

Formation of Pluripotent Stem Cells in the Mammalian Embryo Depends on the POU Transcription Factor Oct4

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Summary

Oct4 is a mammalian POU transcription factor expressed by early embryo cells and germ cells. We report that the activity of Oct4 is essential for the identity of the pluripotent founder cell population in the mammalian embryo. Oct4-deficient embryos develop to the blastocyst stage, but the inner cell mass cells are not pluripotent. Instead, they are restricted to differentiation along the extraembryonic trophoblast lineage. Furthermore, in the absence of a true inner cell mass, trophoblast proliferation is not maintained in Oct4^{-/-} embryos. Expansion of trophoblast precursors is restored, however, by an Oct4 target gene product, fibroblast growth factor-4. Therefore, Oct4 also determines paracrine growth factor signaling from stem cells to the trophectoderm.

Introduction

Embryonic development in mammals begins with a series of cleavage divisions to generate a population of equivalent blastomeres. Maternal gene products deposited in the oocyte dictate a characteristic number and timing of cleavages for each species. During the cleavage process, the zygotic genome is activated and progressively takes control of subsequent development. Cellular differentiation and segregation of developmental lineage commence at the end of cleavage with compaction leading to formation of the blastocyst (Gardner and Papaioannou, 1975; Papaioannou, 1982). The outer layer of cells form an epithelium, the trophectoderm, descendants of which are restricted to generation of the trophoblast components of the placenta. The interior, inner cell mass (ICM), cells develop into pluripotent progenitors of nontrophoblast extraembryonic tissues and of all fetal

cell types, including germ cells. The ICM and its successor the epiblast are highly regulative stem cell populations that can adjust to various perturbations including major alterations in cell number and cell position (Hogan et al., 1994). The pool of stem cells remains pluripotent until late gastrulation. Although it normally persists only transiently in the embryo, the pluripotent stem cell population is intrinsically immortal. Thus, these cells can form stem cell tumors, teratocarcinomas, at high frequency when grafted ectopically (Solter et al., 1970; Diwan and Stevens, 1976). Most significantly, explant cultures of ICM or epiblast can produce pluripotent embryonic stem (ES) cell lines (Evans and Kaufman, 1981; Martin, 1981; Brook and Gardner, 1997). Understanding the molecular basis of the pluripotent phenotype is likely to be critical to efforts to isolate and propagate stem cells from other species, including humans.

Continued interaction with the ICM, and subsequently the epiblast, is required to maintain proliferation of the trophectoderm and produce a trophoblast stem cell compartment, the extraembryonic ectoderm (Gardner and Johnson, 1972; Ansell and Snow, 1975; Rossant and Ofer, 1977; Papaioannou, 1982; Gardner, 1983). Reciprocal signaling from trophectoderm may similarly contribute to sustain the ICM/epiblast (Nichols et al., 1996; J. N. et al., unpublished). The diversification and expansion of the trophoblast and ICM lineages thus involves, first, the specification of distinct developmental potentials and, second, the production of paracrine growth signals. Elucidation of the molecular mechanisms that govern and interconnect these processes in the developing blastocyst will provide a paradigm for understanding more complex tissue differentiation and growth control in later development.

The POU factor Oct4 (Schöler et al., 1990a; Schöler, 1991) (also known as Oct3; Okamoto et al., 1990; Rosner et al., 1990) is distinguished by exclusive expression in blastomeres, pluripotent early embryo cells, and the germ cell lineage (Rosner et al., 1990; Schöler et al., 1990b; Yeom et al., 1996; Pesce et al., 1998). In the mouse blastocyst, Oct4 mRNA and protein are present in the ICM but not in the trophectoderm (Palmieri et al., 1994). In vitro Oct4 is found only in undifferentiated embryonic carcinoma (EC), embryonic stem (ES), and embryonic germ (EG) cells (Okamoto et al., 1990; Rosner et al., 1990; Yeom et al., 1996). Oct4 can act either to repress or to activate target gene transcription (Lenardo et al., 1989; Schöler et al., 1991; Liu and Roberts, 1996; Ben-Shushan et al., 1998; Botquin et al., 1998). It regulates expression of multiple genes (Saijoh et al., 1996) via interactions with at least two other transcription factors present in pluripotent cells, the so-called E1A-like activity (Schöler et al., 1991) and the HMG-box protein Sox2 (Yuan et al., 1996). One of the most interesting candidate targets of Oct4 is the gene encoding fibroblast growth factor-4 (FGF4). The *Fgf4* gene has an octamer-containing enhancer in its 3' noncoding region and has been demonstrated to respond to Oct4 in a Sox2-dependent fashion (Curatola and Basilico, 1990; Yuan et al., 1996; Ambrosetti et al., 1997). FGF4 is coexpressed with Oct4

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in the ICM and epiblast (Ma et al., 1992; Niswander and Martin, 1992).

In this paper, we demonstrate through the use of targeted gene deletion that Oct4 has a pivotal function in the birth of the pluripotential ICM lineage. In addition, we show that Oct4-directed expression of FGF4 provides a paracrine signal that couples expansion of the extraembryonic trophoblast lineage with development of the embryonic primordium.

Results

Embryonic Lethality of *Oct4* Mutants

A mutant allele of *Oct4* was generated via homologous recombination in ES cells. Exons 2–5 of the *Oct4* gene were replaced by an internal ribosome entry site (IRES)- β geo cassette as described previously (Mountford et al., 1994). The effect of this is to delete sequences encoding the two DNA-binding domains and the carboxy terminal transactivation region of the protein. In addition, a selectable marker/reporter gene is introduced into the locus. Germline transmission of the targeted allele was obtained from two independent ES clones. The phenotype described below was found in animals generated from both clones and was fully penetrant on pure strain 129, hybrid-inbred (129 \times CBA), and hybrid-outbred (129 \times MF1) genetic backgrounds.

Male and female animals heterozygous for the *Oct4* deletion were fertile and transmitted the mutant allele to approximately 50% of their progeny. However, there was a total absence of homozygous *Oct4*-deficient pups in 11 litters (89 pups) produced by heterozygous intercross matings. Furthermore, homozygous embryos could not be found amongst 34 embryos genotyped at midgestation. Significantly, however, high numbers of resorption sites were detected at these and earlier stages. Six females were sacrificed at 5.5 days after intercross mating for examination of conceptuses prior to gastrulation. Sixty-one implantation sites were analyzed. A total of 43 prestreak embryos were recovered. The embryos were dissected free of maternal tissue and genotyped by PCR; 11 were wild type, 32 heterozygous, and none homozygous for the mutant *Oct4* allele. The remaining 18 implantation sites did not contain any discernible yolk sac or embryonic structure. They consisted of apparently normal decidual swellings with some trophoblast cells evident. No material could be recovered free of maternal tissue for reliable genotyping, but it is highly likely that this high proportion of empty decidua arise from implantations of *Oct4* null embryos. In a parallel analysis of heterozygote matings to wild-type siblings, only one empty site was found amongst 54 implantations analyzed. The wild-type and heterozygous embryos from these intercrosses were at the stage of proamniotic cavity development and epithelialization of the epiblast (Hogan et al., 1994). Thus, the absence of *Oct4* results in peri-implantation lethality before egg cylinder formation.

PCR-based genotype determination confirmed that homozygous mutant preimplantation embryos were present at close to expected Mendelian frequency at 3.5 days (156/627; 24.8%), although apparently slightly underrepresented at 4.5 days (12/75; 16%). Histochemical staining for β -galactosidase activity revealed three

classes of embryo: nonstained, faintly stained, and moderately stained (Figure 1A). PCR genotyping of several embryos after staining confirmed that this profile corresponded to wild-type, heterozygous, and homozygous mutant genotypes, respectively. Immunohistochemical staining established the absence of Oct4 protein in approaching one-quarter (7/32) of blastocysts from intercross matings (Figure 1B). Freshly isolated 3.5-day mutant embryos resembled early blastocyst structures in morphology. The presence of dividing cells in mutant embryos was detected by confocal microscopy. This did not reveal overt signs of cellular degeneration or increased cell death (Figure 1C). Although partial cavitation was often evident, the mutant embryos were rarely fully expanded in contrast to the majority of wild-type or heterozygous embryos. However, six out of six mutant embryos cultured in vitro from the morula stage expanded fully within 48 hr, and four hatched from the zona. The formation of an intact trophectoderm epithelium and the capacity to induce uterine decidualization indicate that any impairment of initial trophectodermal differentiation or function in the absence of Oct4 is relatively minor.

Oct4-Negative Embryos Are Not Growth-Retarded

POU factors have been reported to promote cell proliferation both indirectly and directly (reviewed in Ryan and Rosenfeld, 1997). The possibility that *Oct4* null embryos were merely growth retarded was examined. Cell numbers in 3.5-day embryos were counted after differential labeling of outside and inside cells (Handyside and Hunter, 1984). The data presented in Figure 1D demonstrate that 3.5-day mutant embryos have normal numbers of cells in both populations. In light of this, we examined the possibility that cleavage development might be sustained by maternal Oct4, which is abundant in oocytes (Rosner et al., 1990). Nuclear localized Oct4 was detected from the 8-cell stage onward in all (37/37) embryos from wild-type matings. In contrast, 5 of 27 intercross 8-cell embryos and 3 of 12 intercross morulae lacked detectable Oct4 protein (Figure 1E). Therefore, maternal Oct4 does not persist through cleavage in *Oct4*^{-/-} embryos, consistent with our previous observation of decay by the late 2-cell stage in wild-type embryos (Palmieri et al., 1994). Thus, both the initial distribution to outside (presumptive trophectoderm) and inside (presumptive ICM) compartments, and subsequent proliferation and cell survival to the mid-blastocyst stage proceed in the complete absence of Oct4.

The preimplantation period was extended to determine whether the mutant embryos had any capacity for further development in vivo. Plugged females were ovariectomized 2.5 days after copulation to prevent implantation. Under these conditions, embryos mature to the expanded blastocyst stage and then enter developmental arrest (Robertson, 1987). After 5 days of implantation delay, 24/24 wild-type and heterozygous blastocysts were fully expanded with well-defined ICMs. Homozygous *Oct4*-deficient mutants survived this period, but in 6/6 cases, the embryos were collapsed structures with no evident ICM.

In order to determine the fate of mutant blastocysts, newly implanted embryos from intercross matings were

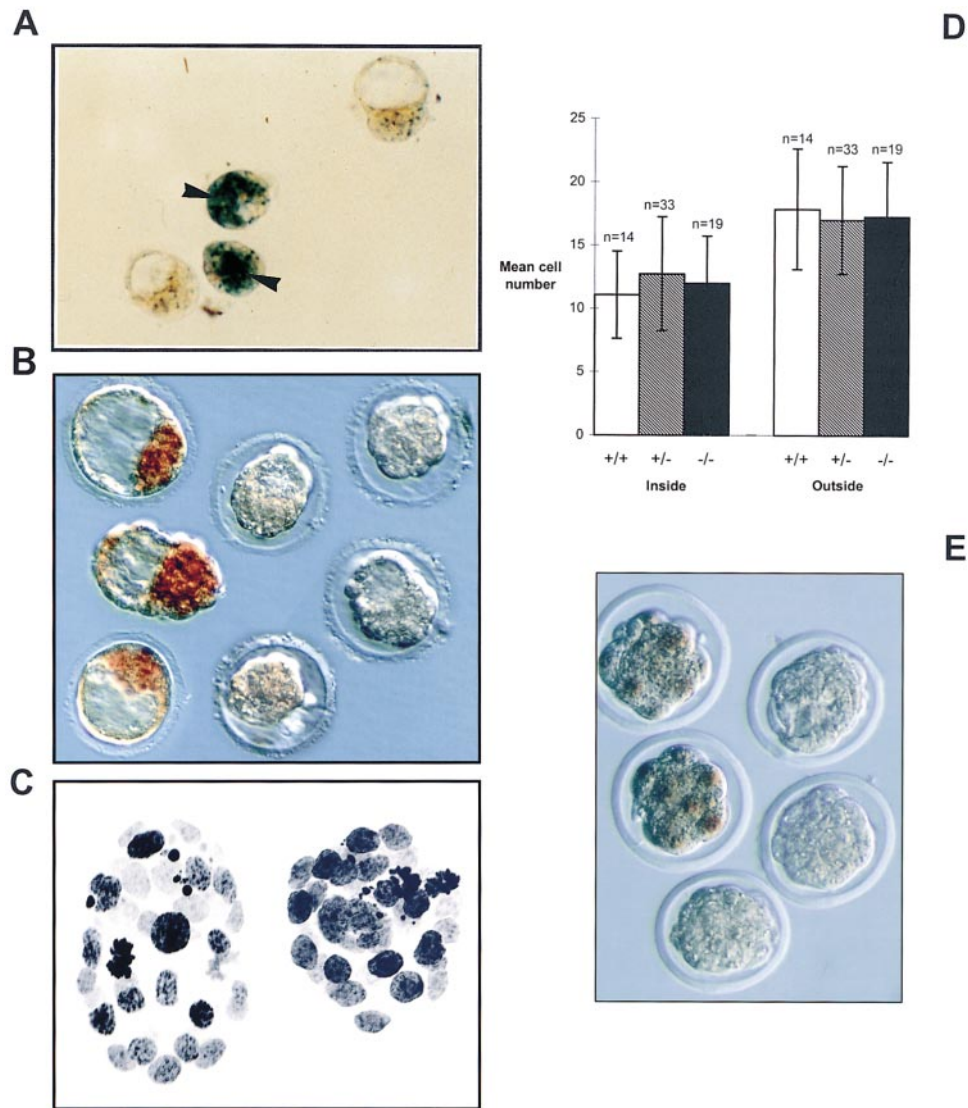


Figure 1. Morphology, Staining, and Cell Numbers of Embryos from Intercross Matings of *Oct4* Mutants

(A) β -galactosidase staining of 3.5-day blastocysts. Two embryos show light staining characteristic of heterozygotes and two show the darker staining associated with homozygous mutants. Note the staining of the inner cells in the latter (arrow).

(B) Immunostaining of 3.5-day blastocysts for *Oct4*. The panel shows three embryos with immunoreactive ICMs and four nonstaining embryos. Seven out of 32 intercross embryos examined at this stage failed to stain, whereas the inner cell mass was strongly immunoreactive in all of more than 50 blastocysts examined from matings of wild-type mice.

(C) Confocal images after propidium iodide staining of two of the embryos shown in (B), *Oct4*-positive (left) and *Oct4*-deficient (right).

(D) Inside and outside cell numbers determined after differential labeling of freshly isolated 3.5-day embryos. Individual specimens were recovered from the slides for genotype determination by PCR. Data are means \pm SEM. There are no significant differences within the groups (t test, $P > 0.75$).

(E) Immunostaining of early morulae (2.5-day) for *Oct4*. Note the nuclear localization in the two positively staining embryos. Three out of 15 intercross morulae failed to stain, whereas 21/21 control embryos gave specific nuclear staining.

isolated from nascent decidual swellings at 5.0–5.25 days. From 51 implantation sites, 37 embryos were recovered prior to proamniotic cavity formation. These all showed well-defined embryonic and abembryonic poles (Figure 2A). They were determined to be either wild type (18) or heterozygous (19). Eight implantations were empty. The remaining six contained small, unstructured fragments that lacked a discernible embryonic compartment. These fragments were genotyped by PCR and in all cases were homozygous for the mutated allele. After overnight culture, wild-type and heterozygous embryos

exhibited prominent rind and core structures characteristic of extraembryonic endoderm overlying epiblast (Figure 2B). All mutant embryos, in contrast, produced trophoblast cells only (Figure 2C).

In Vitro Development of *Oct4*-Deficient Embryos

The preceding observations suggested that there was a specific defect in the viability or developmental potential of the ICM in mutant embryos. The developmental status of mutant blastocysts was therefore examined further by in vitro culture experiments. Blastocysts were

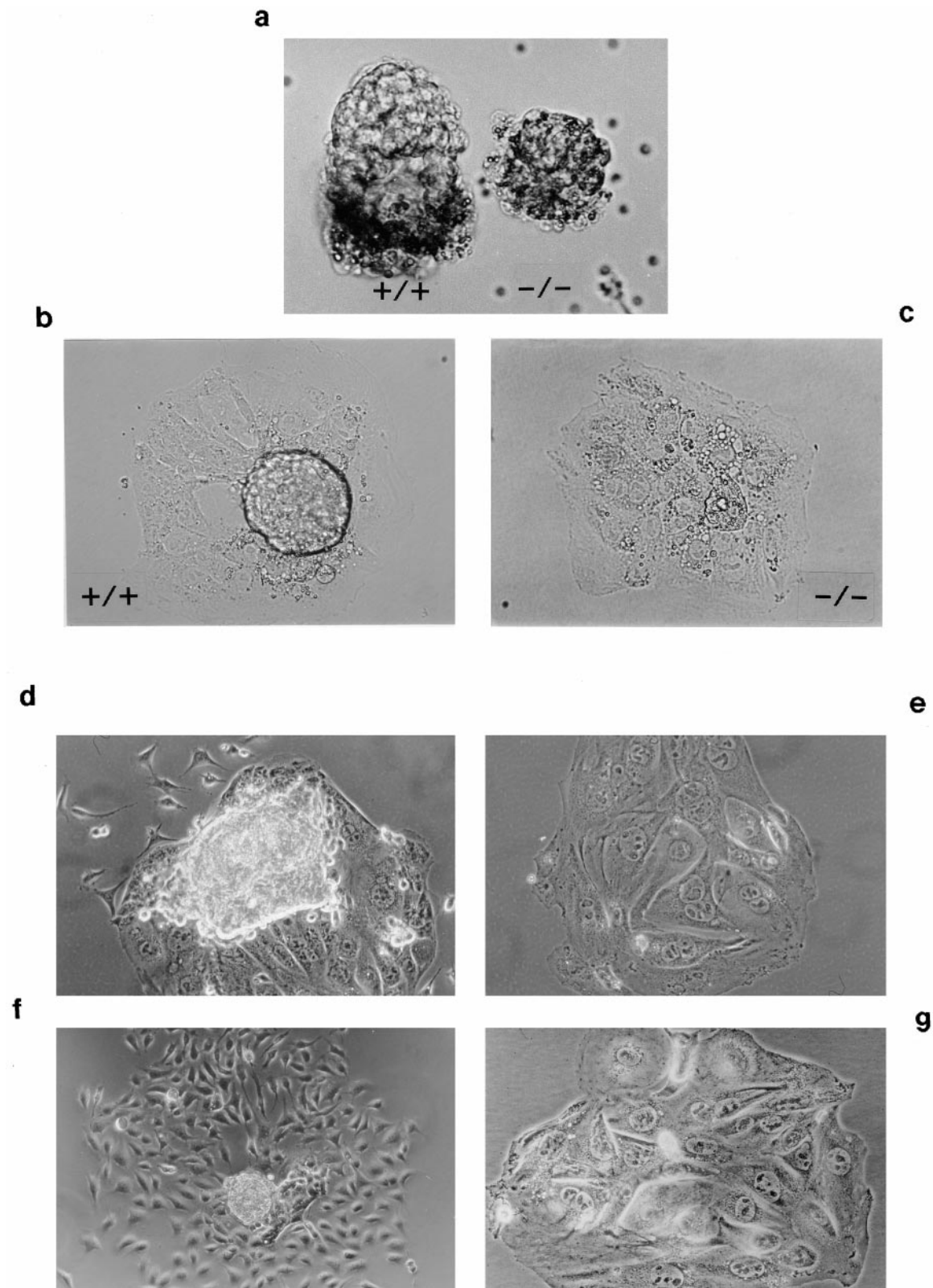


Figure 2. Outgrowth Cultures of Intercross Embryos

(A–C) Peri-implantation stage (5.25 days) wild-type (+/+) and homozygous *Oct4* mutant embryos (-/-) freshly dissected from nascent implantation sites (A) after overnight culture (B and C). (D and E) Whole 3.5-day blastocyst cultures after 4 days. (F and G) Cultures of immunosurgically isolated internal cells after 4 days. (D and F), Wild type; (E and G), homozygous *Oct4* mutant.

Objective magnification: (A) $\times 4$; (B–G) $\times 10$.

Table 1. In Vitro Development of Embryos from Intercross Matings of *Oct4* Heterozygotes

(A) Cultures in Serum-Containing Medium					
Development	Genotype	Genotype			Not typed
		+/+	+/-	-/-	
Whole blastocysts	ICM and/or endoderm	17	34	0	3
	Trophoblast only	0	3	9	3
Internal cells	ICM and/or endoderm	9	19	0	0
	Trophoblast only	1	1	7	0

(B) Cultures in Serum-Containing Medium Supplemented with FGF4					
Development	Genotype	Genotype			Not typed
		+/+	+/-	-/-	
Whole blastocysts	ICM and/or endoderm	15	27	0	0
	Trophoblast only	0	0	13	2
Internal cells	ICM and/or endoderm	78	134	2	10
	Trophoblast only	0	2	9	2
	Trophoblast + "ExEct"	1	1	53	4

Outgrowths were cultured for 4–6 days in Glasgow modification of Eagle's medium supplemented with 10^{-4} M 2-mercaptoethanol, 20% fetal calf serum, and 100 U/ml LIF. FGF4 was added to a final concentration of 2.1 ng/ml in the presence of heparin. "ExEct" refers to the emergence of clusters of cells with morphology of extraembryonic ectoderm. The two ICM outgrowths apparently arising from *Oct4*^{-/-} embryos most likely represent errors in PCR genotyping or handling of samples.

placed in culture in ES cell derivation medium containing 20% fetal calf serum and recombinant leukemia inhibitory factor (LIF) (Nichols et al., 1990). All embryos outgrew a layer of trophoblast giant cells. Wild-type and heterozygous embryos in addition maintained distinctive ICM-derived cell masses that increased in size during the culture period. Migratory parietal endoderm cells differentiated in most cultures (Figure 2D; Table 2). In contrast, homozygous *Oct4*-deficient embryos yielded only trophoblast giant cells (Figure 2E). They did not

contain a recognizable ICM-derived structure nor produce parietal endoderm at any stage in the culture period.

The absence of morphologically identifiable ICMS even in the earliest stages of adherent culture of mutant blastocysts prompted more detailed investigation. Immunosurgery was employed to remove the outer trophoblast layer (Solter and Knowles, 1975) and isolate the internal population of cells from 3.5-day embryos. Genotypes were determined by PCR analysis of the

Table 2. Second Round Targeting of the *Oct4* Gene

(A) Introduction of <i>Oct4iresph</i> construct into ES cells heterozygous for <i>Oct4iresβgeo</i> allele					
Selection	Colonies	X-Gal – ve	Integration Event		
			Random	Retargeting	2nd allele
Hyg	12	9	3	9	0
Hyg + G418	4	0	4	0	0

(B) Introduction of <i>Oct4iresβgeo</i> construct into ES cells heterozygous for <i>Oct4iresph</i> allele					
Selection	Colonies	Xgal + ve	Integration Event		
			Random	Retargeting	2nd allele
G418	8	6	2	6	0
G418 + Hyg	21	ND	21	0	0
G418 + Hyg ^a	96	8	8	0	0

(C) Introduction of <i>Oct4iresph</i> construct into ES cells heterozygous for <i>Oct4ireszeo</i> allele and vice versa					
Second vector	Selection	Colonies	Integration Event		
			Random	Retargeting	2nd allele
<i>hph</i>	zeo + hyg	20	20	0	0
<i>zeo</i>	zeo	3	0	3	0
<i>zeo</i>	zeo + hyg	21	21	0	0

Oct4 targeting constructs were introduced into parental CGR8 ES cells or previously targeted heterozygous derivatives by electroporation and grown up under selection in G418, hygromycin B (hyg), or zeocin (zeo) as indicated. Clones were analyzed as appropriate by X-Gal staining for retention of the *Oct4iresβgeo* allele and by DNA hybridization with *Oct4* genomic probes as described (Mountford et al., 1994).

^a Only clones showing stem cell restricted X-Gal staining were analyzed by DNA hybridization.

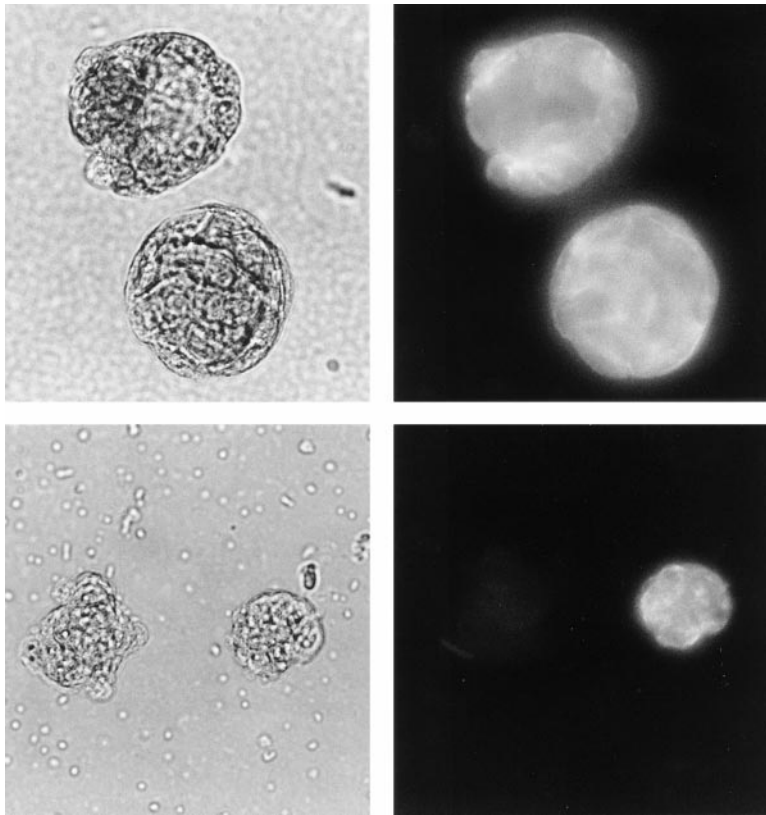


Figure 3. Cytokeratin Expression by Internal Cells from Wild-Type and *Oct4* Mutant Blastocysts

Upper panel, Bright field and fluorescence photographs of intact 3.5-day blastocysts showing Troma-1 immunoreactivity in the trophoblast layer.

Lower panel, Bright field and fluorescence photographs of immunosurgically isolated internal cells from intercross 4.5-day blastocysts. No staining is evident in the heterozygous ICM, whereas expression is apparent throughout the *Oct4*-deficient population. Genotypes of the internal cell clumps were determined by PCR analysis after observation and photomicrography.

Objective magnification: $\times 40$.

trophoblast lysate. The internal cell clumps were cultured to determine their viability and developmental potential. Cultures from wild-type and heterozygous embryos maintained a distinctive central clump of cells with a rind and core structure. In almost all cases they generated appreciable amounts of parietal endoderm and often also patches of visceral endoderm (Figure 2F). Cells from homozygous mutant embryos also survived in culture. However, an ICM-derived rind and core structure was not evident. Instead, the mutant cells flattened out on the substratum within 24 hr of attachment and invariably differentiated into trophoblast giant cells (Figure 2G). They produced neither ICM-like growths nor extraembryonic endoderm (Table 1A).

These findings establish that *Oct4*-deficient cells in the interior of the developing blastocyst remain viable but do not progress to become mature ICM cells. *Oct4* thus appears to be essential for the establishment of pluripotency in the ICM.

Inactivation of *Oct4* in Stem Cell Lines

In order to assess whether there is a continuous requirement for *Oct4* in the maintenance of pluripotent cells, we attempted to inactivate both alleles in stem cell lines. Homozygous *Oct4*-negative clones could not be isolated by subjecting heterozygous targeted CGR8 ES cells or P19 EC cells to selection in high levels of G418 (Mortensen et al., 1992). Further targeting vectors were therefore prepared in which the IRES β geo cassette (Mountford et al., 1994) was substituted by either an IRES hph or an IRES zeo marker. In parental ES cells,

these vectors gave a comparable high frequency (>50%) of homologous recombinants as the β geo construct. In heterozygous cells, this frequency was maintained, but recombination was always at the previously targeted allele (Table 2). When selection was applied for both markers, only random integrants were isolated. This was true regardless of the order in which the targeting constructs were introduced. CGR8 ES cells are derived from pure inbred strain 129OlaHsd mice, and the frequency of homologous recombination is expected to be identical at both alleles. We conclude that a functional *Oct4* allele is likely to be indispensable for maintenance of the self-renewing undifferentiated ES cell phenotype.

Oct4-Negative Internal Cells Are Diverted to a Trophoblast Fate

Differentiation into trophoblast of cultured inner cells from *Oct4*-negative embryos could reflect prior commitment to the extraembryonic lineage. Alternatively, the mutant cells may remain developmentally naive in situ and initiate differentiation only in response to externalization, attachment to a substratum, or some other inductive cue from the culture environment. To attempt to distinguish between these two possibilities, the phenotype of mutant cells was investigated immediately after immunosurgical isolation. The monoclonal antibody Troma-1 reacts with intermediate filaments, which are first expressed in nascent trophoblast and are not found in ICM cells (Brulet et al., 1980). Inner cell clumps were immunostained using FITC-conjugated

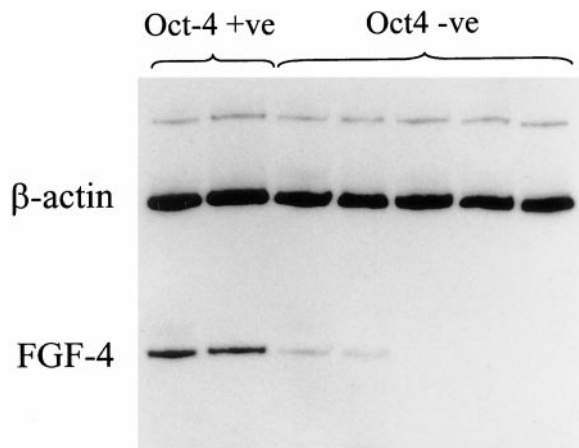


Figure 4. Reverse Transcription PCR Analysis of *Fgf4* Transcripts at the Blastocyst Stage

Aliquots of cDNA prepared from individual 3.5-day blastocysts were first analyzed for amplification of *Oct4* sequence. Further aliquots were then assayed for amplification of *Fgf4* sequence, using incorporation of [³²P]-dCTP to maximize sensitivity. Amplification of β -actin sequence was employed as a control in both reactions. In the representative panel shown, three of the *Oct4*-negative samples produce no detectable FGF4 amplification product and two yield a band that is <25% the intensity of that produced from the *Oct4*-positive samples.

secondary antibody, examined by fluorescence microscopy, and then genotyped by PCR. As expected, whole blastocysts at either 3.5 days or 4.5 days gave a strong signal localized to the trophoderm (Figure 3 and data not shown), but ICMs from wild-type or heterozygous embryos showed no reaction (Figure 3). In contrast, *Oct4* null inner cells were immunoreactive with Troma-1 in half of 3.5-day embryos (9 out of 18) and in all 4.5-day embryos (5 out of 5) examined (Figure 3). These observations indicate that the mutant cells do not remain indeterminate *in vivo* but divert into the trophoblast lineage and begin to differentiate whilst in an internal location.

Reduced Expression of Fibroblast Growth Factor-4 in *Oct4* Mutant Embryos

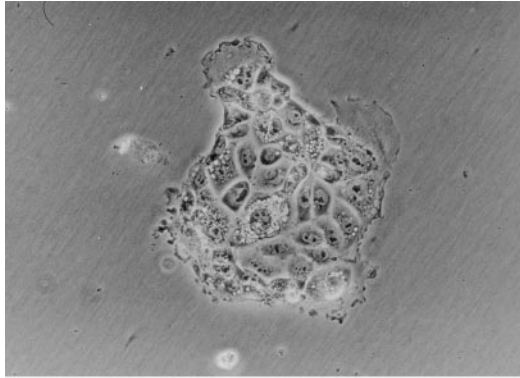
Mouse embryos lacking FGF4 form blastocysts but do not develop significantly beyond implantation (Feldman et al., 1995). The possible contribution of a loss of FGF4 expression to the *Oct4* mutant phenotype was therefore investigated. RT-PCR analysis was used to determine the presence or absence of *Oct4*, *Fgf4*, and β -actin transcripts in 3.5-day embryos from intercross matings. All embryos contained β -actin mRNA, but *Oct4* mRNA was absent in 15 out of 60 (25%). *Fgf4* mRNA was present in all the *Oct4*-positive samples. Of the 15 *Oct4*-negative embryos, 6 yielded no detectable FGF4 amplification product, and 9 had a greatly reduced level of *Fgf4* mRNA that was detectable only by incorporating radiolabeled nucleotides in the PCR reaction (Figure 4). These results are consistent with *in vitro* evidence that expression of FGF4 is, at least in part, dependent on Oct4 (Curatola and Basilico, 1990; Yuan et al., 1996; Ambrosetti et al.,

1997). The low levels of FGF4 transcripts detected in some of the mutants may arise from variation in embryo age and reflect the persistence of maternal *Fgf4* mRNA (Rappolee et al., 1994) in less advanced embryos. Alternatively, there may be a degree of Oct4-independent transcription of *Fgf4* in the early embryo as occurs in later development (Niswander and Martin, 1992).

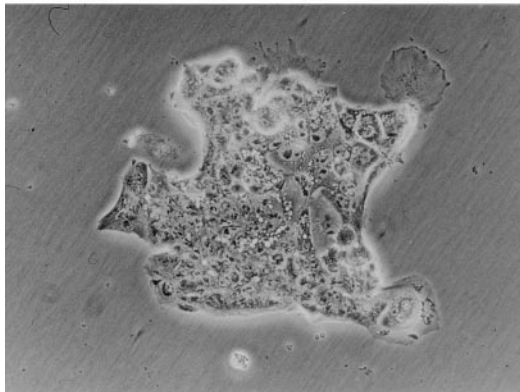
FGF4 Does Not Restore ICM Pluripotency but Promotes Proliferation of Trophoderm Precursors

Recombinant FGF4 was added to cultures of *Oct4* mutant embryos. No effect was discernible on intact blastocysts (Table 1B). Under these conditions, however, access of growth factor to the target cells inside the embryo may be restricted by the overlying trophoderm. FGF4 was therefore also added to cultures of immunosurgically isolated internal cells. In this case, a marked effect was apparent in the majority of mutant cultures (Table 1B). Clusters of rounded, morphologically unspecialized cells emerged from the monolayer of trophoderm cells after 3–4 days of culture (Figure 5). They did not originate from an ICM-like clump of cells but grew directly out from the trophoderm layer. These cells remained viable for at least 2 weeks, although proliferation slowed after the first few days. The clusters were distinct in appearance from ICM-derived structures and significantly did not give rise to either parietal or visceral endoderm cells (compare with Figure 2F). This effect was reproducibly observed on addition of FGF4 to inner cells isolated from either 3.5-day or 4.5-day mutant blastocysts. The response could also be induced by FGF2 (not shown). It was specific to members of the FGF family, however, since it was not induced by serum alone, nor by two growth factors implicated in trophoblast development, epidermal growth factor and colony stimulating factor-1.

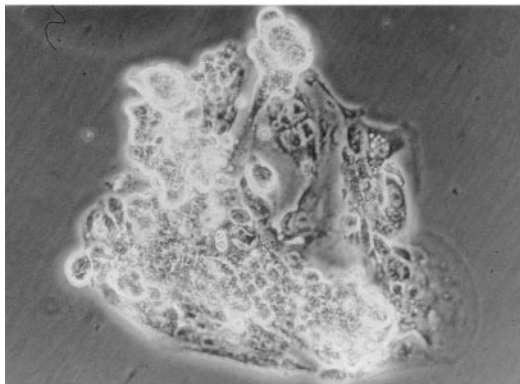
In situ hybridization analysis of marker gene expression was employed to investigate the identity of the FGF4-induced cells in mutant cultures. *Sox2* and *H19* transcripts show reciprocal expression patterns in the early embryo. *Sox2* is expressed in a similar pattern to *Oct4* in the ICM and epiblast (Robin Lovell-Badge, personal communication) whilst *H19* is expressed in trophoblast and extraembryonic ectoderm (Poirier et al., 1991). Neither gene is expressed in mature parietal endoderm. This specificity of expression is maintained in culture (Figure 6 and data not shown). No expression of *Sox2* was detected in the FGF4-induced cell growths in mutant cultures (Figure 6B). These cells did, however, express *H19* at high level (Figure 6C). This confirms that they are not ICM cells and strongly suggests that they are trophoblastic. In appearance, these growths resemble primary cultures of isolated extraembryonic ectoderm that also show high expression of *H19* (not shown). Expression of *Mash2* mRNA, a specific marker of diploid trophoblast cells (Guillemot et al., 1994), was then investigated. No hybridization was observed in wild-type ICM cultures. However, many of the rounded cells induced by FGF4 treatment of *Oct4* mutant cultures gave a strong signal (Figure 6D).



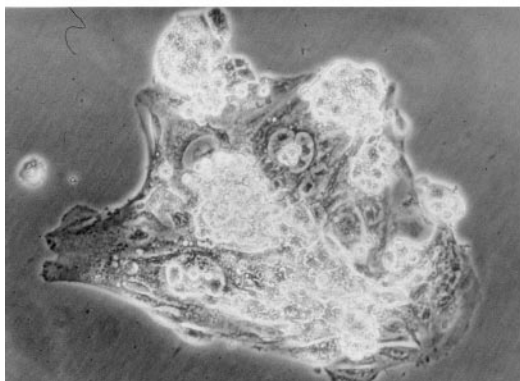
48 hrs



72 hrs



96 hrs



120 hrs

Figure 5. Response of Internal Cells from *Oct4* Mutant Blastocysts to FGF4

Immunosurgically isolated internal cells were cultured for 5 days in the presence of FGF4 (2.1 ng/ml) plus heparin and individual cultures photographed at daily intervals. Genotypes were determined by PCR analysis of trophoctoderm lysates.

Objective magnification: 48 and 72 hr, $\times 15$; 96 and 120 hr, $\times 10$.

These data demonstrate that FGF4 cannot rescue ICM pluripotency in *Oct4* mutants. Unexpectedly, however, the results indicate that FGF4 promotes the expansion of diploid trophoblast precursors from mutant embryos.

FGF4 Is a Growth/Survival Factor for Normal Diploid Trophoctoderm

The preceding observations suggested that FGF4 might be a candidate for the hitherto unidentified paracrine

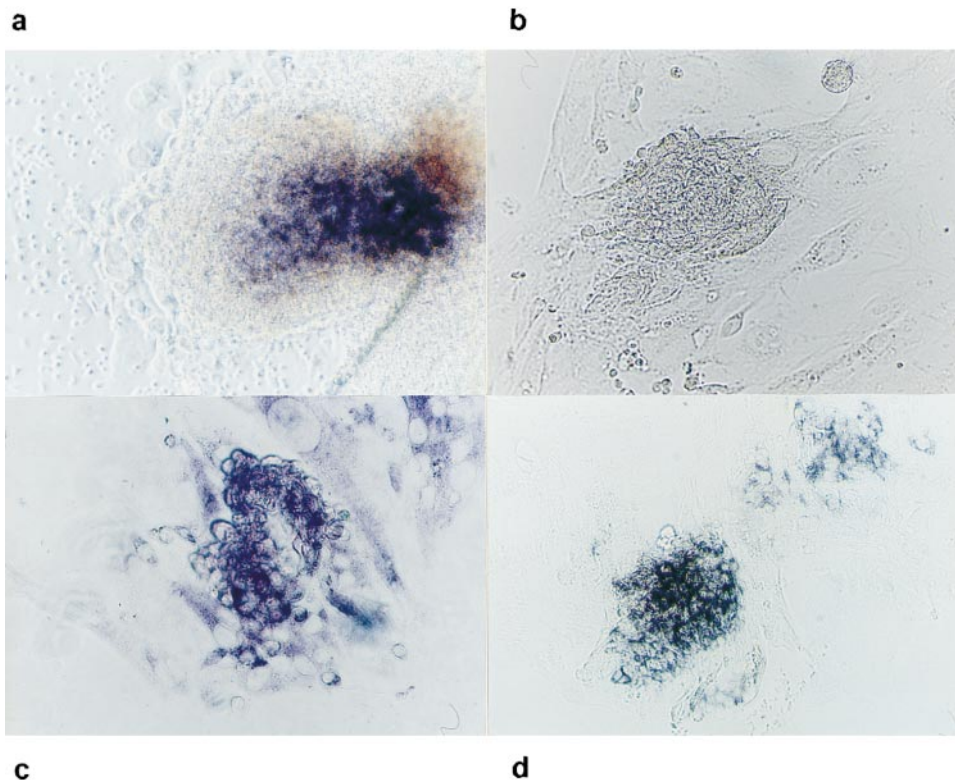


Figure 6. Expression of *H19*, *Sox2*, and *Mash2* RNAs in Outgrowths of Wild-Type and *Oct4*-Deficient Embryos Cultured in the Presence of FGF4

Immunosurgically isolated internal cells from wild-type (A) or homozygous *Oct4* mutant (B–D) 3.5-day blastocysts were cultured for 4 days in the presence of FGF4 plus heparin, then fixed and hybridized with antisense *Sox2* (A and B), *H19* (C), or *Mash2* (D) probes. Wild-type or heterozygous ICMs were positive for *Sox2* (4 out of 4), either negative (2 out of 5) or with patchy surface expression (3 out of 5) for *H19*, and negative for *Mash2* (2 out of 2). Clusters of rounded cells derived from mutant embryos were *Sox2* negative (7 out of 7), uniformly positive for *H19* (10 out of 10), and positive for *Mash2* (5 out of 6). Objective magnification: $\times 10$.

growth signal from the ICM/epiblast that is required to maintain proliferative diploid trophectoderm (Gardner, 1983). In order to investigate this further, the effect of FGF4 on wild-type trophectoderm cells was investigated. Extraembryonic ectoderms were microsurgically isolated from 5.5-day embryos and placed in culture in defined medium or in serum-containing medium in either the presence or absence of FGF4. In both conditions, a pronounced effect of FGF4 was evident (Figure 7). In the absence of serum and FGF4, extraembryonic ectoderms are viable but differentiate almost entirely into trophoblast giant cells within 3–4 days. Addition of FGF4 suppresses trophoblast differentiation and maintains or even expands the population of undifferentiated cells. In the presence of serum the findings are similar, although small clusters of undifferentiated cells do persist in the absence of FGF4 and some giant cell differentiation occurs in its presence. These findings show that FGF4 can support the maintenance of normal trophoblast precursors.

Discussion

The ICM is established at the first differentiation event in mammalian embryogenesis and is the precursor of all

fetal cell types. Although the morphogenetic processes and changes in cell physiology associated with ICM development are well characterized (Hogan et al., 1994), initiating molecular events remain obscure. In particular, no gene has previously been shown to play a specific causal role in the formation of the pluripotent stem cell lineage. The findings reported here demonstrate such a critical requirement for the POU factor Oct4.

Oct4 is abundant in the oocyte (Rosner et al., 1990), but we have previously shown that maternal protein is degraded before the end of the 2-cell stage (Palmieri et al., 1994). Consistent with this decay of maternal product, Oct4 protein was absent in 8 out of 42 cleavage and early morula stage intercross embryos examined. Nonetheless, Oct4-deficient embryos progress through cleavage and compaction to form blastocyst-like structures. Normal numbers of cells are distributed to the prospective ICM. These internal cells are viable, but they are unable to produce extraembryonic endoderm or other differentiated derivatives of the mature ICM in vivo or in vitro. Interestingly, however, they appear to retain activity of the *Oct4* promoter. β -galactosidase activity is evident in internal cells of homozygous mutant embryos at 3.5 days when it is already down-regulated in trophectoderm (Figure 1A). This suggests that they

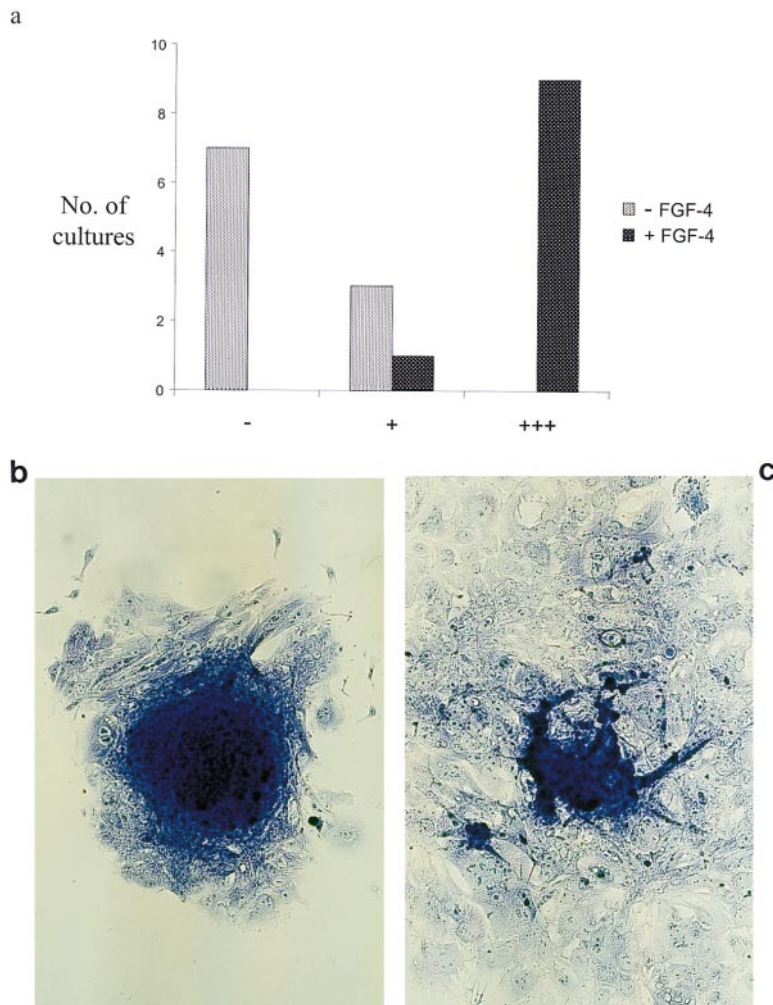


Figure 7. Maintenance of Extraembryonic Ectoderm by FGF4

Extraembryonic ectoderms were dissected from 5.5-day CBA×C57BL/6 F2 embryos and cultured for 5 days in the absence or presence of FGF4 plus heparin.

(A) Cultures maintained in the absence of serum in DMEM/F12 with N2 supplement (GIBCO) were scored for differentiation into giant cells (–) or the presence of a small (+) or large (+++) colony of morphologically undifferentiated extraembryonic ectoderm.

(B and C) Cultures maintained in serum-containing medium were fixed and stained with Leishman's reagent to discriminate faintly staining trophoblast giant cells and dark staining, undifferentiated, extraembryonic ectoderm cells. (B) Representative culture in the presence of FGF4 showing large area of undifferentiated cells and reduced number of giant cells. (C) A typical culture in the absence of FGF4 showing a large area of giant cell differentiation with a small clump of undifferentiated cells. The area of undifferentiated cells was determined from camera lucida drawings. This confirmed a statistically significant increase in the presence of FGF4 (Student t test, $P < 0.001$; sample size, 12 embryos in each group). Objective magnification: ×10.

are receptive to cues that they are inside rather than outside cells. Internal location should result in ICM development (Tarkowski and Wroblewska, 1967; Gardner, 1983), but in the absence of Oct4 the cells cannot proceed along this path. Consequently, it is possible that Oct4-deficient inner cells may transiently be in an indeterminate state. The observation that Troma-1 positivity is only expressed by 50% of mutant embryos at 3.5 days may be reflective of such a transient status of the inner cells. Even if this is the case, however, it is relatively rapidly superseded by commitment to trophoblast differentiation, as revealed by cytokeratin expression *in vivo* and overt trophoblast differentiation in culture. Thus, in the absence of Oct4, developmental potential is restricted to the trophoblast lineage. To our knowledge, this phenotype has not been described amongst the many mutants that disrupt early mouse development. Although mutations have been described that perturb the subsequent growth, morphogenesis, or differentiation of the epiblast (Haegel et al., 1995; Stephens et al., 1995; Hakem et al., 1996; Sirard et al., 1998; Waldrip et al., 1998), a specific failure to establish the ICM has not been reported before.

The inner cell population in mutant embryos is still

present at 4.5 days, as shown by immunosurgical isolation. However, the inner clumps from mutant embryos were noticeably smaller than most of those from wild-type or heterozygous sister embryos at this stage. This suggests that mutant cells exhibit reduced or even arrested proliferation, which would be consistent with their altered identity.

The failure of FGF4 to restore pluripotency to Oct4-deficient embryos is noteworthy because the *Fgf4* gene is regarded as a key Oct4 target and *Fgf4* mutant embryos die at the peri-implantation stage. This phenotype has been ascribed to a failure in ICM development, although extraembryonic endoderm clearly forms (Feldman et al., 1995). Recently, in fact, the notion of an autocrine function for FGF4 in the ICM/epiblast lineage has been thrown into question by the finding that its expression is not required for ES cell propagation (Wilder et al., 1997). Our data do not preclude a role for FGF4 in ICM expansion but do demonstrate that FGF4 is not sufficient to confer pluripotential identity in the absence of Oct4.

FGF receptors are expressed in trophoblast and extraembryonic ectoderm (Holdener et al., 1994; Rappolee et al., 1994; Arman et al., 1998), consistent with

the suggestion that FGF4 has a key function in trophectoderm development. Proximity to the ICM/epiblast is known to be required for maintenance of diploid trophoblast cells in the mouse embryo (Gardner and Johnson, 1972; Ansell and Snow, 1975), but the molecular basis of this effect has remained elusive (Gardner, 1983). We suggest that Oct4-directed secretion of FGF4 by the ICM/epiblast may constitute the signal that sustains diploid trophoblast. This same mechanism may also be used to amplify extraembryonic endoderm cells following the second lineage segregation event in preimplantation development (Rappolee et al., 1994; Arman et al., 1998). Significantly, Wilder et al. (1997) did present evidence that FGF4 functions as a paracrine regulator of differentiated cell growth in ES cell cultures.

In the developing embryo, down-regulation of *Oct4* expression correlates precisely with loss of potential to form germ cells. This has led to the notion that *Oct4* may be an essential determinant of the germ line (Pesce et al., 1998). A premature loss of *Oct4* expression in *Smad2*-deficient embryos is associated with precocious differentiation of the epiblast (Waldrip et al., 1998), further suggesting that Oct4 is an exclusive marker of pluripotency. The evidence presented here that expression of Oct4 is crucial to the establishment of pluripotential identity in nascent ICM cells and probably also for maintaining such identity in ES cells is consistent with this hypothesis.

This demonstration of a functional association with pluripotency confers on Oct4 a preeminent position in the hierarchy of transcriptional regulators of mammalian cell fate. POU factors are complex multifunctional proteins that mediate pleiotropic control of gene expression. Typically, they participate in multiple protein-protein interactions and can either activate or repress transcription, depending on context (Schöler, 1991; Anweiler et al., 1992; Cleary and Herr, 1995; Ryan and Rosenfeld, 1997; Ben-Shushan et al., 1998; Botquin et al., 1998). The pluripotent germ line-competent phenotype is likely to be defined by multiple Oct4 target genes (Saijoh et al., 1996). Evidence that Oct4 can directly repress expression of human chorionic gonadotrophin (hCG) expression (Liu and Roberts, 1996) is intriguing, as it suggests that a key aspect of Oct4 function may be to suppress expression of differentiation genes. In addition, the example of FGF4 indicates that the activity of some Oct4 targets may not be directed at the pluripotent cells themselves. Much interest will now focus on identification of the key genes downstream of Oct4 and also on defining the mechanisms that regulate the activity and expression of Oct4 itself.

Experimental Procedures

ES Cell Culture and Embryo Manipulation

CGR8 ES cells (Mountford et al., 1994) and derivatives were cultured without feeders in LIF-supplemented medium (Smith, 1991). Chimeras were produced by microinjection into C57BL/6 blastocysts (Schwartzberg et al., 1989) and crossed with outbred MF1 females. Germline chimeras were subsequently mated directly with CBA/Ca or 129/OlaHsd mice to generate hybrid inbred lines. Mice were backcrossed at least once before analysis of heterozygous intercrosses. Transmission of the deleted *Oct4* allele was monitored by

Southern hybridization with probes external to the recombination construct (Mountford et al., 1994).

All embryos were generated by natural matings. Differential cell counts were determined following immunolysis of the trophectoderm and combined propidium iodide and Hoechst staining (Handyside and Hunter, 1984). Immunosurgery was carried out as described (Solter and Knowles, 1975) with collection of trophectoderm lysates for genotype determination by PCR. Extraembryonic ectoderms were isolated from 5.5-day embryos separated from primary trophoblast and Reichert's membrane. Each embryo was bisected along the embryonic/extraembryonic junction with siliconized glass needles, the ectoplacental cone was removed, and the visceral endoderm was stripped off by drawing through a fine bore pipette.

Embryo explant cultures were carried out in ES cell derivation medium (Glasgow modification of Eagle's medium supplemented with 10^{-4} M 2-mercaptoethanol, 20% fetal calf serum, and 100 U/ml LIF) (Nichols et al., 1994), except for serum-free culture of extraembryonic ectoderm, which was performed in DMEM/F12 (50:50) with N2 supplement (GIBCO). Recombinant human FGF4, mouse FGF2, mouse EGF, and mouse CSF-1 (M-CSF) were purchased from Sigma. FGFs were used in the presence of heparin at 1 μ g/ml.

β -galactosidase expression was visualized by staining with 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) (Beddington and Robertson, 1989). *Oct4* immunostaining was carried out as described (Palmieri et al., 1994). Fluorescence confocal microscopy was performed on propidium iodide-stained specimens and images constructed using EMBL in-house software. Whole mount in situ hybridization was carried out on paraformaldehyde-fixed embryo outgrowths using digoxigenin-labeled antisense RNA probes (Rosen and Beddington, 1993).

PCR Genotyping

PCR-mediated amplification of the wild-type and mutant *Oct 4* alleles was performed using the oligonucleotide pairs 5'-TTGGGCTCCCTTCTTGCT-3'/5'-AATGGGAACAGGAAACAT-3', product 844 bp, and 5'-TGACCGCTTCTCTGTGCTTACG-3'/5'-GCCTTCCTTATAGGTTGGGCTCC-3', product 545 bp, respectively. Samples were lysed in 10–20 μ l 50 mM KCl, 2.5 mM MgCl₂, 0.1 μ g/ml gelatin, 0.45% NP40, 0.45% Tween20, 10 mM Tris.Cl (pH 8.3), with 200 μ g/ml proteinase K, and incubated at 55°C for 1 hr. Following proteinase K inactivation (95°C, 10 min), amplification was carried out on 1–7 μ l of DNA for 30 cycles of 94°C, 45 sec; 55°C, 12 sec; 72°C, 60 sec, with a final extension at 72°C for 10 min. Reaction products were resolved by agarose gel electrophoresis, transferred to nylon membrane, and hybridized to random hexamer-labeled probes derived from *Oct4* and β -*geo* coding sequences.

Indirect Immunofluorescence

Groups of immunosurgically isolated internal cells from 3.5-day and 4.5-day blastocysts were fixed in precooled methanol, washed in PBS, and incubated in Troma-1 supernatant (1/5 dilution). After rinsing through PBS, FITC-conjugated goat anti-rat Ig (Sigma) was applied and staining observed and photographed using a fluorescence microscope. Specimens were then processed individually for genotype determination by PCR.

Reverse Transcription PCR

RNA isolated from individual 3.5-day embryos using RNazol was converted into cDNA using oligo-dT and MMLV reverse transcriptase and stored at –20°C in a final volume of 20 μ l. PCR was carried out on 5 μ l aliquots in a total reaction volume of 25 μ l. Primer sets were as follows: β -actin 5'-GGCCAGAGCAAGAGAGGTA TCC-3'/5'-ACGCACGATTCCCTCTCAGC-3', product 460 bp; Oct4 5'-GGCGTTCTCTTTGAAAGGTGTTTC-3'/5'-CTCGAACCACATCC TTCT-3', product 312 bp; Fgf4 5'-AGCGAGGCGTGGTGAGCA TCTT-3'/5'-TGGTCCGCCCGTTCTTACTGAG-3', product 175 bp. Amplification was achieved by 45 cycles of 94°C, 30 sec; annealing T, 60 sec; 72°C, 60 sec, followed by a final incubation for 5 min at 72°C. Two primer sets were used in each reaction and each embryo cDNA was analyzed with all three combinations: β -actin/Oct4 (annealing at 62°C), β -actin/Fgf4 (annealing at 64°C), and Fgf4/Oct4

(annealing at 63°C). Products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. For quantitation, PCR was carried out in the presence [³²P]-dCTP and gel-separated products quantified by phosphoimage analysis.

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