) and the difference between delta-Ct and the Ct value of the calibrator sample (ES cells) was labeled delta-delta-Ct. The $\log_2$(delta-delta-Ct) gave the relative quantitation value of the Oct4 gene expression with ES cell expression designated as one. The cycling conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles at 94°C for 15 sec, 60°C for 30 sec , and 72°C for 30 seconds. Control wells containing SYBR Green PCR master mix and primers without sample cDNA emitted no fluorescence after 40 cycles.

Table 1. List of primers used in this study

Gene	Primers (5'→3')		Product size	Gene Bank Accession No.	
	Forward	Reverse		Mouse	Rat
Oct4				NM_013633	XM_228354
Mouse-specific	ggcgttctctttggaaaggtgttc	actcgaaccacatccttctct	314		
Rat-specific	ggtggaggaagctgacaacaac	ggcaatgctagtgatctgctgc	172		
Dual-specific	gagggatggcatactgtggac	ggtgtaccccaaggtgatcc	272		
Nanog	tatcccagcatccattgcag	gtcctccccgaagttatggag	252	AB126939	AB162852
Sox2	ccaagacgctcatgaagaagg	ctgatcatgtcccggaggtc	478	NM_011443	XM_574919
Fgf4	tctactgcaacgtgggcatc	tggtccgcccgttcttac	285	NM_010202	NM_053809
Rex1	tggagtacatgacaaaggggac	gcagccatcaaaaggacacac	509	NM_009556	XM_224882
Gata6	gccgggagcaccagtaca	gtgacagttggcacaggacag	419	AF179425	NM_019185
Hnf4α	gtgctgctcctaggcaatgac	cttgacgatggtggtgatgg	651	NM_008261	NM_022180
FoxA2	agccccaacaagatgctgac	tggttgaaggcgtaatggtg	602	NM_010446	NM_012743
Ihh	cctgtcagctgtaaagccagg	ggagcataggacccaaggg	336	NM_010544	AF175209
LamininB	actacaccacgggccacaac	gcccaggtaattgcagacacac	440	NM_008482	XM_216679
Dab2	ccacaggacaacctgcagtc	gccacagatgtggtaggacac	325	BC016887	NM_024159
Sparc	attgcaaacatggcaaggtg	gccagtggacagggaagatg	474	NM_009242	NM_012656
Cdx2	gcgaggactggaatggctac	tccttggctctgcggttc	499	NM_007673	NM_023963
Eomesodermin	cggcaaagcggacaataac	gttgtcccggaagcctttg	361	NM_010136	AY457971
Placental lactogen	ctgcttccatccatactccaga	gacaactcggcacctcaaga	410	XM_225307	NM_172156
Hprt				NM_013556	NM_012583
Mouse-specific	gcttgctggtgaaaaggacctct	ggaaatcgagagcttcagactcgtc	584		
Rat-specific	gcttgctggtgaaaaggacctct	ccacaggactagaacgtctgctagttc	251		
Dual-specific	cagtcccagcgtcgtgattag	atccagcaggtcagcaaagaac	229		

Northern blot

Northern blotting was performed using established procedures without significant modifications (Sambrook and Russell, 2001). Total RNA was isolated with TRIZOL (Invitrogen), and 5ug per lane were electrophoresed. The gels were blotted onto Hybond (Amersham) membranes, and hybridizations were performed using probes that were produced by RT-PCR and labeled with ^{32}P-dCTP by the random priming procedure. The sources of the probe cDNAs were ES cells (Nanog) or rat XEN cells (Gata6 and Oct4), using the primers indicated in Table 1 for Nanog and Gata6 or primer pair ggagggatggcatactgtgg and accagggtctccgatttgc for Oct4. rRNA was used to ensure equal loading of RNA samples.

Western blot

Protein extraction and western blotting were carried out as described in Sambrook and Russel (2001). Cells were trypsinized, centrifuged, suspended in PBS and pelleted by centrifugation. Pellets were then resuspended in 10% SDS, and boiled at 95^0C for 10 min. Protein concentration was determined using the BCA kit (Pierce) and equal amounts of protein samples (50µg/lane) were separated through 10% polyacrylamide by SDS-polyacrylamide gel electrophoresis. The gel was equilibrated in transfer buffer and electroblotted onto nitrocellulose filters (Biorad) for 1 hr at 350mA or overnight at 90mA. The membranes were then blocked with TBS/Tween/5% milk powder for 1 hr at room temperature. They were then incubated for 2 hrs at room temperature with a monoclonal anti-oct4 antibody (Santa Cruz) at a dilution of 1:200.

After washing the membranes 3x (each 5min) with TBST, they were incubated for 1 hr with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Santa Cruz) diluted at 1:1000. After 3x washing with TBST, the blots were developed using the Western Blue substrate (Promega).

Immunocytochemistry

Cells were seeded into Nunc 4-well plates at regular passaging density or at low density (100 cells/well). At the time point of interest, immunocytochemistry was performed at room temperature. The wells were washed twice with PBS, fixed in 4% paraformaldehyde (10-15 minutes) and rinsed 3x with PBS. For intracellular antigens (Oct4, Gata6), the cells were then permeabilized with 0.2% Triton X-100 in PBS (15-20 minutes) and rinsed 3x with PBS. Cells were blocked with 5% goat serum (Santa Cruz) in PBS (1 hr), incubated with primary antibodies overnight, rinsed 3x with 1% goat serum, and incubated with the secondary antibody conjugated to either FITC, TR or TRITC for 1 hr in the dark. For dual-color immunofluorescence, species-specific secondary antibodies conjugated with FITC or TR was used. After secondary antibody incubation, the cells were washed 3x with 1% goat serum, incubated with 1 μg/ml of Hoechst dye in PBS, and photographed under epifluorescence. The antibodies and their dilutions are listed in Table 2.

Table 2. List of antibodies used in this study

Antigen	Primary antibody and dilution*	Secondary antibody and dilution*
Oct4	SC-5279; 1:100	Goat anti-mouse IgG-FITC, 1:200, SC-3699
Oct4	SC-9081, 1:200	Goat anti-rabbit IgG-TR, 1:300, SC-3842
Gata6	SC- 9055, 1:200	Goat anti-rabbit IgG-TR, 1:300, SC-3842
SSEA-1	DSHB MC-480, 1:500	Goat anti-mouse IgG-FITC, 1:200, SC-3699
SSEA-3	DSHB MC-631, 1:100	Goat anti-mouse IgG-TRITC, 1:100, Invitrogen 81-6514
Laminin B2	DSHB D18, 1:400	Goat anti-mouse IgG-FITC, 1:200, SC-3699
Collagen IV	DSHB M3F7, 1:200	Goat anti-mouse IgG-FITC, 1:200, SC-3699

* SC, Santa Cruz Biotechnology; DHSB, Developmental Studies Hybridoma Bank, University of Iowa.

Alkaline phosphatase stain

Alkaline phosphatase staining was performed as described (Nagy et al., 2002). Briefly, cells were grown overnight in 4-well plates, were fixed in 4% paraformaldehyde for 15 min and then were washed three times with PBS. About 300µl of alkaline phosphatase staining solution, consisting final concentrations of 25mM Tris-maleate (pH 9.0), 0.4mg/ml α-naphtyl phosphate, 1mg/ml Fast-Red TR salt, and 8mM $MgCl_2$, was added to each well and incubated for 30 min to 1 hr at room temperature. After removing the solution the cells were washed with PBS and photographed with IPX70 microscopy.

Vector production and GFP labeling of rat XEN lines

Recombinant lentivirus vector and packaging constructs were generously provided from Dr. David Baltimore (Caltech). The vector construct consists of a human immunodeficiency virus (HIV)-based self-inactivating (SIN) replication-defective lentivirus transfer vector, pFUGW, expressing a green fluorescent protein (GFP) reporter

gene driven by the human ubiquitin-C promoter (Lois et al., 2002). The helper plasmid constructs are the packaging plasmid, pCMVΔR8.91, and the envelope plasmid, pMD.G encoding VSV-G. For virus production, 293T cells were seeded at 2 x 10^6 cells per 100-mm dish the day before transfection, and each dish was co-transfected with 10 µg of pFUGW, 10 µg of pCMVΔR8.91 and 6µg VSV-G plasmid using lipofectamine 2000 (Invitrogen) following manufacturer's instructions. After 24 hrs of incubation, cells were gently washed 2x with serum-free media. The cells were then incubated with media containing serum. The culture supernatants containing virus particles were harvested 24 hrs later, centrifuged at 500 g for 5 min to remove cell debris and filtered through a 0.45µM pore-size filter. The supernatant was concentrated using Centricon Plus-20 ultrafiltration device (Millipore, Bedford, MA) according to the manufacturer's instructions. The concentrated virus was stored at -80°C. The titers of vector stocks were determined by serial dilutions on 293T cells.

For transduction, rat XEN cells were seeded in 4-well plates (Nunc) at a density of 5 x10^4 cells per well and incubated overnight. The media was changed 2 hrs before transduction. Transductions were carried out in the presence of 8 µg of Polybrene (Sigma) per ml of media at MOI of 1. After incubation at 37°C for 24 hrs, the transduction medium was replaced with fresh DMEM containing serum. The percentage of cells exhibiting GFP fluorescence was quantified using fluorescence microscope. The cells were then expanded and sorted for GFP before injections.

Microinjection experiments

These experiments were done in the laboratory of our collaborators, Drs. Iannaccone and Galat, at Northwestern University, Chicago. All cells were manipulated in cell culture media mixed with an equal volume of M2 and kept on ice before injection. Micromanipulations were performed with a Leica system essentially as described (Stewart, 1993; Iannaccone and Galat, 2002). For the in vitro experiments, 1-3 rat XEN cells were injected into rat or mouse blastocysts or morulae, or the cells were aggregated with, or sandwiched between, rat morulae freed from the zona pellucida by acid Tyrode solution (Sigma). Groups of 3-10 embryos were then cultured in 30 μl-drops of KSOM (Specialty Media) under pre-equilibrated mineral oil (Sigma) for 24-48 hrs at 37^{o} C in 5% CO2. For the in vivo experiments, 3-5 rat XEN cells were injected per blastocyst, followed by transfer into the uteri of pseudo-pregnant females, and the conceptuses were recovered and dissected at 6.5-10.5 dpc. After in vitro culture or in vivo development, the injected GFP-labeled rat XEN cells or their offspring were located by fluorescence microscopy and confocal images acquired using a Zeiss LSM 510 META Laser Scanning Microscope System (Thornmood, NY).

Results

Cultured rat XEN cells express Oct4. When rat blastocysts were explanted on mitotically inactivated primary embryo fibroblast feeders, smooth, compact outgrowths grew rapidly and morphologically resembled ES cell colonies. The expression of Oct4 mRNA was followed in the resulting outgrowths by RT-PCR. Oct4 mRNA was lost in

the outgrowths within 6 days, but after ~10 days, the outgrowths stopped enlarging and abruptly converted into cells with the characteristic XEN morphology and simultaneously started re-expressing Oct4 mRNA at a level comparable to that in blastocysts (Fig. 5).

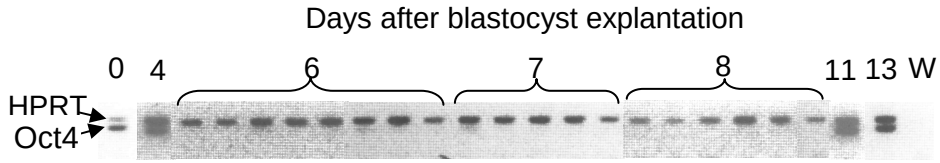

Fig. 5. Loss and re-emergence of Oct4 expression in rat blastocyst outgrowths. Individual outgrowths were analyzed at the indicated culture days by RT-PCR for Oct4 (rat-specific primer pair) and hprt (housekeeping gene). Day 0= blastocyst. The outgrowths showed compact, ICM-like morphology before day 10, but XEN morphology thereafter. W, water control. (Contributed by Dr. B. Binas)

Intrigued by the expression of the ICM marker Oct4 which was not studied in previously isolated rat XEN cell lines (Notarianni and Flechon, 2001; Ouhibi et al., 1995) and is absent from mouse XEN cell lines (Kunath et al., 2005), three rat XEN cell lines were established (CX1, CX5 - strain WKY; CX2 –strain BDIX). For comparison, three mouse XEN lines (CX3, CX4 - strain NMRI; CX6 – strain BalbcxB6 F1) were isolated; all strains were randomly selected.

All rat XEN lines and the mouse ES cells, but neither the mouse XEN lines nor rat TE-like cells expressed Oct4 mRNA. Despite this, all XEN cell lines showed the characteristic XEN morphology (Fig. 6A) and expressed the parietal endoderm markers, laminin B and Sparc (Fig. 6B). However, the rat XEN cells expressed higher levels of the pan-XEN marker Gata6 and of the VE markers, Dab2 and Foxa2. Also, rat XEN and

mouse ES cells, but not mouse XEN cells, expressed the TE marker Eomesodermin and ICM/ES marker Rex1. However, the TE markers Cdx2 and placental lactogen, and the ES markers Nanog, Fgf4, and Sox2 were absent from the rat XEN cells (Fig. 6B).

Since Oct4 gene expression in XEN cells has not been reported before and is the central finding underlying our study, we validated this result with several independent methods. Using dual-specific (rat=mouse) primers, we compared the Oct4 mRNA levels of whole cultures and arbitrarily picked microsamples (<500 cells) with mouse ES cells (=100%) by qRT-PCR. Normalized to HPRT mRNA, the Oct4 mRNA contents ranged from 30-84% in whole cultures and from 19-210% in microsamples (Table 3). Importantly, Table 3 also shows that high oct4 mRNA expression is not limited to one XEN cell line or rat strain, since independently derived XEN cell lines from the same strain (CX5, strain WKY) and another arbitrarily chosen rat strain (CX2, BDIX) also expressed oct4 mRNA at significant levels.

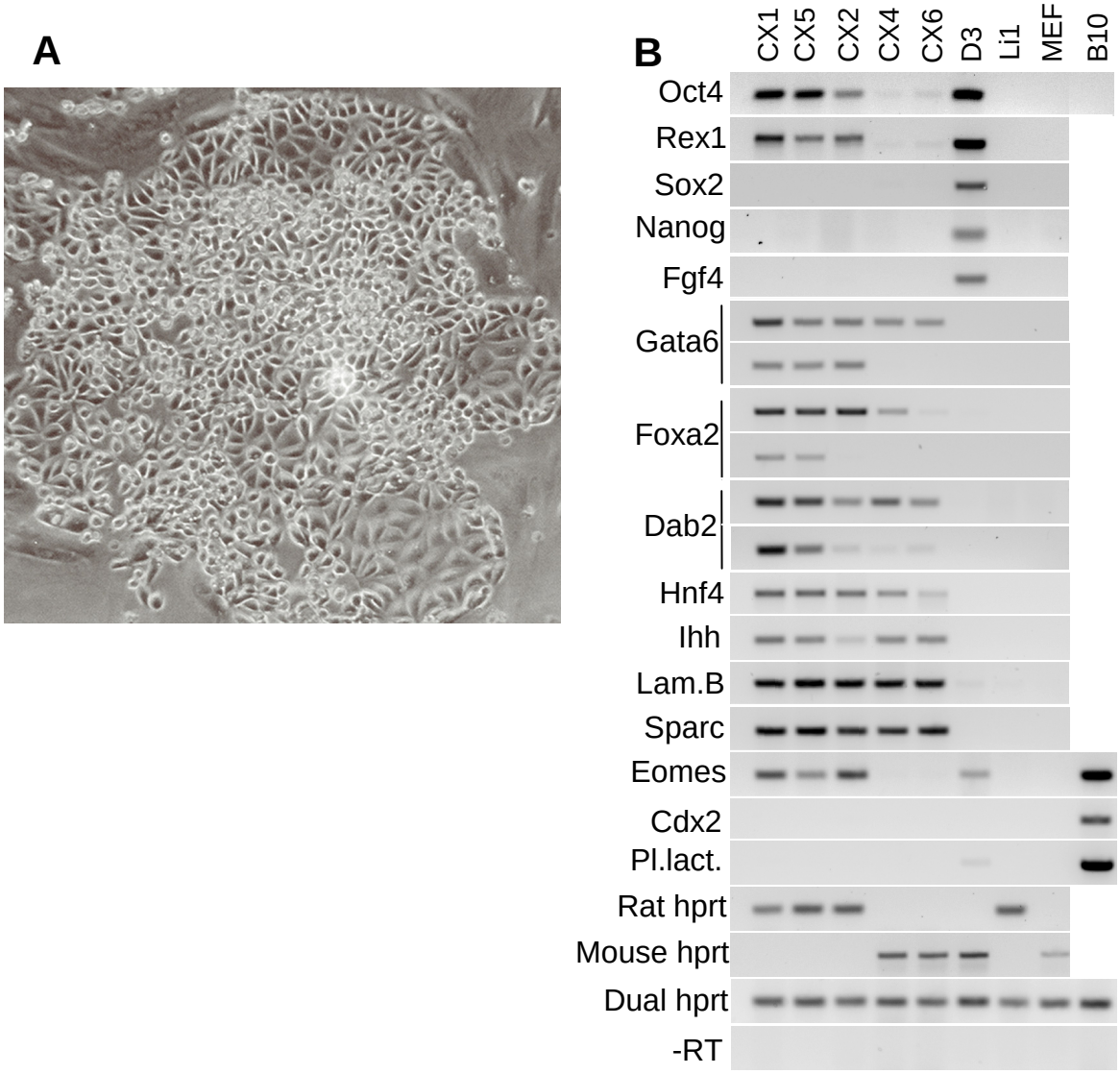

Fig. 6. Cultured rat XEN cells express ICM and XEN markers. (**A**) Phase contrast photo showing characteristic morphology of rat XEN cells growing on rat embryo fibroblast feeder. Colonies obtained by low-density plating typically contained round, refractile cells at their fringes and epithelial cells inside. (**B**) RT-PCR analysis showing that cultured rat XEN cells exhibit a mixed embryonic lineage marker profile. Rat XEN cells (lines CX1, CX2, CX5) were compared with mouse XEN cells (lines CX4, CX6), a mouse ES cell line (D3), a TE-like rat cell line (B10), a rat embryo fibroblast cell line (Li1) used as feeder for rat XEN cells, and primary mouse embryo fibroblasts (MEF) used as feeders for mouse XEN and ES cells. Note that line B10 was previously suspected to be ES-like (Fandrich et al., 2002) but is in fact rather TE-like, as shown in this figure and in line with findings by Buehr et al. (2003). For Gata6, Foxa2, and Dab2, two dilutions of the RT reaction were subjected to PCR for semi-quantitative comparison. See Table 1 for primer sequences.

Table 3. Quantitative RT-PCR for Oct4 expression in XEN cells

Cells	Species	Strain	Line	Oct4 mRNA enrichment
XEN	Rat	WKY	CX1	[A] 2.10±0.41[§] (7)
				[B] 0.19±0.05[§] (3)
				0.84[*]/0.30[*] (2)
				[C] 0.62*/0.67* (2)
			CX5	0.47[*] (2)
		BDIX	CX2	0.22±0.1[§] (4)
	Mouse	NMRI	CX4	0.003±0.001(4)[*]
		B6xBalb/c	CX6	0.0003[*] (2)
ES	Mouse	129	D3	1.00[*] (reference)

([§])Microsamples, ([*]) whole culture samples. Samples (numbers in brackets) were RNA-extracted, RNA preparations were DNAse-treated and quantified in duplicate by real-time RT-PCR using dual-specific (mouse=rat) primers; controls without reverse transcriptase did not yield a product. Data (Means±SEM) were normalized to hprt mRNA and expressed as fold of the level in ES cells, i.e. ES cell level is set as 1. [A], [B], two groups of microsamples with high and moderate oct4 mRNA expression, respectively. Two experiments labeled [C] were corrected for feeder RNA; the other measurements are underestimates.

We then chose the rat XEN cell line CX1 for closer examination of Oct4 gene expression. Northern blotting showed that the Oct4 mRNAs in rat XEN and mouse ES cells were of the same length; in agreement with the qRT-PCR, the average rat XEN Oct4 mRNA level was somewhat lower than in ES cells (Fig. 7A). The figure also confirms that along with Oct4, rat XEN cells express Gata6 and lack Nanog, i.e. show the hallmark expression pattern of the XEN-precursor (Chazaud et al., 2006). In order to examine whether the Oct4 mRNA is translated into a protein, we performed western blotting. Indeed, we found that the rat XEN Oct4 mRNA is translated into a protein of the same length as in mouse ES cells (Fig. 7B). However, with a dilution series of ES

cells, we estimated the Oct4 content in the rat XEN cells as ~5% of the ES cell level (Fig. 7C), which is lower than one would expect from their average Oct4 mRNA level (30-80% of ES cells on a whole culture level, Table 3). Because of this discrepancy, we wondered whether in addition to the regular Oct4 mRNA, a pseudogene-encoded RNA is expressed by rat XEN cells, as recently seen in human tumors and pluripotent cells (Suo et al., 2005; Pain et al., 2005). A BLAST search of the rat genome database revealed two retrotransposons, one highly homologous and full-length (chromosome 13) and one moderately homologous and truncated (chromosome X), in addition to the Oct4 gene (chromosome 20). We verified that the oct4 mRNA is not pseudogene-encoded by using a *PstI* restriction-length polymorphism (Fig. 7D). Since according to the known rat genome sequence, the oct4 cDNA and the chromosome 13 pseudogene amplicon differ by a *PstI* restriction site, we digested the cDNA product (obtained from a DNAse-treated RNA sample) in parallel with the putative pseudogene product. As shown in Fig. 7D, the cDNA and genomic products produced different *PstI* digestion patterns, exactly as expected from the Oct4 gene and the chromosome 13 pseudogene, respectively. That is, digestion of the cDNA product yielded 3 bands consistent with the expected pattern of 141, 70, and 61 bp, while the genomic product yielded 2 bands consistent with the expected pattern of 211 and 61 bp. This result established conclusively that the XEN oct4 mRNA is not encoded by a retrotransposon.

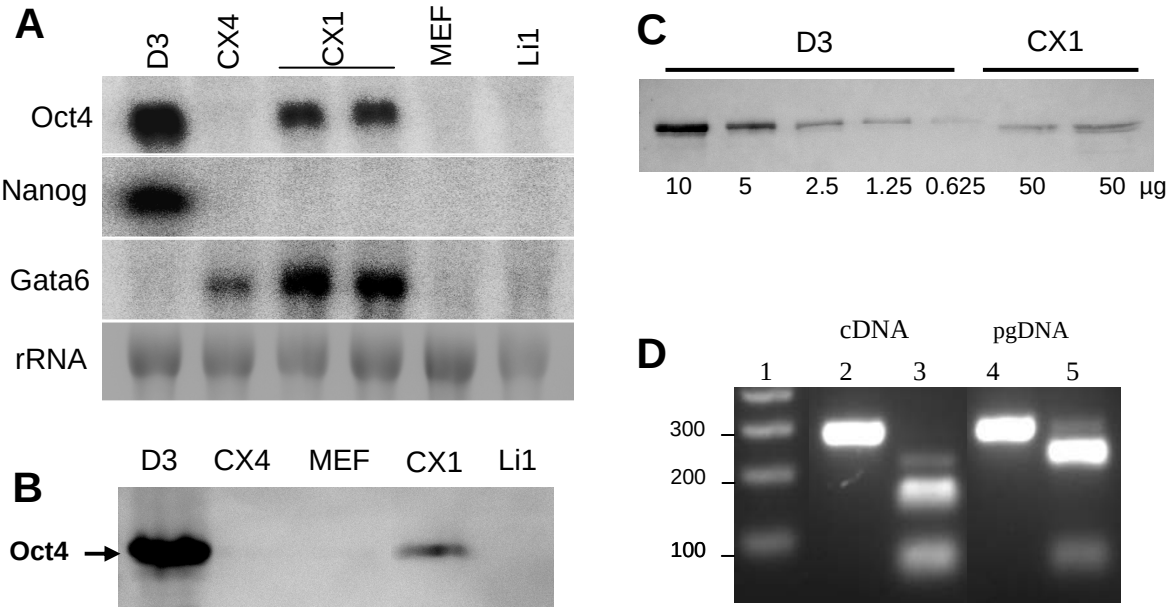

Fig. 7. Cultured rat XEN cell lines express true Oct4 gene. (**A**) Northern blot analysis of rat XEN (CX1, different passages), mouse XEN (CX4), mouse ES (D3) cells, MEF (mouse embryonic fibroblast feeder) and Li1 (rat feeder cell line) (Contributed by S.Adhikari). (**B)** Western blot for Oct4 in rat XEN (CX1), mouse XEN (CX4), mouse ES (D3) cells, and rat feeder cell line (Li1) using a monoclonal anti-oct4 antibody; 50 µg of protein loaded. (**C**) Estimate of Oct4 protein content in two independent rat XEN cell cultures; the indicated amounts of cell protein were loaded. (**D**) *PstI* restriction length polymorphism showing that the XEN oct4 cDNA amplified by RT-PCR corresponds to the predicted gene rather than the pseudogene. Dual-specific (rat=mouse) primers were used for PCR with cDNA from DNAse-treated rat XEN cell RNA (lane 2, 3) and genomic XEN cell DNA (lane 4, 5). The amplicons were digested with *PstI* (cDNA digest, lane 3; genomic DNA digest, lane 5). Lane 1, molecular weight marker (100-bp ladder). The detected bands correspond to the patterns expected from the digestion of the oct4 gene (chromosome 20)-derived cDNA product (141, 70, and 61 bp on lane 3) and a pseudogene (chromosome 13)-derived genomic product (211 and 61 bp on lane 5), respectively. pgDNA, pseudogene DNA.

Rat XEN cell lines are heterogeneous. In order to know whether the low Oct4 protein level resulted from a generally low expression or from heterogeneity of rat XEN cells, we performed immunocytochemistry. We used line CX1 and found that Oct4 was expressed at high, ES-like levels in 5-15% of rat XEN cells while the remaining rat XEN

cells exhibited very low but detectable amounts of Oct4. Mouse ES cells were homogeneously positive whereas mouse XEN cells were free of Oct4 (Fig. 8A). In addition, immunocytochemistry for Gata6 confirmed that mouse ES cells were free of Gata6 while both mouse XEN cells and rat XEN cells expressed it (Fig. 8B). Interestingly, many rat XEN cells showed a higher Gata6 level than mouse XEN cells, in line with the results of the northern blot and RT-PCR. We extended the analysis of heterogeneity by studying the ICM/ES cell marker SSEA1 (Solter and Knowles, 1978), and the PrE/VE marker SSEA3 (Shevinsky et al., 1982). As expected, a minority fraction of rat but not mouse XEN cells expressed SSEA1 at a proportion comparable with the Oct4-positive fraction (Fig. 8C). SSEA3 was expressed in the majority of rat XEN cells but was missing from the mouse XEN and ES cells (Fig. 8D); this is in line with the RT-PCR results that had shown a greater abundance of VE markers in rat than mouse XEN cells. Similar to Oct4 and SSEA1, alkaline phosphatase, another ICM/ES marker (Hahnel et al., 1990; Strickland and Mahdavi, 1978), was expressed by many round cells in the rat XEN cultures while it was absent from mouse XEN cells (Fig. 8E), confirming heterogeneity. To see whether Oct4 and SSEA1 are reproducibly expressed in all rat XEN cell lines, we examined line CX2 for expression of Oct4 and SSEA1 and line CX5 for SSEA1 expression. As shown in Fig. 9**,** these cell lines also expressed Oct4 and SSEA1 but at lower levels than line CX1.

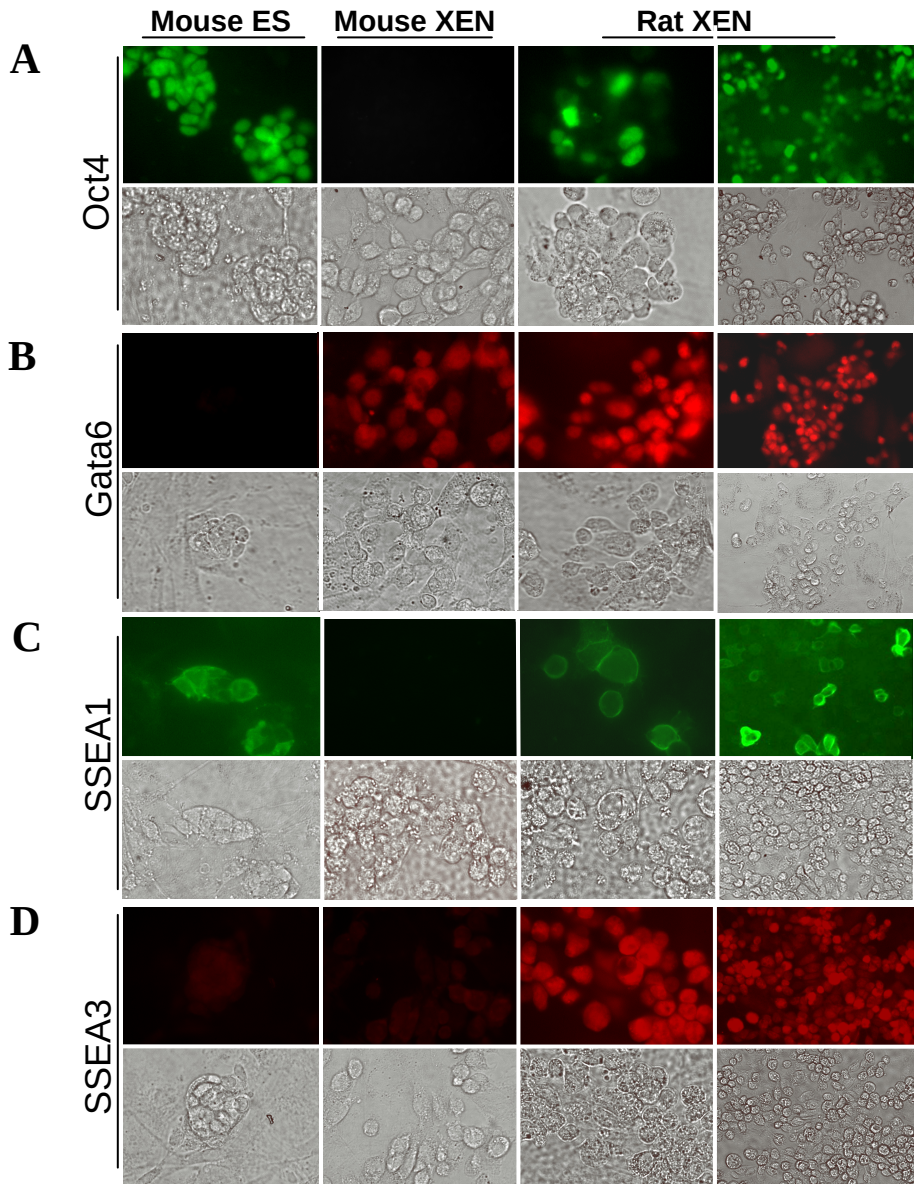

Fig. 8. Heterogeneous expression of embryonic marker genes in rat XEN cells. Rat XEN (line CX1), mouse XEN (line CX4), and mouse ES (line D3) cell lines were analyzed by indirect immunofluorescence with antibodies specifically recognizing Oct4 (**A**), Gata6 (**B**), SSEA1 (**C**), or SSEA3 (**D)**, or they were stained for alkaline phosphatase activity (**E**). Controls in which primary antibodies were omitted were negative and are not shown. Upper rows show immunofluorescence, lower rows show bright field images.

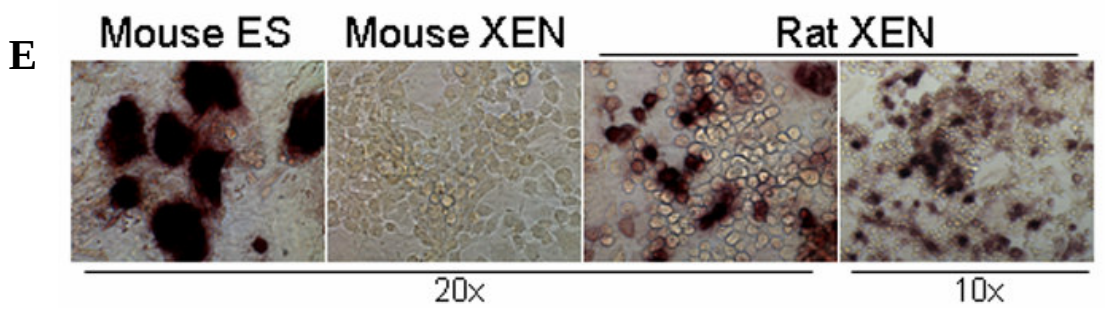

Fig. 8. Continued.

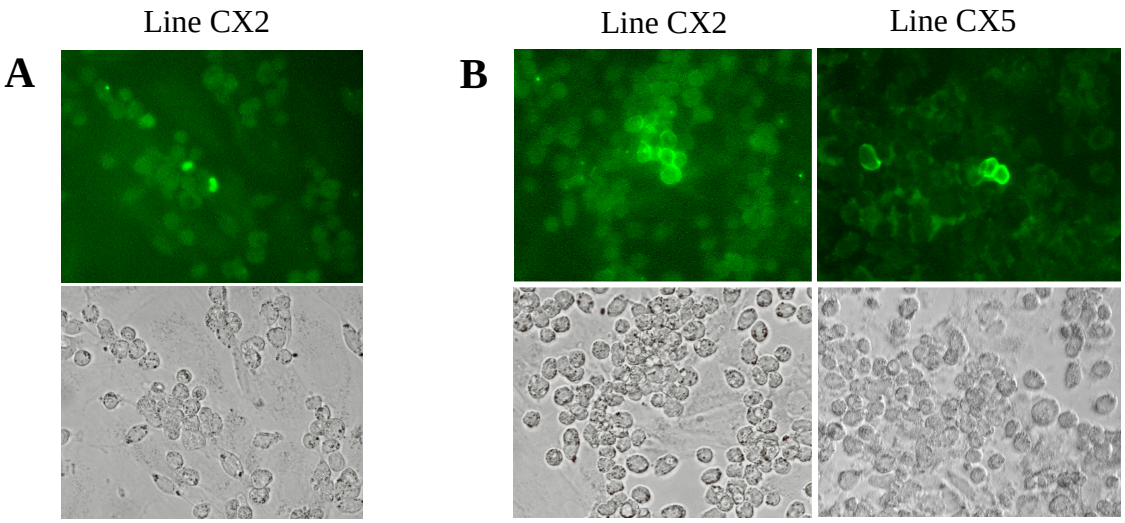

Fig. 9. Expression of Oct4 and SSEA1 by other rat XEN cell lines. (**A**) Line CX2 expressed Oct4 when analyzed by immunocytochemistry. (**B**) SSEA1 expression in Lines CX2 and CX5. Expression in both lines was lower than line CX1 (Compare Fig. 8A, B) Upper row, immunofluorescence; lower row, bright field (original magnification 20x).

Heterogeneity of rat XEN cells is intrinsic. In view of the heterogeneity, we wondered whether our cultures contain cells that individually express several of the markers that define the ICM-stage XEN-precursor; and whether the culture heterogeneity results from

differentiation of such cells or is a result of the parallel proliferation of a mix of lines representing different stages. We therefore performed two types of analyses.

In the first approach, we plated the cells at clonal density and studied the resulting colonies over time by immunocytochemistry for expression of the ICM markers Oct4 and SSEA1, the pan-XEN marker Gata6, the PrE/VE marker SSEA3, and the PE markers laminin B and collagen IV (Strickland et al., 1980; Gardner, 1983). Strikingly, nearly all colonies consisted initially (2-3 days after plating) entirely or almost entirely of round cells that highly co-expressed Oct4, Gata6, and SSEA1 (Fig. 10 A,B) while lacking the PrE/VE marker SSEA3 (Fig. 10C) as well as the basement membrane components laminin B (Fig. 10D) and collagen IV (Fig. 10E) that are characteristically produced by differentiated XEN cells and especially PE. The young colonies were poorly adherent and very easily lost during washing steps. By contrast, in older, larger colonies (4-7 days after seeding), the inner cells became epithelial, and many round as well as the epithelial cells were negative for SSEA1 and very low in Oct4. Rather, many of the round cells now expressed SSEA3 (Fig. 10C), and the epithelial areas contained abundant extracellular laminin B (Fig. 10D) and collagen IV (Fig. 10E). Notably, however, Oct4/SSEA1-positive cells always persisted at the colony fringes and showed also higher Gata6 levels than the rest of the colony (Fig. 10 A,B). With further evolution of the colony (7-14 days after seeding), the round fringe cells kept proliferating and piled up on top of the colonies (Fig. 11) before eventually (10-20 days after seeding) converting into bridge-like ductal structures that were not investigated.

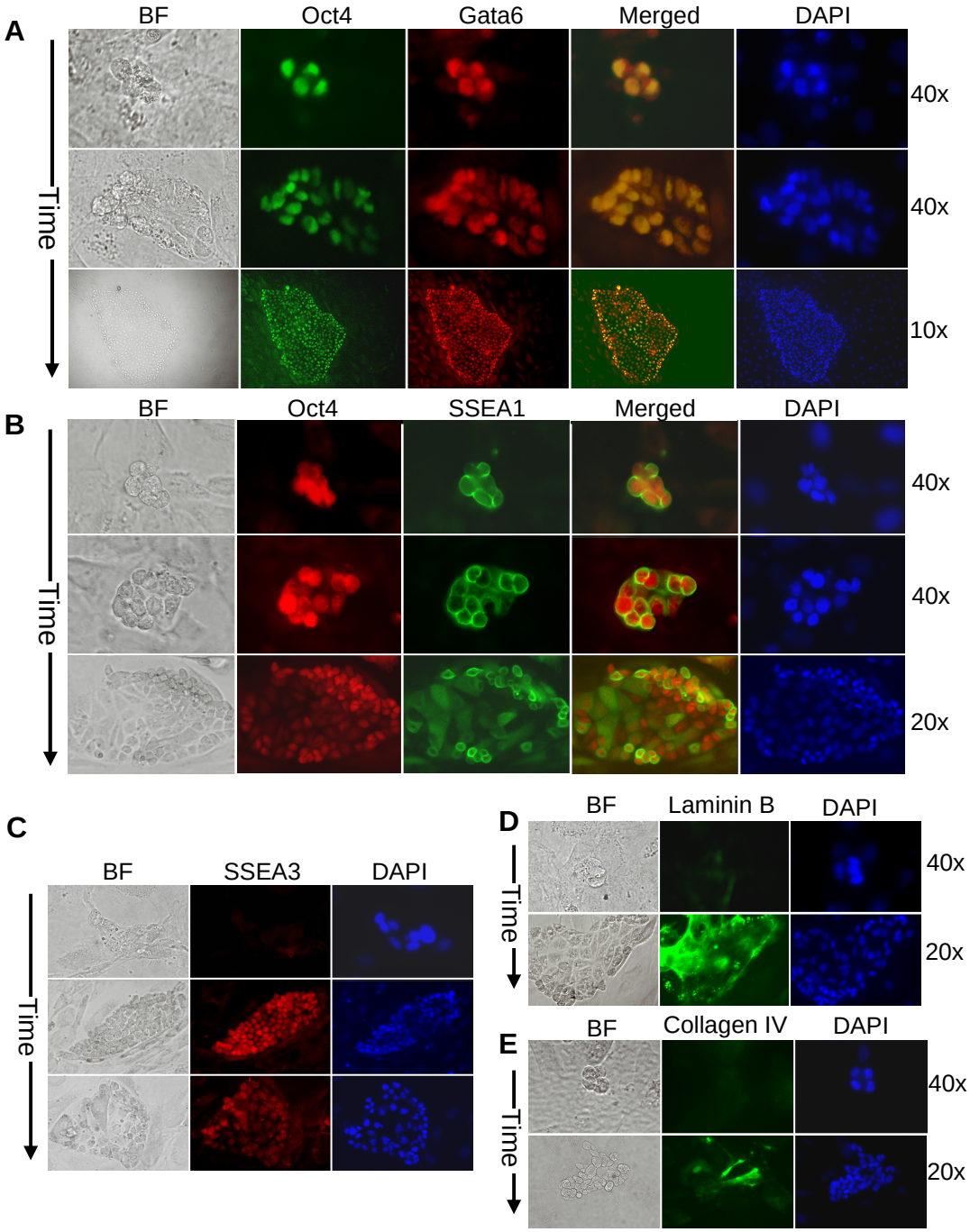

Fig. 10. Colony-level analysis of rat XEN cell lines for selected lineage markers. Rat XEN cells (line CX1) were plated at low density. At different time points, the resulting colonies were stained with the indicated antibodies and counterstained with Hoechst dye. (**A**) Double staining for Oct4 (green) and Gata6 (red).(**B**) Double staining for Oct4 (red) and SSEA1 (green). (**C**) Staining for SSEA3. (**D**) Staining for laminin B. (**E**) Staining for collagen IV. BF, bright field.

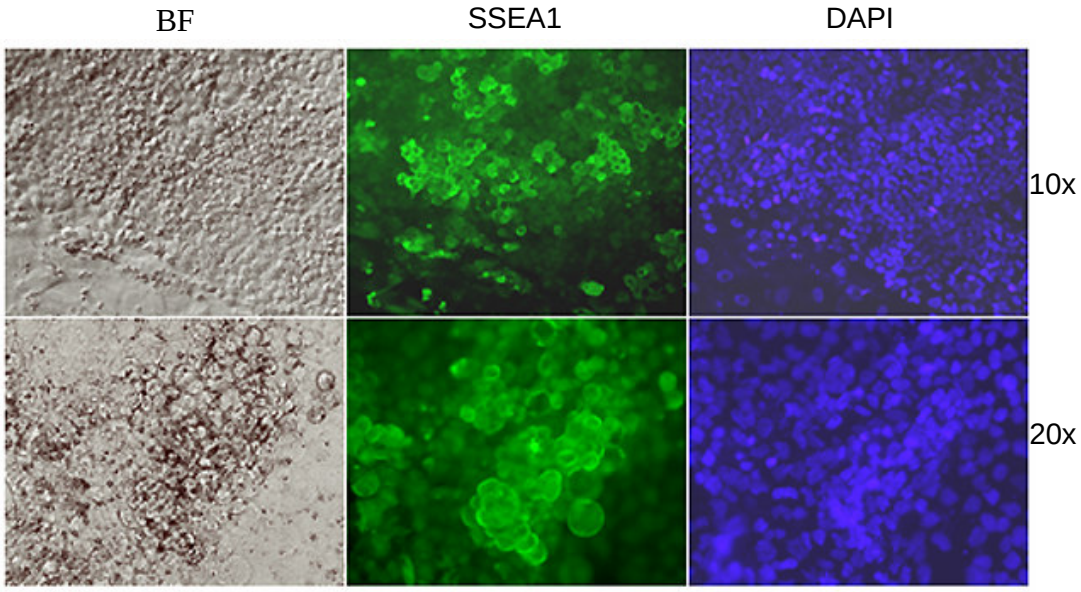

Fig. 11. Continued proliferation and preferential accumulation of SSEA1-positive cells in old rat XEN cell colonies. Two magnifications of a representative 16-days old colony (line CX1) are shown. Control omitting primary antibody was negative and is not shown. BF, bright field.

In the second approach, we transfected the rat XEN cells stably with a neomycin resistance marker, a method that ensures single-cell origin despite problems to trypsinize the XEN cells to homogeneous single cell suspensions. We obtained and expanded these single-cell derived colonies and randomly selected three of them for analysis by immunocytochemistry and RT-PCR using known markers of the different stages of XEN. All the resulting clones showed identical heterogeneous morphology (round-epithelial), the same heterogeneous expression patterns of Oct4, Gata6, SSEA1, and SSEA3 (Fig. 12A) as well as the same mixed ICM/PrE/VE/PE marker gene expression (Fig. 12B) as the parent line.

Rat XEN cells repopulate both visceral and parietal yolk sacs. In order to test the developmental potential of rat XEN cells, we transduced rat XEN cell lines CX1 and CX5 with the GFP-expressing lentiviral vector FUGW (Lois et al., 2002) and sent them to our collaborators at Northwestern University, Chicago. There the labeled cells were plated at low density to enrich for the XEN-precursor cells and then were injected into rat and mouse morulae and blastocysts, followed by culture or uterine transfer. Upon injection into rat and mouse morulae and blastocysts, labeled cells moved into the ICM (9%), onto the ICM (22%), and to the remaining inner surface of the blastocyst cavity (20%), positions that are compatible with XEN-precursor, PrE/VE, and PE identities, respectively. Unexpectedly, we also observed incorporations into the trophectodermal layer (50%), more often in mouse than rat embryos (Fig. 13A, Table 4)**.** After implantation, the labeled cells proliferated and contributed to the PE (79%) and VE (18%) layers of rat and mouse yolk sacs (Fig. 13B, Table 5). Thus, the cultured rat XEN cells contributed more than sporadically to the VE, although they did more often contribute to the PE (Table 5). No integration into fetal tissue was observed.

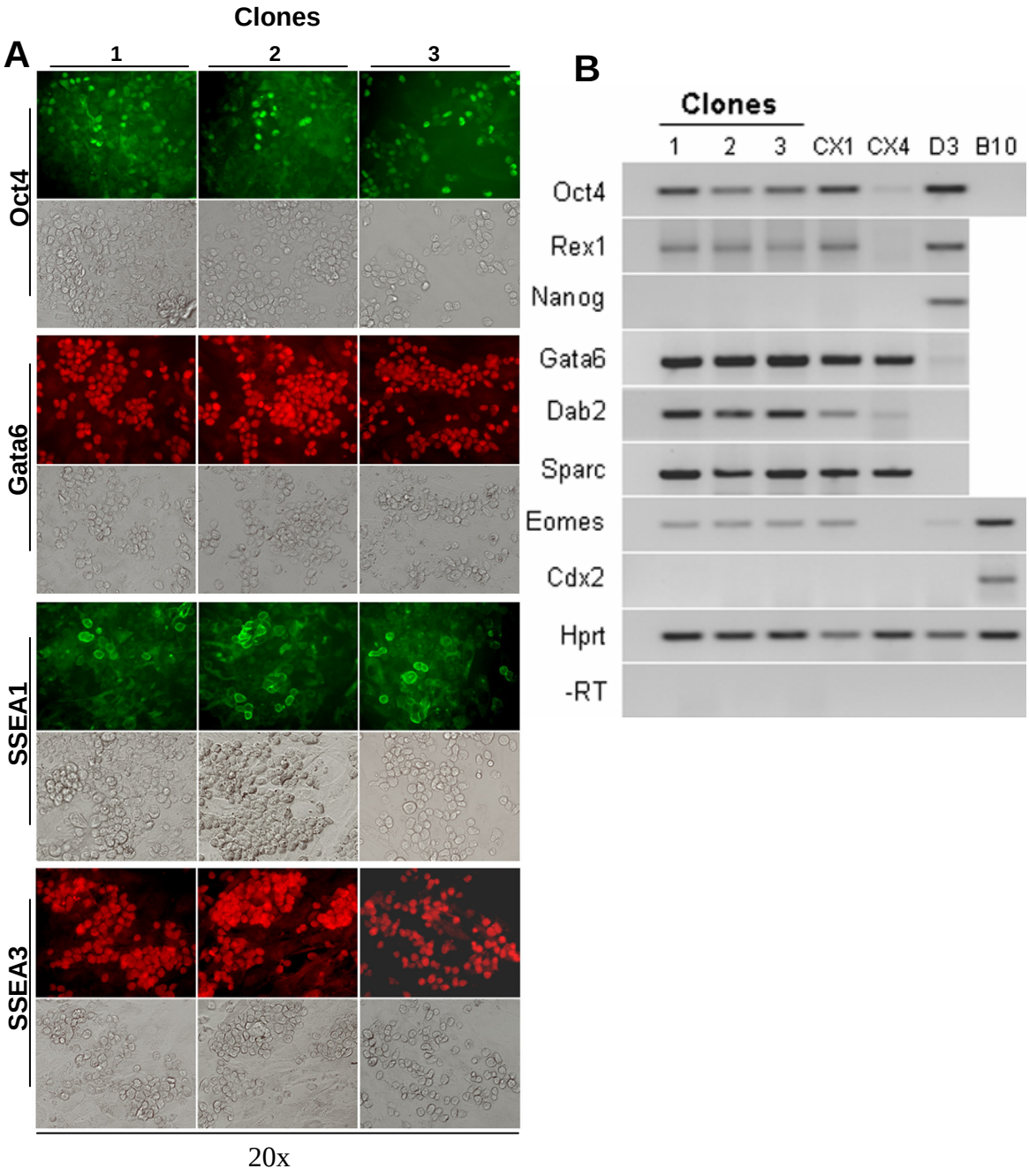

Fig. 12. Lineage marker expression in clonal rat XEN cell lines. Three arbitrarily picked subclones of line CX1 were analyzed by (**A**) indirect immunofluorescence for Oct4, Gata6, SSEA1and SSEA3 (Compare to Fig. 8) or (**B**) RT-PCR for expression of selected lineage markers. Cells used: rat XEN (CX1), mouse XEN (CX4), mouse ES (D3) cells and rat TE-like cells (B10). (Compare to Fig. 6B).

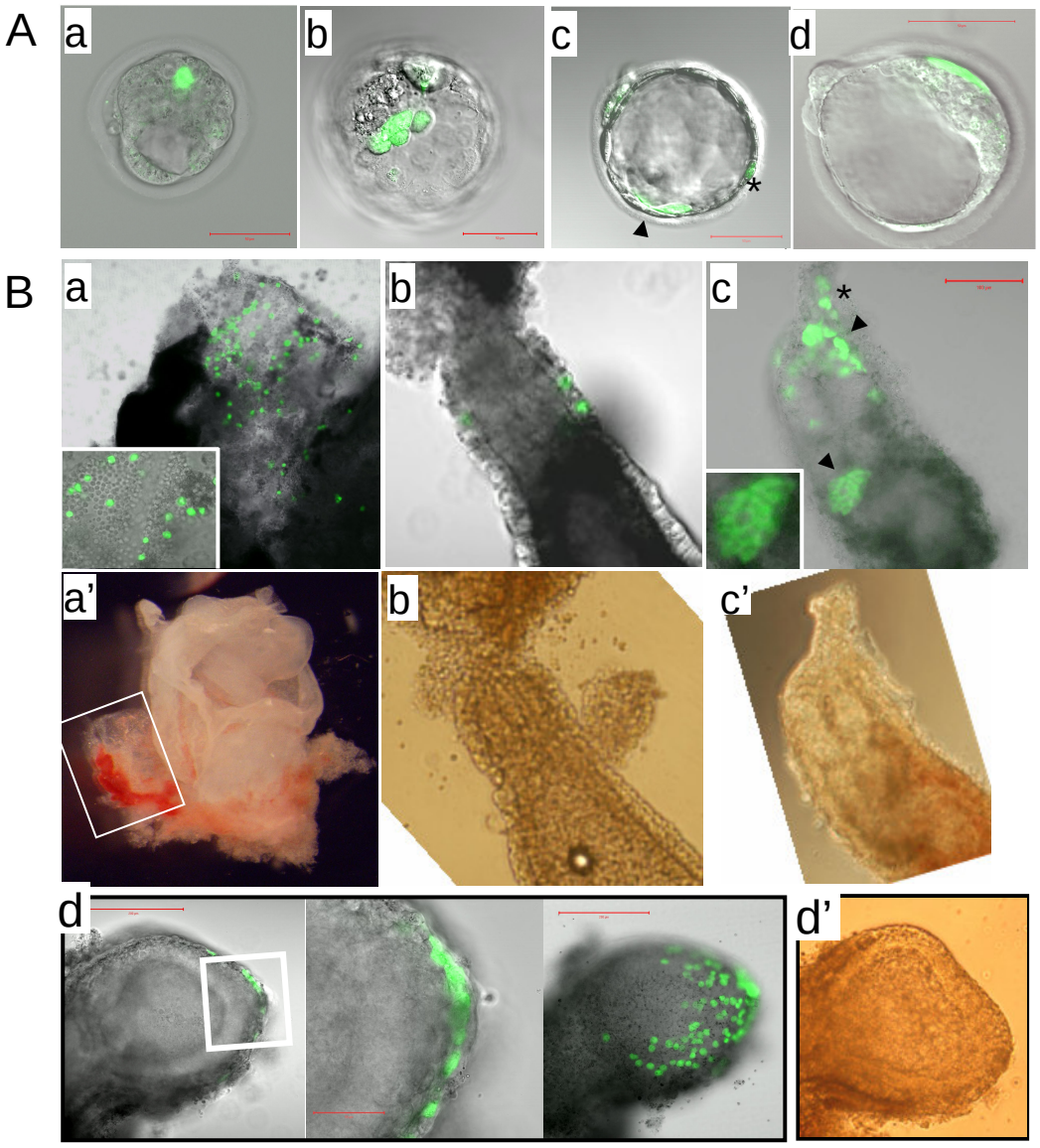

Fig. 13. Contributions of cultured rat XEN cells to preimplantation and postimplantation embryos. (**A**) Representative photographs showing in vitro incorporation of microinjected rat XEN cells into blastocyst regions that correspond to (**a**) ICM (mouse morula injected); (**b**) PrE/VE on top of ICM (rat blastocyst injected); (**c**) PE (arrowhead) and mural TE (asterisk) (mouse blastocyst injected); (**d**) polar TE (rat morula injected). (**B**) Representative fluorescence (a-d) and bright field (a'-d') photographs demonstrating in vivo contributions of microinjected rat XEN cells to (**a, a'**) parietal yolk sac of a 9.5 dpc rat conceptus (inset showing magnification; (**b, b'**) visceral endoderm of a 7.5 dpc rat conceptus; (**c, c'**) visceral endoderm (arrowheads; one patch magnified in inset) and parietal endoderm (asterisk) of a 6.5 dpc mouse conceptus, and (**d, d'**) visceral endoderm of a 7.5 mouse conceptus. (Contributed by Dr. V.Galat)

Table 4. Incorporation of cultured rat XEN cells into blastocysts

No. of Expts*	Cell line	Host species	Injected embryos	Stage	Developed to blast.	ICM	PrE/ VE	PE	TE
1	CX1	Rat	12	Blast.	n.a.	1	2	2	2
2	CX5		12	Morula	11	1	4	2	2
1	CX1	Mouse	11	Morula	10	1			8
2			9	Blast.	n.a.		1	2	8
1	CX5		8	Morula	6				1
2			24	Blast.	n.a.	1	3	3	2
Total (%)						4 (8.7)	10 (21.7)	9 (19.6)	23 (50)

* A total of 66 rat and 57 mouse embryos were injected in 7 experiments each. Included in the table are only those experiments in which after in vitro culture, labeled cells could be traced to blastocyst structures, which include inner cell mass (ICM), primitive or visceral endoderm (PrE/VE), parietal endoderm (PE), and trophectoderm (TE). (Contributed by Dr. V.Galat)

Table 5. Incorporation of cultured rat XEN cells into postimplantation conceptuses

No. of Expts*	Cell line	Host species	Injected embryos	Implantat -ion sites	Regressed embryos	Recovery day	VYS	PYS	Unclear
3	CX1	Rat	30	24	5	8.0-9.0	2	1	
4	CX5	Rat	59	39	9	8.5-9.5		7	
3	CX1	Mouse	34	25	15	7.0-8.0	3	6	1
4	CX5	Mouse	59	37	20	7.5-8.5		7	
Total (%)							5 (19)	21 (78)	1 (3.7)

* A total of 145 rat and 168 mouse embryos were injected and transferred in 13 and 15 experiments, respectively. Included in the table are only those experiments in which labeled cells could be recovered in a structure of the intact conceptus, including visceral yolk sac (VYS) endoderm and parietal yolk sac (PYS) endoderm (Contributed by Dr. V.Galat).

Discussion

We present here rat XEN cell lines that exhibited the molecular signature (Oct4+ Gata6+ SSEA1+ Nanog-Cdx2- Sox2-) expected from the XEN-precursor and give rise to its expected products, namely PrE/VE and PE in vitro and the respective yolk sac membranes in vivo. Our results show that cultured rat XEN cells highly express the key pluripotency marker Oct4 while mouse XEN cells did not contain significant Oct4 mRNA levels, as known before (Kunath et al., 2005). The expression of Oct4 is associated with cells having totipotent or pluripotent differentiation ability (Rosner et al., 1990; Scholer et al., 1990b; Yeom et al., 1996). However, Oct4 mRNA has previously been detected in the PrE layer of blastocysts (Rosner et al., 1990) and in the PrE/VE layer of embryoid bodies (Hamazaki et al., 2004). Although the average Oct4 mRNA levels in the rat XEN cells were fairly variable and somewhat lower than in ES cells, peak Oct4 mRNA levels in rat XEN cell colonies were even higher than those of mouse ES cells using qRT-PCR (Table 3). This is in line with the observation that over-expression of Oct4 in undifferentiated ES cells leads to endodermal differentiation (Niwa et al., 2000) and that a transient surge of Oct4 mRNA level accompanies the RA differentiation of F9 EC cells into endoderm (Botquin et al., 1998). However, Nanog, Sox2, and Fgf4, which are known Oct4 target genes and commonly used EPI markers, were not expressed in the rat XEN cells excluding the presence of ES-like cells. By contrast, the Rex1 gene, which is also an EPI marker and Oct4 target gene (Hosler et al., 1993; Ben-Shushan et al., 1998; Shi et al., 2006), was expressed in rat but not mouse XEN cells suggesting that Oct4 in the rat XEN cells is functional. This result parallels

the co-expression of Oct4 and Rex1 in Nanog-deficient mouse ES cells, which are XEN-like (Mitsui et al., 2003). Moreover, our results revealed that the rat XEN, but not mouse XEN, cells expressed the ICM/ES cell markers SSEA1 and alkaline phosphatase in a significant minority of the cells similar to Oct4 expression.

The findings that cultured rat XEN cells show a cluster of features traditionally considered as characteristic of mouse ES cells and ICM, in addition to expressing XEN markers strongly suggest that cultured rat XEN cells represent the ICM-stage XEN precursor (Chazaud et al., 2006; Kurimoto et al., 2006). More precisely, ~5-15% of the rat XEN cells (those with high Oct4 protein levels) are XEN-precursor cells while a larger fraction appears to represent the PrE stage, which is also missing in mouse XEN cell cultures. Indeed, we observed that there is a higher Oct4 mRNA level in the rat XEN cell cultures than reflected by the amount of protein, implying that some of the rat XEN cells that are low in Oct4 protein still express the mRNA, similar to what has previously been observed in the XEN layer on the surface of mouse ES cell-derived embryoid bodies (Hamazaki et al., 2004). In line with this, the majority of cultured rat XEN cells express SSEA-3, which is considered a PrE marker (Shevisnky et al., 1982) while mouse XEN cells that are PE-like (Kunath et al., 2005) lack SSEA3. Interestingly, we also noted that our rat XEN cell cultures showed higher levels of the pan-XEN marker Gata6 and of the VE/PrE markers Foxa2 and Dab2 than mouse XEN cells. Further, rat but not mouse XEN cells also expressed Eomesodermin, which marks the TE and the anterior visceral endoderm (Ciruna and Rossant, 1999) while Cdx2 and placental lactogen were not expressed in all XEN cell lines excluding TS-like cells in the culture. In summary,

while the mouse XEN cell lines were largely PE-like as expected (Kunath et al., 2005), the rat XEN cell lines expressed markers of all stages of the XEN lineage (XEN-precursor, PrE/VE, and PE).

The analysis of cells at low density showed that young/small colonies co-expressed Oct4, Gata6 and SSEA1 (Fig. 10A,B). Thus, young colonies show the key marker pattern of the XEN-precursor (Chazaud et al., 2006; Kurimoto et al., 2006). These colonies were poorly adherent and very easily lost during washing steps, in line with lack of expression of the basement membranes laminin B and collagen IV. This might explain why the young colonies that survived washings tended to have one or two differentiating cells that anchored them, and implies that the immunocytochemistry experiments with whole cultures (Fig. 8) underestimated the true percent of Oct4 and SSEA1 positive cells. By contrast, the majority of cells in older/large colonies expressed no or low Oct4, and lower Gata6 levels but additionally expressed differentiated XEN markers SSEA3 (PrE/VE)(Shevinsky et al., 1982) and laminin and collagen IV (PE) (Strickland et al., 1980; Gardner, 1983). In line with the high deposition of the basement membranes (Fig. 10D, E), older colonies became firmly adherent and contained a large fraction of epithelial cells. In further experiments, single-cell derived clones expanded to a whole culture level were able to give rise to the heterogeneous culture known from rat XEN cells providing formal evidence that one XEN-precursor cell can generate the whole culture heterogeneity.

Taken together, the gene expression studies in combination with colony and clonal analyses demonstrate that (i) Our rat XEN cell lines contain XEN-precursor cells (Oct4+

Gata6+ SSEA1+ Nanog- Cdx2-Sox2-); (ii) The XEN-precursor cells are the principal self-renewing entity under our current culture conditions, since they give rise to most, if not all, colonies; (iii) Culture heterogeneity is intrinsic and results from XEN-precursor cell differentiation into PrE/VE (SSEA3+) and PE (laminin+, collagen IV+). In addition, these data suggest that XEN-precursor cells can be enriched by SSEA1 sorting. The findings that our rat XEN cultures contain XEN-precursor cells that undergo both self-renewal and differentiation to the next XEN stages resembles cellular hierarchies recently discovered within human ES cell cultures that involve heterogeneity and posttranscriptional downregulation of Oct4 (Stewart et al., 2006).

Investigation of the developmental potential of rat XEN cells showed that they retain the capacity to contribute to visceral and parietal endoderm in vivo in chimeras, confirming to their XEN lineage origin. Although the overall contribution was biased towards the PE, line CX1 that showed a higher percentage of SSEA1-positive cells than CX5 (Figs. 8B, 9B) contributed at a relatively high frequency to the VE. This observation is in line with the previous findings that freshly isolated ICM cells contribute well to both VE and PE while freshly isolated PrE and VE cells mainly go to the PE (Gardner, 1982; Gardner, 1984). The predominant incorporation to the PE could also be a result of the blastocyst injection procedure used to generate chimers that may have provided an environment that promotes PE and hinders VE differentiation. In post-implantation embryos, no contribution into fetal or trophoblast tissue was detected. However, integrations into the TE layer of the blastocysts were observed (Fig. 13Ad). This TE-integration was unlikely due to contamination with TE-committed cells,

because our rat XEN cells lack Cdx2 that is required for TE commitment and expressed in the early TE as well as in rat and mouse TE-like and TS cell lines (Tanaka et al., 1998; Buehr et al., 2003; Strumpf et al., 2005; Niwa et al., 2005). Besides, TS cell expression pattern was not seen in single-cell derived clones. It is important to emphasize here that given all cell types in the XEN cultures originate from a single cell in vitro (Fig. 12B), all phenotypes seen in vivo derive at least indirectly from cultured XEN precursor cells. In line with the molecular analyses described above and the capacity to differentiate in vitro, the comparable ability of cultured rat XEN cells to integrate into rat and mouse embryos indicates that these cultures contain cells that are not principally different from primary XEN-precursor cells known from the mouse (Chazaud et al., 2006).

The availability of the XEN-precursor cell lines, which can differentiate into XEN subtypes in vitro and contribute to normal development in chimeras in vivo, provide a unique model for an early mammalian lineage that will complement the established ES and TS cell lines. In vitro combinations of ES, TS and XEN-precursor cells may help model the in vivo interactions between embryonic and extraembryonic lineages important for embryonic patterning (Kunath et al., 2005; Rossant, 2007). Using these cell lines, insights will be gained about the nature of the XEN-precursor, its differentiation and potential plasticity, and the developmental roles of the XEN lineage. It will also be interesting to compare the XEN-precursor with their better studied ES cell siblings for specific molecular features, such as overall epigenetic and X-chromosome

status, role and regulation of Oct4, and the signaling pathways involved in self-renewal (Niwa, 2007; Surani et al., 2007; Yeom et al., 1996; Chambers and smith, 2004).

CHAPTER III

REGULATION OF OCT4 GENE EXPRESSION IN RAT YOLK SAC ENDODERM STEM CELL LINES

Overview

The transcription factor Oct4 is a key regulator of pluripotency that is regarded as a hallmark of ES cells. It has been shown that the Oct4 gene contains a distal enhancer that drives specific expression in preimplantation embryos and ES cells and a proximal enhancer that activates expression in the epiblast and EC cells. But so far Oct4 regulation has not been described in the XEN lineage. We used the Oct4-expressing XEN-precursor cell lines, which were isolated in our laboratory, to identify regulatory elements responsible for Oct4 expression in the XEN cells. We divided the 5' flank of the mouse Oct4 gene into Region I (~2.7 kb) that contains the known enhancers and Region II (~1.9 kb) that is unexplored before and is upstream of Region I. We found that the rat XEN cells express reporter genes driven by Region (I+II) but do not express a reporter gene driven only by Region I. Our findings show the existence of differential Oct4 regulation between mouse ES and rat XEN cells and that a novel enhancer drives high-level gene expression in the rat XEN cells.

Introduction

Oct4 is a crucial determinant of pluripotency in ES cells (Nichols et al., 1998; Niwa, 2007) and is one of only four factors required to induce ES-like pluripotency in

fibroblasts (Yamanaka and Takahashi, 2006; Okita et al., 2007; Wernig et al., 2007). Oct4 is also essential for the differentiation of ES cells (Niwa et al., 2000). A critical level of Oct4 is required to maintain stem cell renewal in ES cells, and the level of Oct4 expression need to be tightly regulated in order to determine pluripotency and cell fates in preimplantation development (Niwa et al., 2000; Niwa, 2007). Therefore, investigation of the mechanisms that regulate Oct4 levels is important for understanding early embryonic development, pluripotency and differentiation.

Previous studies have defined three regulatory regions that are important for driving Oct4 expression in stem cells and in developing embryos (Okazawa et al., 1991; Sylvester and Scholer, 1994; Yeom et al., 1996). These regulatory regions include the TATA-less proximal/minimal promoter that mediates Oct4 basal promoter activity (Okazawa et al., 1991; Sylvester and Scholer, 1994) and two enhancer elements (distal enhancer and proximal enhancer) located in a 3-kb region upstream of the proximal promoter. These regulatory elements have been discussed in detail in chapter I under the subtitle ‘regulation of Oct4 gene expression’.

The observation that the distal enhancer is the principal Oct4 enhancer in the ICM and ES cells (Yeom et al., 1996) was made before it became apparent that the ICM is a heterogeneous population consisting of the EPI-precursor and XEN-precursor cells that both express Oct4 (Chazaud et al., 2006; Kurimoto et al., 2006). ES cells are now thought to be the in vitro equivalents of the EPI-precursor (Chazaud et al., 2006; Yamanaka et al., 2006) while our laboratory has isolated and characterized cell lines representing the XEN-precursor cells. Unlike in ES cells where the regulatory elements

that drive Oct4 expression are well characterized, it is unknown whether such elements exist in the rat, and which one of the known enhancers would be active in the Oct4-expressing rat XEN cells. In this study we show that the 5' region of the mouse Oct4 gene is active in the rat XEN cells. Further examination of this region revealed a previously unknown enhancer that drives Oct4 expression in rat XEN cells and that mediates differential regulation in mouse ES and rat XEN cells.

Materials and methods

Cell lines and cell culture

Mouse ES (line D3), mouse XEN (lines CX4, CX6) and rat XEN (lines CX1,CX2, CX5) cells were grown in DMEM containing glutamine and sodium pyruvate, 15% (v/v) ES-qualified serum (Invitrogen), 0.1 mM β-mercaptoethanol, 100 U/ml of Penicillin and 100 ug/ml streptomycin, and 1000 u/ml of LIF. Cells were seeded by trypsinization (0.25% trypsin-EDTA) at 37 °C and 5% CO_2. Passaging was performed every other day. Rat feeder cell line (Li1) and primary mouse embryonic fibroblasts were grown in DMEM with glutamine and sodium pyruvate, 100 U/ml of penicillin and 100ug/ml streptomycin and 10% fetal calf serum. The feeders were passaged at 1:4 to 1:6 split (as needed) in a 10-cm plate. Both mouse and rat feeders were inactivated using mitomycin (10µg/ml) i.e., by adding 10µl of mitomycin per 10ml of media in a 10-cm tissue culture plate and incubating it overnight at 37^{o}C. Following mitomycin treatment, the plates were washed 2X with PBS and were either trypsinized and seeded or stored in

the incubator until use. A 10-cm feeder dish was commonly seeded into 4x6-cm plates (about 1,000,000 cells per 6-cm plate).

Reporter gene constructions

The schematic description of the reporter constructs used in this study is shown in Fig. 14. In order to generate a fragment containing the two known Oct4 gene enhancers (distal and proximal enhancers), we used a bacterial artificial chromosome (BAC) clone (RP23-75C13 - obtained from CHORI BACPAC resource center, Oakland, CA) that contains the mouse Oct4 locus. We amplified *fragment 1* (-2695/+1; the numbering of nucleotides in this study is according to Nordhoff et al. (Nordhoff et al., 2001), i.e. the position relative to the translational start site) using the primer sequences: 5'-cacccggcccaaagtgactc-3' and 5'-aggtgggcaccccgagc-3' and a high-fidelity enzyme Accuprime Pfx (Invitrogen) by 7 PCR cycles (94 °C, 30 sec; 65 °C, 30 sec; 68 °C, 3:30 min). *Fragment 1* was then cloned into the SmaI site of the pGL3-Basic vector upstream of the firefly luciferase gene (Promega). The resulting construct (called Region I) was verified by sequence analysis. Region I was then used to further reconstruct another luciferase reporter gene which contains only the minimal Oct4 promoter (called here Proximal promoter). This construct was made by replacing the NheI/XbaI fragment of pGL3-Basic vector with the XbaI/XbaI fragment from Region I construct. The proximal promoter served as a background control. In order to determine if Oct4 in the rat XEN cells is regulated independently of mouse ES cell enhancers, another fragment (-4614/-2641, called here *fragment 2*) was amplified using Sahara DNA polymerase (Bioline, MA, USA) from plasmid GOF-9 (kindly provided by Dr. Yeom; Yeom et al.,

1996) that contains the mouse Oct4 gene plus a LacZ reporter "knocked in" the endogenous start codon. For this amplification, we used primers which contained the MluI restriction site (bold) (5'-att**acgcgt**tcctagcggggagac-3' and 5'-cat**acgcgt**atatgtgccactctgcc-3') and 15 total PCR cycles (5 cycles of 94 °C, 30 sec; 53°C, 30 sec; 72 °C, 3 min; and 10 cycles of 94 °C, 30 sec; 66°C, 30 sec; 72 °C, 3 min). *Fragment 2* was then cloned into the MluI site of the Proximal promoter and we designated this construct Region II **(Fig. 14).** The last construct (called Region (I+II)) was generated by cloning the PCR *fragment 2* into the MluI site of Region I. For all constructs the right orientation was confirmed by PCR and restriction enzyme digestion.

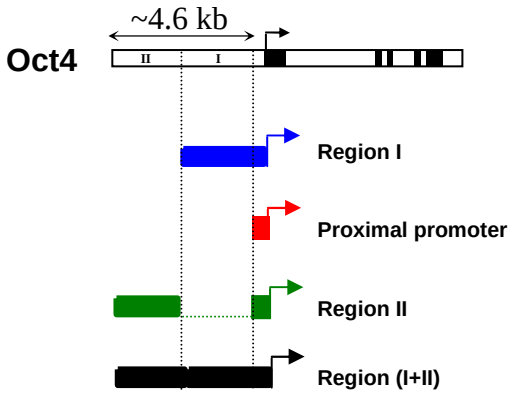

Fig. 14. Scheme of Oct4 gene depicting the main fragments analyzed in the reporter gene assays. Oct4 gene exons are symbolized with black boxes and the transcription start site with a bent arrow.

Lacz assay with histochemical stain

We seeded mouse ES, Mouse XEN, rat XEN cells as well as rat feeder cells in 4-well plates overnight and transfected them with 1µg/well of plasmid GOF-9 (Yeom et al., 1996) using lipofectamine 2000 reagent (Invitrogen, CA). Non-transfected controls

were included for each cell type. After 48 hrs, the β-galacosidase activity was visualized in situ by the histochemical stain method described (Sambrook and Russell, 2001). Briefly, media was removed and cells rinsed twice with PBS. Cells were then fixed with cell fixative (2% formaldehyde, 0.2% glutaraldehyde, 1x PBS) for 5min at room temperature. After washing with PBS three times, cells were stained with a histochemical stain (5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$, 1mg/ml X-gal, 1x PBS), incubated for 24 hrs at 37^{o}C, rinsed with PBS and photographed under a bright field microscope.

Quantification of β-galactosidase activity

We used the β-galactosidase Enzyme Assay System (Promega) for quantification of the β-galactosidase activity in transfected mouse ES, mouse XEN, rat XEN and rat feeder cell lines. Cells were seeded overnight in 6-well plates and were co-transfected with a mix (2µg/well, molar ratio 200:1) of GOF-9 construct and an SV40-driven pGL3-control vector (Promega)-to normalize transfection efficiency. After 48 hrs, the cells were washed twice with PBS and lysed with the reporter lysis buffer for 15 min at room temperature. The cells were then collected with a scraper and centrifuged to remove cell debris. The cell lysate was split and assayed for β-galactosidase and luciferase assay systems. The β-galactosidase activity was assayed (in duplicate) by mixing 50µl of the cell lysate in a 96-well plate with an equal volume of 2x assay buffer and was incubated at 37^{o}C for 2-3 hrs, followed by measurement of the absorbance (415 nm) with a plate reader. The luciferase activity was measured (in triplicate) using the luciferse assay

system (Promega) in a 96-well Luminocount luminometer (Packard Instruments). The β-galactosidase activity was then divided by luciferase activity in order to correct for differences in transfection efficiency.

Luciferase assays

Mouse ES (2.0×10^4), mouse XEN (4.0×10^4), rat XEN (4.0×10^4) and rat feeder (4.0×10^4) cells were seeded in 96-well bottom-clear tissue culture plates (PerkinElmer, MA, USA) for 24 hrs. 100 ng of the firefly luciferase constructs along with 2 ng of the Renilla luciferase (pRL/TK, Promega) was transfected using Fugene HD (Roche). The pRL/TK plasmid served as an internal control for normalizing the transfection efficiency. The cells were lysed 48 hrs after transfection and firefly and Renilla luciferase activities were measured with the Dual-Glo Luciferase Assay System (Promega). Because of problems with lysis when following the manufacturer's procedure, we modified it by removing the medium, freezing the plates at -80^oC for 5 min, equilibrating to room temperature, adding the media back and then an equal volume of the dual-glo luciferase assay reagent. Plates were read in a Luminocount 96-well luminometer from Packard Instruments. The luciferase activity of each construct was calculated relative to the pGL3-Basic vector, which lacks a promoter and enhancer region. All transfection experiments were repeated in triplicate and promoter activities are reported as means ± SEM. In order to correct for variations in transfection efficiency, readouts of firefly luciferase-encoding Oct4 reporter plasmids were divided by the values determined for the Renilla luciferase-encoding plasmid pRL-TK which had been

co-transfected at a ratio of 1 molecule pRL-TK per 50 molecules of Oct4 gene reporter construct.

Results

A mouse Oct4 reporter gene is active in rat XEN cells. In order to test whether the mouse Oct4 gene confers reporter gene expression in the rat XEN cells, we transiently transfected the GOF-9 reporter gene construct that includes the Oct4 gene and a LacZ cDNA cloned into the start codon (Yeom et al., 1996). The cells were stained using a histochemical stain 48 hrs after transfection. As shown in Fig. 15, GOF-9 stained positively for beta galactosidase not only in mouse ES cells but also in rat XEN cells while mouse XEN cells that do not express Oct4 (Fig. 6B; Kunath et al., 2005) and rat fibroblasts were negative.

We then quantified the beta galactosidase activity using β-galactosidase Enzyme Assay System after transfecting the cells with GOF-9. Similar to the qualitative histochemical stain, GOF-9 was active in mouse ES cells and rat XEN cells but no activity was seen in mouse XEN cells and in rat fibroblasts (Fig. 15B). The average relative level of GOF-9 expression in the rat XEN cells was ~30% of the level in ES cells. This was comparable with the average relative level of endogenous Oct4 mRNA. The lower average beta galactosidase activity in the rat XEN vs. the mouse ES cells could be partly attributed to the heterogeneity observed in rat XEN with respect to Oct4 mRNA levels; Not only are the differentiated cells lowly expressing Oct4 but the

construct that corrects for transfection efficiency will also be incorporated into such cells which likely underestimates the Oct4 reporter gene activity.

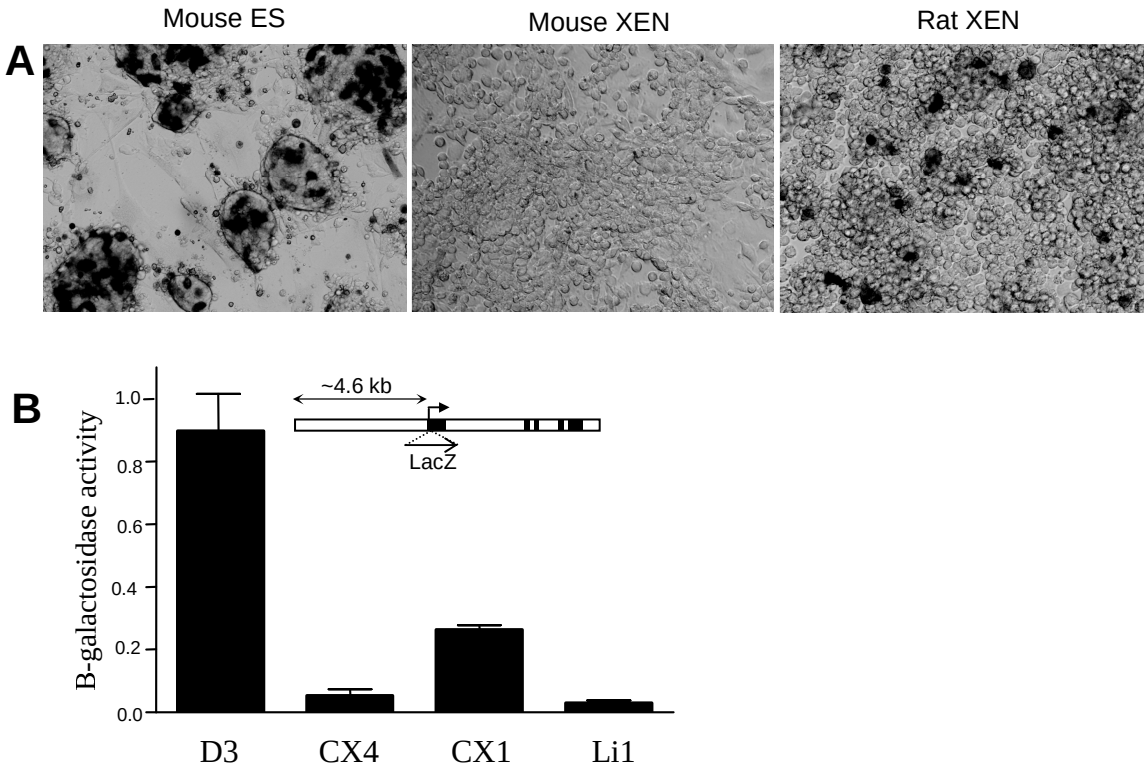

Fig. 15. Cultured rat XEN cells express reporter genes driven by mouse Oct4 regulatory region. Cells used: D3 mouse ES cells, CX4 mouse XEN cells, CX1 rat XEN cells and Li1 rat embryo fibroblast. (**A**) Histochemical staining for B-galactosidase activity after transient transfection with GOF-9 (Yeom et al., 1996). (**B**) Quantification of the reporter gene activity normalized to SV-40 driven luciferase. Non-transfected cells did not show LacZ staining.

The Oct4 enhancer sequence that is active in cultured rat XEN cells was derived from the mouse Oct4 gene construct GOF-9 (Yeom et al., 1996). Although GOF-9 contains an upstream and downstream region separated by a LacZ reporter placed at the start codon, in this study we generated the Oct4-Luc fragments only from the sequences of the upstream region because in previous experiment (Yeom et al., 1996), a reporter

transgene containing the downstream sequences plus the Proximal promoter was inactive in the ICM, whereas the upstream regions lead to expression.

A novel enhancer drives Oct4 expression in rat XEN cells. Having known that the mouse Oct4 reporter gene is active in rat XEN cells, we sought to determine whether the known regulatory elements (the distal and proximal enhancers) that have previously been shown to restrict reporter gene expression to Oct4-expressing cell types (Yeom et al., 1996) are also responsible for driving Oct4 expression in rat XEN cells. We first tested Region I (see Fig. 14 for scheme), an Oct4-Luc reporter construct containing both distal and proximal enhancers, for reporter gene expression in the rat XEN cells. As shown in Fig 16, Region I drove robust luciferase expression in mouse ES cells but only background expression in rat XEN, mouse XEN and rat fibroblast cells. Thus, Region I is insufficient for Oct4 expression in XEN cells. We then tested the other Oct4-Luc reporter constructs including Region II, Region (I +II) and the Proximal promoter. We found that Region (I+II) construct was active in mouse ES and rat XEN cells but not in mouse XEN and rat fibroblast cells while Region II and the Proximal promoter constructs did not confer reporter gene expression in any of the cells (Fig. 17). Thus, Region I is necessary and sufficient for Oct4 expression in ES cells. But neither Region I nor Region II is sufficient, and both regions are necessary for expression in rat XEN cells. This shows that a novel or previously unknown transcriptional enhancer is required for Oct4 expression in rat XEN cells and that there is differential regulation between mouse ES and rat XEN cells.

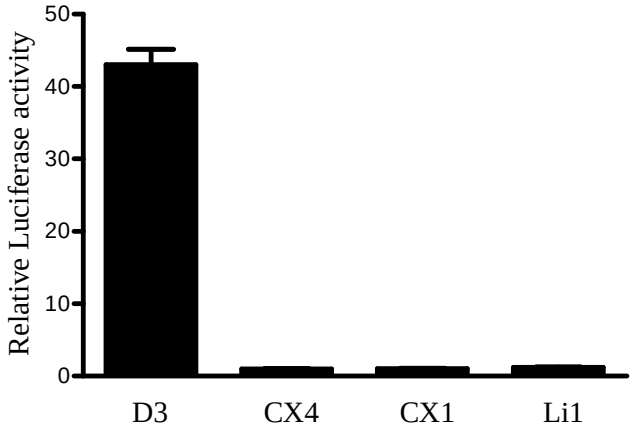

Fig. 16. Previously known mouse ES cell enhancer is insufficient to drive Oct4 expression in rat XEN cells. Bars show expression levels of Region I luciferase reporter construct, normalized for transfection efficiency Cells used: D3 mouse ES cells, CX4 mouse XEN cells, CX1 rat XEN cells and Li1 rat embryo fibroblast.

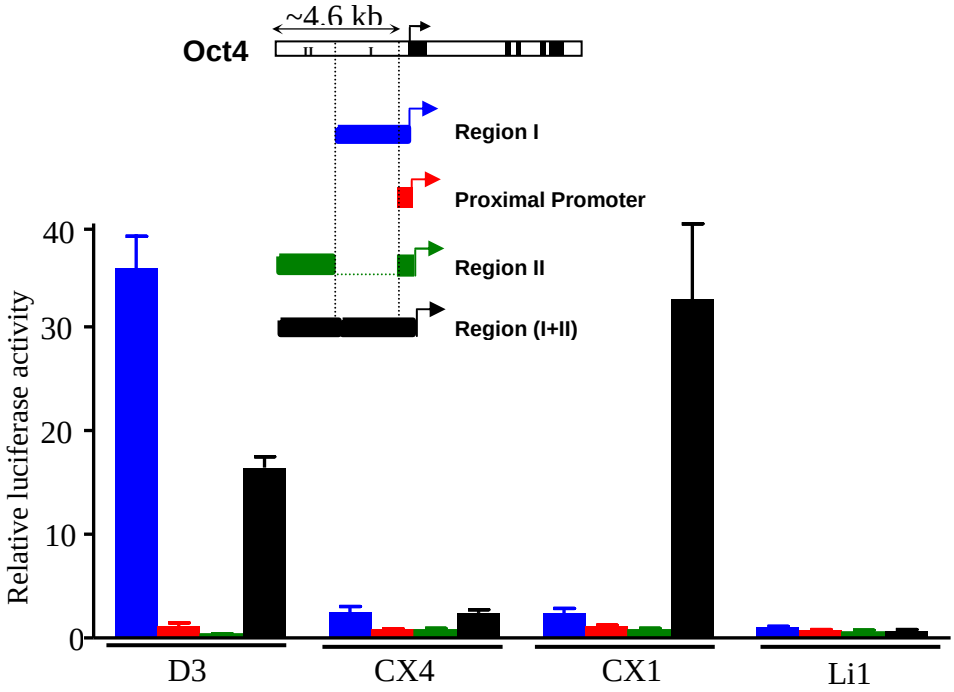

Fig. 17. A novel transcriptional enhancer drives Oct4 expression in rat XEN cells. Cells used: D3 mouse ES cells, CX4 mouse XEN cells, CX1 rat XEN cells and Li1 rat embryo fibroblast. While Region I is sufficient in ES cells, Region (I+II) is required for Oct4 expression in rat XEN cells. Bars show luciferase expression levels of the luciferase reporter constructs, normalized for transfection efficiency with pRL-TK.

Discussion

Two enhancers, both located within 3-kb upstream of the transcription start site, have previously been shown to confer tissue-specific Oct4 gene expression: the distal enhancer is active in the ICM and germline in vivo and in ES cells and EG cells in vitro, while the proximal enhancer is active in the epiblast in vivo and in EC cells in vitro (Yeom et al., 1996). Here we discovered that the 5' region of the mouse Oct4 gene contains previously unknown cis-acting elements that appear to mediate differential regulation of Oct4 in mouse ES cells and rat XEN cells. In support of our findings, there were indications that Oct4 might be regulated differently in the XEN cells: an increased dosage of Oct4 can convert ES cells into XEN cells (Niwa et al., 2000) and the differentiation of murine EC cells into XEN cells involves a transient increase of Oct4 gene transcription (Botquin et al., 1998). Moreover, one report claims that cells of the newly forming primitive endoderm expressed Oct4 protein at a level that appears to be higher than that seen in the ICM population (Palmieri et al., 1994) although this finding was not confirmed by others (Strumpf et al., 2005). Differential Oct4 regulation in mouse ES and rat XEN cells might suggest that a significant epigenetic difference is established during, if not before, the second embryonic lineage choice when the ICM converts to EPI and XEN lineages.

The reason that previous reporter gene constructs (Yeom et al., 1996; Buehr et al., 2003) did not reveal Oct4 gene expression in the XEN-precursor cells in vivo might be because the activity of the XEN-enhancer was obscured by the activity of the known ICM-enhancer (i.e. the distal enhancer). But with the discovery of the heterogeneity of

the ICM (Chazaud et al., 2006) and our finding of a novel XEN-enhancer in the Oct4-expressing rat XEN cells, the view that the distal enhancer is the only Oct4 enhancer used in the ICM may have to be re-interpreted such that both XEN enhancer and ES enhancer contributed to the reporter gene expression in the different subpopulations of the ICM.

A systematic dissection and precise identification of the cis-acting element(s) driving Oct4 expression in the XEN cells as was done in ES cells (Okumura-Nakanishi et al., 2005; Chew et al., 2005; Yeom et al., 1996) might allow the selective inactivation of the Oct4 gene enhancer in XEN-precursor cells without affecting it in the EPI-precursor cells. This will enable us to study the lineage-specific functions of Oct4, identify trans-acting factors and optimize the derivation, maintenance and differentiation of pluripotent ES cells for therapeutic purposes.

CHAPTER IV

SUMMARY AND CONCLUSIONS

During the first embryonic lineage choice, the outer cells of the mammalian morula become committed towards the TE lineage while the remaining inner cells become the ICM. It has previously been thought that the ICM is composed of a homogenous population of cells, and ES cells were usually portrayed as representing the ICM. However, we have learned only very recently (Chazaud et al., 2006; Kurimoto et al., 2006) that the ICM, during the early blastocyst (E3.5 in the mouse) stage, is composed of two intermingled, morphologically indistinguishable populations of cells: the EPI-precursor that founds all fetal lineages and the XEN-precursor that founds the yolk sac endoderm.

The EPI-precursor has been well characterized because it is represented in vitro by the well known ES cell lines that have been available since1981 (Evans and Kaufman, 1981; Martin, 1981). By contrast, cell lines representing the XEN-precursor cells are missing. Mouse XEN cell lines described recently (Kunath et al., 2005) do not express Oct4 (the hallmark of ICM cells) and do not contribute efficiently to all XEN cell types in vivo, and thus they do not represent the XEN-precursor. Our laboratory has derived XEN cell lines from rat blastocysts that express the key ICM and pluripotency marker Oct4 and we hypothesized that these cell lines represent the XEN-precursor. The goals of the work presented here were i) to define the molecular identity of these Oct4-

expressing rat XEN cells using molecular and differentiation markers, and ii) to study the regulation of Oct4 expression in the rat XEN cells and compare it with that in mouse ES cells.

To achieve our first goal, we used known molecular markers that define the three fundamental tissue lineages of the early developing embryo (TE, EPI, and XEN) and compared our rat XEN cell lines with mouse XEN, mouse ES and rat TE-like cells. We easily detected Oct4 mRNA in cultured rat XEN cells but not in mouse XEN cell lines, confirming Kunath et al. (Kunath et al., 2005). In addition to expressing known XEN markers and Oct4, rat XEN cells also expressed Rex1, an ICM marker and a target gene of Oct4 but they lacked other ES cell markers and TE markers, excluding their contamination with ES/TS cells. In colony samples, the Oct4 mRNA in rat XEN cells reached levels >2 fold of those in mouse ES cells, reminiscent of the 2.5 fold increase of Oct4 mRNA in murine EC cells differentiating into XEN cells (Botquin et al., 1998). But the average Oct4 mRNA from a whole culture ranged from 30-84% indicating heterogeneity in the culture. In line with this, we found that only a minority fraction (5-15%) of Oct4 mRNA-containing rat XEN cells express Oct4 protein at comparable levels with that in mouse ES cells.

We studied colonies over time in order to understand the identity of the Oct4-expressing XEN cells and examine whether heterogeneity in rat XEN cells is arising from differentiation of each XEN cell to the next developmental stage (s). Very young colonies showed expression patterns expected from the XEN-precursor. That is, they co-expressed Oct4/SSEA1 and Gata6 while the PrE/VE marker SSEA3 and the PE markers

laminin and collagen IV were absent. As colonies get older, however, most of the cells were negative for SSEA1 and Oct4 but rather expressed SSEA3, laminin and collagen IV. Yet, the older colonies always retained high Oct4/SSEA1 and Gata6-expressing cells at their fringes. By expanding single-cell derived clones into a whole culture level and analyzing three of them with immunocytochemistry and RT-PCR, we found a heterogeneous morphology, heterogeneous Oct4/SSEA1/SSEA3 staining, and mixed XEN-precursor/PrE/VE/PE marker expression. The in vivo developmental potential of the rat XEN cells was studied by injecting GFP-labeled cells into rat and mouse blastocysts. The injected rat XEN cells showed exclusive contribution to the XEN cell types, including both the visceral and parietal endoderm.

In summary, our results show that rat XEN cells contain the ICM-stage XEN-precursor, and only XEN-precursor cells gave rise to colonies under our culture conditions. These cells can be maintained in vitro as a self-renewable entity just like the ICM-stage EPI-precursor of other species can be maintained in the form of ES cells. Our results also demonstrate that culture heterogeneity results from the differentiation of XEN-precursor cells into PrE/VE and PE and thus, provide a superb in vitro model of early differentiation along the XEN pathway.

The second goal, which is on identifying regulatory elements responsible for Oct4 expression in the XEN cells, was accomplished by dissecting the 5’ flank of the mouse Oct4 gene sequences (~5 kb) into Proximal promoter, Region I that consists of previously known ES cell and epiblast enhancers, Region II that is unexplored before and is upstream of Region I, and Region (I+II). We found that Region (I+II) drove

luciferase gene expression while neither Region I nor Region II is sufficient for reporter gene expression in rat XEN cells. Thus, a previously unknown transcriptional enhancer drives Oct4 expression in rat XEN cells and differential Oct4 regulation exists in mouse ES vs. rat XEN cells.

In summary, we here present for the first time cell lines that based on their molecular signature and differentiation potential represent the XEN-precursor as known from mouse ICM. These cell lines offer new possibilities for examining the nature, differentiation, and plasticity of the XEN-precursor and the biological roles of the XEN. In particular, determining the role and regulation of Oct4 in XEN-precursor cells will help to understand their nature and relationship with the EPI-precursor (Chazaud et al., 2006) and provide knowledge useful for methods to induce pluripotency (Takahashi and Yamanaka, 2006); this is because Oct4 can both protect the identity of ES cells and induce their differentiation, including differentiation into XEN (Niwa et al., 2000). Likewise, it will be instructive to compare ES and XEN-precursor cells with respect to their epigenetic status (Niwa, 2007; Surani et al., 2007) and the signaling pathways involved in self-renewal (Chambers and Smith, 2004). Furthermore, our rat XEN cell lines offer the first in vitro differentiation model in which XEN cells are generated from XEN-precursor cells. This mimics the natural process but contrasts with the traditional in vitro model in which XEN cells are generated from ES cells (Doetschman et al., 1985), which likely involves trans-differentiation (Chazaud et al., 2006). This work has also uncovered new facets on the regulation of Oct4 gene expression at a stage of embryonic development that is poorly accessible. Future studies on the new Oct4 gene enhancer

will benefit from answering questions such as where exactly is this enhancer located (promoter bashing)? Where and when this enhancer is active in vivo? Does the differential regulation observed in vitro between XEN-precursor and EPI-precursor (ES cells) cells also exist in vivo? Would inactivation of this enhancer in the XEN cells have developmental consequences?

In conclusion, this work provides XEN-precursor cell lines and the newly identified enhancer as new tools that will advance understanding of the molecular basis of early embryogenesis, and improve the derivation and directed differentiation of pluripotent and therapeutic stem cells.

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