Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst

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Summary
Blastocyst formation marks the segregation of the first two cell lineages in the mammalian preimplantation embryo: the inner cell mass (ICM) that will form the embryo proper and the trophectoderm (TE) that gives rise to the trophoblast lineage. Commitment to ICM lineage is attributed to the function of the two transcription factors, Oct4 (encoded by Pou5f1) and Nanog. However, a positive regulator of TE cell fate has not been described. The T-box protein eomesodermin (Eomes) and the caudal-type homeodomain protein Cdx2 are expressed in the TE, and both Eomes and Cdx2 homozygous mutant embryos die around the time of implantation. A block in early TE differentiation occurs in Eomes mutant blastocysts. However, Eomes mutant blastocysts implant, and Cdx2 and Oct4 expression are correctly restricted to the ICM TE. Blastocoeol formation initiates in Cdx2 mutants but epithelial integrity is not maintained and embryos fail to implant. Loss of Cdx2 results in failure to downregulate Oct4 and Nanog in outer cells of the blastocyst and subsequent death of those cells. Thus, Cdx2 is essential for segregation of the ICM and TE lineages at the blastocyst stage by ensuring repression of Oct4 and Nanog in the TE.

Key words: Trophoblast, Stem cells, Pou5f1, Oct4, Eomesodermin, Nanog, Mouse

Introduction
Preimplantation stages of early mammalian embryonic development lead to the formation of the blastocyst, which is composed of the trophectoderm (TE), a single epithelial layer that surrounds a fluid filled cavity (the blastocoel), and the inner cell mass (ICM). One day prior to blastocyst formation, the eight-cell embryo undergoes compaction, a morphogenetic change characterized by the flattening of blastomeres against each other and establishment of E-cadherin-dependent basolateral cell-cell adhesion. Blastomeres acquire apicobasal polarity typified by apical localization of microvilli and acquisition of cytoplasmic polarity (Fleming et al., 2001; Pratt et al., 1982; Reeve and Ziomek, 1981). In subsequent cell divisions, outer cells remain polarized and generate the TE epithelium. During blastocyst formation, the TE cells form a transporting epithelium, with functional adherens and tight junctions (Fleming et al., 2001). The activity of sodium pumps (Na+, K+-ATPases) leads to establishment of ionic gradients across the TE and accumulation of fluid in the forming blastocoel cavity (Watson, 1992). In addition, aquaporins contribute to water movements across the TE, leading to blastocoel expansion (Barcroft et al., 2003). After hatching from the zona pellucida, a subpopulation of TE cells – the mural TE cells – exhibit increased protrusive activity and undergo changes in cell polarity through relocation of proteins such as integrin α5β1 and integrin α7β1 from basal to apical domains (reviewed by Sutherland et al., 2003). At this stage, cells in the ICM have segregated into the epiblast, or embryonic lineage, and the primitive endoderm (PE), or extraembryonic endoderm lineage.

Following implantation, cells overlying the ICM – the polar TE – continue to proliferate and form the extra-embryonic ectoderm (ExE) that contains trophoblast stem (TS) cells (Tanaka et al., 1998) and the diploid ectoplacental cone (EPC), while the mural cells cease division and form trophoblast giant cells. Further differentiation of the trophoblast lineage generates the labyrinth, spongiosotrophoblast and glycogen cells of the mature chorioallantoic placenta (Rossant and Cross, 2001).

Identification of the molecular components required for the initial segregation of the TE and ICM lineages has been elusive, as few mutations have been found to cause early lineage-specific defects. Oct4 (Pou5f1), a POU domain transcription factor (TF), is required for maintenance of ICM fate and pluripotency of ES cells (Nichols et al., 1998; Niwa et al., 2000). Oct4 is expressed in all blastomeres of the cleavage stage embryo, but becomes restricted to the ICM after initiation of blastocyst formation (Palmieri et al., 1994). Homozygous mutant Oct4 embryos develop to the blastocyst stage, but their isolated ICM cells express trophectoderm markers when outgrown in vitro (Nichols et al., 1998). Furthermore, conditional repression of Oct4 in ES cells leads
to differentiation into trophoblast morphology and an increase in expression of trophoblast-specific markers (Niwa et al., 2000; Hay et al., 2004). Culturing these cells under conditions that promote trophoblast proliferation generated cells apparently equivalent to TS cells (Niwa et al., 2000).

* Nanog, a homeobox gene, is also expressed in the ICM at 3.5 days post-coitum (dpc) and becomes epiblast-specific in the implanting blastocyst. Nanog maintains ES cell pluripotency independent of LIF signalling, and in the absence of Nanog, ES cells and ICMs both differentiate into extra-embryonic endoderm (Chambers et al., 2003; Mitsu et al., 2003). Thus, Nanog has been implicated in repressing the extra-embryonic endoderm or PE fate, while Oct4 may function as a repressor of the trophoblast cell fate.

Only a few TFs show TE-specific expression at the blastocyst stage (Beck et al., 1995; Hancock et al., 1999; Luo et al., 1997; Rossant et al., 1998; Russ et al., 2000), and none so far has shown absence of TE formation when mutated (Guillemot et al., 1994; Luo et al., 1997; Russ et al., 2000). It has been proposed that the TE develops by default in the absence of Oct4 (Pesece and Scholer, 2001). However, TE differentiation begins prior to downregulation of Oct4 in the outer cells of the nascent blastocyst, suggesting that there should also be positive acting factors promoting TE fate. Cdx2, a caudal-type homeodomain TF, has been reported to be specifically expressed in TE at blastocyst stage, and expression is maintained within the proliferating ExE (Beck et al., 1995). Heterozygous Cdx2 mutants show homeotic transformation defects and homozygous mutants die at the peri-implantation stage (Chawengsaksophak et al., 1997; Tamai et al., 1999). Eomesodermin (Eomes), a T-box TF, is also expressed specifically in the TE at the blastocyst stage, and, like Cdx2, is expressed at later stages in the ExE (Ciruna and Rossant, 1999; Hancock et al., 1999; Russ et al., 2000). *Eomes* mutants have also been reported to show early defects in trophoblast proliferation (Russ et al., 2000).

In this paper, we compare the *Cdx2*−/− and *Eomes*−/− mutant phenotypes in more detail, and show that *Cdx2* mutant blastocysts fail to maintain trophoblast differentiation and fail to implant. Interestingly, loss of *Cdx2* is associated with failure to downregulate Oct4 and Nanog in outer cells of the blastocyst and results in subsequent death of outer cells. By contrast, *Eomes* mutants form blastocysts, display ICM-restricted Oct4 expression and TE-specific *Cdx2* expression, but trophoblast does not differentiate further. Thus, *Cdx2* is the earliest TF identified so far to be involved in specification of TE fate, and *Cdx2* is required for repression of Oct4/Nanog and normal blastocyst development.

### Materials and methods

#### Targeting and inactivation of Eomesodermin gene

Mouse *Eomes* genomic clones were isolated from a strain 129/sv genomic library. To generate the deletion/replacement knockout construct, a 3.4 kb *EcoRI* fragment encompassing exons 1 and 2 was used as left homologous arm, and a 2.1 kb *HindIII/XbaI* fragment containing 3′ UTR as right arm (as shown in Fig. 8A). Electroporated R1 ES cell clones were doubly selected with G418 and ganciclovir, and screened for homologous recombination by Southern blot using a 500 bp 5′ external probe and verified by a 3′ internal probe (not shown). Two positive clones were aggregated with ICR morulae to produce heterozygotes. Heterozygous knockout mice were maintained in either a pure 129 or a 129/ICR mixed background; no difference in phenotype was observed.

#### Mouse breeding, embryo collection and genotyping

Mice heterozygous for the *Cdx2* targeted mutation *Cdx2*tm1Fbe (MGI:1857928) (Chawengsaksophak et al., 1997) were maintained by crossing to outbred ICR strain mice. Embryos were collected from intercrossed *Cdx2* or *Eomes* heterozygotes. Genotyping was performed on individually isolated embryos directly or after observation in culture or following in situ hybridization or antibody staining. All genotyping was performed blind by PCR with primers that produced both mutant and wild-type bands, thus ensuring unequivocal identification of genotypes. The conditions of PCR genotyping of mice and embryos for *Cdx2* targeted mutation *Cdx2*tm1Fbe were as previously described (Chawengsaksophak et al., 1997). *Eomes* targeted mutants were genotyped using the following primers (see also Fig. 8A): primer A, (forward primer) 5′-GAAGAGCCGCTGTCTCCACGCCC-3′; primer B, (reverse primer) 5′-AACACTCTCGTGTCCTCTCCAGTCAGC-3′; primer C, (Neo′ forward primer) 5′-CAGGCTCATCGCCCTTCATGCCGC-3′; A 440 bp amplification product is generated from the mutant allele between primer A and B; a 350 bp product is amplified from the wild-type allele between primers A and B.

#### RNA preparation and RT-PCR

RNA was isolated from single embryos as described (Chomczynski and Sacchi, 1987). cDNA was reverse transcribed using Superscript II reverse transcriptase according to manufacturer (Invitrogen). cDNA was diluted 1/10 for semi-quantitative PCR. Minimum number of cycles required for amplification by a given primer set was determined on cdna from single wild-type RNA preparation under the experimental conditions tested. Primers used were as follows: β-actin, (forward) 5′-gccccagcaacagagatctact-3′ and (reverse) 5′-aagcaaggatctctcag-3′ (30 cycles; product size 460 bp); *Cdx2*, (forward) 5′-gcctctgagagccacagttg3′ and (reverse) 5′-ctctcggagagcccaagtgtg-3′ (35 cycles; product size 162 bp); *Fgf2*, (forward) 5′-gagaaggacacagtcaacc-3′ and (reverse) 5′-ctgccctgaagagacagcc-3′ (40 cycles; product size 217 bp); *Hand1*, (forward) 5′-atgaacggctggggta-3′ and (reverse) 5′-tcctgtggattagctgccac-3′ (40 cycles; product size 550 bp); *Eomes*, (forward) 5′-gctactcaggagttgggag-3′ and (reverse) 5′-ggagggcctgtctctg-3′ (35 cycles; products sizes 350 bp and 340 bp); PIIL (Csh1 – Mouse Genome Informatics) (forward) 5′-atcttcgaaatgcacgtcag-3′ and (reverse) 5′-gacattcgcccagttgc-3′ (40 cycles; product size 336 bp).

#### In situ hybridization

Fluorescent in situ hybridization using digoxigenin- or FITC-labelled RNA probes was performed according to protocol available on the Rossant laboratory website: http://www.mshri.on.ca/rossant/protocols/doubleFluor.html. RNA antisense probes were in vitro transcribed from the following mouse cDNA templates: *Cdx2* (939 bp; Dr Peter Traber, PA); *Oct4* (1336 bp; Dr Hitoshi Niwa, Japan); *Nanog* (981 bp; Dr Austin Smith, UK).

#### Immunohistochemistry

Immunohistochemistry protocols can be found on the Rossant laboratory website (http://www.mshri.on.ca/rossant/protocols/immmunoStain.html). The following antibodies were used at the following dilutions: affinity-purified polyclonal rabbit anti-Cdx2 C-term and CNL (gift of Dr Edmond Rings) (Rings et al., 2001) 1:500-1:1000 of 0.6 mg immunoglobulin/ml [specificity of anti-Cdx2 CNL was confirmed in blocking experiments as described by Silberg et al. (Silberg et al., 2000)]; monoclonal anti-Cdx2 (CDX2-88, BioGenex, CA, USA) 1:200; monoclonal mouse anti-Oct4 (C10; Santa Cruz Biotechnology) 1:100; rabbit anti-Nanog (Mitsu et al., 2003; Dr Yamanaka, NAIST, Japan) 1:400; monoclonal rat anti-integrin α7.
Development

Incubator XL, Heating Insert P and CO2 controller. Temperature and imaged using the Zeiss Axiovert 200 inverted microscope with glass-bottom dishes (MatTek, USA) overlaid with light mineral oil microscopy, embryos were collected and cultured in KSOM-AA in 1:400-1:1000 dilution with 10-20 µg/ml in PBS) or laminin (Sigma; 25 µg/ml in PBS). Outgrowth containing 20% FCS in four-well tissue culture dishes (Nunc, Denmark) untreated or pre-coated with 0.1% gelatin (Sigma), fibronectin (from bovine plasma, Sigma; 20 µg/ml in PBS) or laminin (Sigma; 25 µg/ml in PBS). Outgrowth formation was monitored over 72-120 hours. For digital time-lapse counting multiple stacked optical sections.

Embryo culture

To follow development in vitro, eight-cell stage embryos were flushed from oviducts in M2, treated with acidic Tyrode’s to remove zonae pellucidae (Nagy et al., 2003). The embryos were then cultured in microdrops of KSOM-AA under mineral oil for 48 hours at 37°C, 5% CO2 and transferred into microdrops of RPMI 1640 containing 0.1% BSA and 100 µM non-essential amino acids for an additional 24 hours. For trophoblast outgrowth formation, 3.5 dpc blastocysts from heterozygous Cdx2 or Eomes intercrosses were individually cultured in KSOM-AA overnight and then transferred into RPMI 1640 containing 20% FCS in four-well tissue culture dishes (Nunc, Denmark) untreated or pre-coated with 0.1% gelatin (Sigma), fibronectin (from bovine plasma, Sigma; 20 µg/ml in PBS) or laminin (Sigma; 25 µg/ml in PBS). Outgrowth formation was monitored over 72-120 hours. For digital time-lapse microscopy, embryos were collected and cultured in KSOM-AA in glass-bottom dishes (MatTek, USA) overlaid with light mineral oil and imaged using the Zeiss Axiovert 200 inverted microscope with Incubator XL, Heating Insert P and CO2 controller. Temperature and CO2 were set to 37.5°C and 5.5%, respectively. DIC images were recorded, with halogen lamp voltage (<2.5 V), every 30 minutes using AxioCam MRm with Axiovision 3.1 software.

TS cell culture and derivation

Derivation of trophoblast stem (TS) cell lines from blastocysts from heterozygous Cdx2 or Eomes intercrosses was performed as previously described (Tanaka et al., 1998) and as detailed at http://www.mshri.on.ca/rossant/protocols/TScells.html. Genotyping of cell lines was performed by PCR (for Cdx2) or confirmed by Southern analysis (for Eomes).

TUNEL staining

Eight-cell embryos were cultured in KSOM-AA for 48 hours, then fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature, and washed in PBS+0.1% Tween 20, incubated in TUNEL reaction mixture (Roche) for 60 minutes at 37°C and washed as above. The total number of FITC-labelled/TUNEL-positive nuclei in each embryo was scored as well as their distribution in the ICM or TE by counting multiple stacked optical sections.

Results

Cdx2 expression is detected in the trophectoderm prior to implantation

Cdx2 expression was previously reported in the TE of the blastocyst and the trophoblast lineage during post-implantation stages (Beck et al., 1995). To gain better insight into the role of Cdx2 in early development of the trophoblast lineage, we followed the spatial and temporal dynamics of Cdx2 expression from the early morula stage to 5.5 dpc (Fig. 1). By RT-PCR analysis, Cdx2 mRNA was first detected at the eight-cell stage (not shown), and Cdx2 protein was first clearly detected in the nucleus and cytoplasm of the outer cells of early morula (~16 cells, n=4; Fig. 1A,B) and late morula stages (~25 cells, n=4; Fig. 1C,D). Some low level of expression was seen cytoplasmically throughout the rest of the embryo. Interestingly, at these stages Oct4 protein is present equally in...
all cells of the embryo, and thus is co-expressed with Cdx2 in the outer cells (Fig. 1D). At the early blastocyst stage, Cdx2 expression was more restricted to the outer cell layer (3.5 dpc, \( n=2 \); Fig. 1E). By the expanded blastocyst stage, Cdx2 protein was exclusively localized to the TE nuclei (3.5 dpc, \( n=4 \); Fig. 1F). At 4.5 dpc, by the onset of implantation, Cdx2 protein levels appeared to be reduced in the mural TE, but maintained in the polar TE cells directly overlying the ICM (\( n=3 \); Fig. 1G). At later stages, Cdx2 expression became confined to the polar TE (nascent ExE) cells directly overlying the epiblast (4.75 dpc, \( n=2 \); Fig. 1H). By 5.5 dpc both epiblast and ExE have expanded, and intense Cdx2 expression spanned the first three or four cell rows in the ExE adjacent to the epiblast (\( n=2 \); Fig. 1I). Cdx2 expression is thus associated with the initiation of TE fate, and later specifically delineates the cells of the trophoblast lineage that give rise to TS cells in the peri-implantation embryo (Corson et al., 2003; Tanaka et al., 1998).

A similar expression profile was found for Cdx2 mRNA (see Fig. S1 in the supplementary material).

To determine whether there is any maternal Cdx2 protein in the early embryo, we examined Cdx2+/– embryos at early morulae and blastocyst stages. There was no nuclear Cdx2 protein detectable in Cdx2 mutants at either stage (4 mutant morulae, 5 mutant blastocysts; Fig. 1K,M), compared with prominent nuclear expression in Cdx2+/+ embryos (Fig. 1O). Some low level fluorescence was observable in the cytoplasm of Cdx2+/– embryos but whether this is real signal or background staining is unclear.


**Cdx2+/– blastocysts fail to maintain blastocoel**

Previous analysis revealed that Cdx2 homozygous mutant embryos die around the time of implantation. Dissection and genotyping of post-implantation stages revealed neither embryos die around the time of implantation. Dissection and genotyping of post-implantation stages revealed neither evidence of Cdx2+/– mutants nor any empty deciduae, which is indicative of death prior to implantation (Chawengsaksophak et al., 1997). We examined the morphology of embryos from Cdx2+/– intercrosses by dissection from the uterus at 4.5 dpc. Cdx2+/– or Cdx2+/+ blastocysts were recovered and were fully expanded and hatched from their zonae pellucida by this stage (\( n=10 \)). By contrast, Cdx2+/– embryos recovered were still enclosed in their zonae, and had little or no blastocoelic cavity (\( n=4 \)). Blastocyst formation was monitored by culturing zona-free eight-cell stage embryos from Cdx2+/– intercrosses over a time course of 72 hours (Fig. 2). Between 24 and 48 hours, all embryos had a blastocoe. However, by 72 hours all Cdx2 mutants (\( n=4 \)) showed no blastocoelic cavity and surface morphology was rough. Live imaging of litters from Cdx2+/– intercrosses revealed that the blastocoe of Cdx2 mutants initially expanded, but began to collapse around the time that non-mutant littersmates hatch from the zona (three mutants, 18 non-mutants; see Movie 1 in the supplementary material). This collapse was accompanied by a morphological change in the TE, as cells acquired a rounded, non-epithelial appearance.

**The trophoderm of Cdx2 homozygous mutant blastocysts fails to maintain epithelial integrity**

To evaluate the degree of TE differentiation in Cdx2+/– embryos, we examined the expression of TE-specific markers. One of the characteristics of the TE epithelium is the formation of tight junctions between the TE cells, which facilitate the maintenance of the blastocoel cavity. As Cdx2 homozygous mutants fail to maintain an expanded blastocoe, we examined the expression of the tight junction and adherens junction components in Cdx2 mutant embryos during development. ZO-1α– and ZO-1α+ (encoded by Tjp1) incorporate into tight junctions early and late during TE formation, respectively (Sheth et al., 1997). E-cadherin localization to the basolateral adherens junctions precedes tight junction formation (reviewed by Fleming et al., 2000; Fleming et al., 2001). Both tight and adherens junctions appeared grossly normal in early blastocysts of both mutant and non-mutant genotype, as assessed by expression of ZO-1α+ (four mutants, 15 non-mutants; Fig. 3A,B), ZO-1α– (six mutants, 21 non-mutants; not shown), and E-cadherin (10 mutants, 46 non-mutants; Fig. 3C,D). By contrast, both tight and adherens junctions appeared abnormal in most mutants by the late blastocyst stage. In particular, ZO-1α+ appeared patchy or diffuse compared with littermates (7/8 mutants; 12 non-mutants; Fig. 3E,F), while ZO-1α– was diffuse (3/3 mutants, 5 non-mutants; not shown). E-cadherin expression was still observed in Cdx2+/– blastocysts but appeared to be mislocalized basally in some cells of the TE (6/6 mutants, 13 non-mutants; Fig. 3G,H). These observations suggest that the polarity and integrity of the TE epithelium, while initially normal, is not maintained in the absence of zygotic Cdx2.

Consistent with these observations, the later polarity marker integrin α7 (Itga7) (Klaffky et al., 2001) was undetectable in abnormal, collapsed Cdx2 mutant at 4.5 dpc (Fig. 4E). Interestingly, Oct4 appeared to be expressed in all cells of Cdx2+/– embryos at this stage (Fig. 4E; see below).

**Fig. 2.** Cultured Cdx2+/– embryos fail to form an expanded blastocyst. Bright-field images of Cdx2+/+ embryos (A-D) and Cdx2+/– embryos (E-I). Eight-cell embryos from Cdx2+/– intercrosses were individually cultured, monitored for 72 hours, and images recorded at (A,E) 6 hours; (B,F) 24 hours; (G) 30 hours; (C,H) 48 hours; and (D,I) 72 hours. Scale bars: 25 µm.
Expression of TE markers is compromised in Cdx2−/− embryos

To investigate the fate of TE cells in Cdx2−/− embryos, we followed the expression of additional TE markers by a semi-quantitative RT-PCR analysis from individual in vitro cultured blastocysts from Cdx2+/− intercrosses. Analysis was performed on embryos prior to culture (3.5 dpc), at 24 hours and 72 hours of culture (Fig. 4J).

Cdx2−/− embryos expressed the TE markers, Eomes and Fgfr2 (Haffner-Krausz et al., 1999; Russ et al., 2000), although the expression levels of Eomes were markedly lower in Cdx2−/− presumptive null embryos than in littermates. The expression of Hand1 and Pl1 (Csh – Mouse Genome Informatics) (Cross et al., 1995; Faria et al., 1991), markers of differentiated trophoblast giant cells, was undetectable in Cdx2−/− embryos, although these markers were detected in Cdx2+/+ and Cdx2+/− embryos (Fig. 4J). Altogether, TE-specific marker analysis indicated that, although blastocyst formation begins in Cdx2+/− embryos, trophoblast
differentiation is compromised beyond the expanded blastocyst stage.

**Cdx2 homozygous mutants fail to form trophoblast giant cells or TS cell lines in vitro**

To address whether, under in vitro culture conditions, the TE of Cdx2+/– embryos could be promoted to differentiate, zona-free 3.5 dpc blastocysts from Cdx2+/– intercrosses were cultured in serum-containing medium or specific extracellular matrix substrates (Fig. 4G-I). Although Cdx2+/– or Cdx2+/+ blastocysts attached and initiated TE outgrowth within 24-36 hours after plating in serum-containing medium, Cdx2–/– blastocysts failed to attach regardless of the extracellular matrix substrate used (fibronectin, gelatin, or laminin; see Table 1). By 72 hours of culture, Cdx2–/– or Cdx2+/– blastocysts formed both ICM and trophoblast outgrowths, the latter containing trophoblast giant cells with typical large nuclei and cytoplasm (Fig. 4G). By contrast, Cdx2+/– embryos showed no attachment and giant cell outgrowth, with only occasional parietal endoderm-like cells attached to the substrate. Embryos survived and grew into structures resembling embryoid bodies (Fig. 4H,I). Indeed, ES cells can be derived from Cdx2–/– blastocysts (Chawengsaksophak et al., 2004), indicating that ICM development is not affected in these embryos. When we attempted to derive TS cell lines (Tanaka et al., 1998) from blastocysts obtained from Cdx2–/– blastocysts, including the outer cells of 3.5 dpc blastocysts (2/2 mutants, 4 non-mutants; Fig. 5C,D). It has previously been reported that Nanog is expressed only in inside cells of the late morula and then the ICM (Chambers et al., 2003; Mitsui et al., 2003). If true, this would suggest that loss of Cdx2 leads to ectopic activation of Nanog in outside cells. Using antibody to detect Nanog protein, we found detectable levels of nuclear Nanog in all cells of ~16-cell (n=13) and ~32-cell (n=8) Cdx2+/+ and Cdx2–/– embryos (Fig. 6A; not shown). Nanog was restricted to the ICM of Cdx2+/+ and Cdx2–/– embryos by the early blastocyst stage (n=10; Fig. 6C). At this stage, Nanog was detected at equivalent levels in TE and ICM cells in Cdx2–/– embryos (n=5), (Fig. 6C,D). However, no difference in Nanog expression was observed between non-mutant and Cdx2–/– embryos (n=3) at earlier stages (Fig. 6A,B and not shown). These results indicate that Cdx2 is also required to ensure downregulation of Nanog, as well as Oct4, in outer cells beginning around the early blastocyst stage.

**Increased incidence of programmed cell death in Cdx2–/– embryos at the expanded blastocyst stage**

As mentioned above, the periphery of the 4.5 dpc Cdx2–/– embryos showed a rough appearance with cells delaminating from the embryonic mass surface. Moreover, nuclear staining revealed fragmented, pyknotic nuclei, suggestive of cell death. No such pyknotic nuclei were observed at earlier stages and cell numbers of mutant versus wild-type embryos were similar (not shown). To determine whether cell death is increased in Cdx2–/– embryos, we cultured zona-free eight-cell stage embryos from Cdx2–/– intercrosses, and monitored the degree of cell death by a TUNEL assay after 48 hours of culture (Fig. 7). In Cdx2+/+ or Cdx2+/– embryos (n=16; Fig. 7A) cell death was limited and almost all TUNEL-positive nuclei were within the ICM (Fig. 7C). By contrast, in Cdx2–/– embryos (n=7; Fig. 7B,B’) a significantly higher number of TUNEL-positive nuclei was detected, with an increase of three-fold and eight-

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**Table 1. Cdx2+/– blastocysts plated on different substrata fail to attach and form a trophoblast outgrowth**

<table>
<thead>
<tr>
<th>ECM substrate</th>
<th>+/-</th>
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<tbody>
<tr>
<td>Fibronectin</td>
<td>43/43</td>
<td>75/75</td>
<td>0/29(1)</td>
</tr>
<tr>
<td>Gelatin</td>
<td>5/5</td>
<td>10/10</td>
<td>0/4(1)</td>
</tr>
<tr>
<td>Laminin</td>
<td>12/12</td>
<td>13/13</td>
<td>0/7</td>
</tr>
<tr>
<td>No substrate</td>
<td>2/2</td>
<td>10/10</td>
<td>0/3</td>
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Blastocysts from Cdx2–/– heterozygous intercrosses were plated in serum-containing medium on uncoated tissue culture dishes or pre-coated with fibronectin, gelatin or laminin (see Materials and methods).

*For each substrate and genotype, the number of formed outgrowths/plated embryos is indicated.

†None of the Cdx2–/– embryos formed an outgrowth; number in parentheses indicates number of embryos showing attached parietal endoderm cells.
Development of trophoblast differentiation in Eomes–/– embryos, can induce a decidual response. To evaluate the degree of integrin α implantation was compromised, as measured by the expression of TE-specific markers (Fig. 8). Embryos from Eomes+/– and Cdx2+/– intercrosses were assayed for trophoblast outgrowth formation (Fig. 8E,I). As previously reported (Russ et al., 2000), Eomes+/– blastocysts failed to attach and form a TE outgrowth (Fig. 8I). Morphologically we observed that the Eomes+/– blastocysts remained as fully expanded blastocysts after 96 hours of culture, unlike Cdx2+/– embryos, which eventually collapse. We examined the expression of additional TE markers by a semi-quantitative RT-PCR analysis from individual in vitro cultured embryos following TUNEL staining. The values plotted represent the mean number of TUNEL-positive nuclei (±s.e.m.). The increase in the number of TUNEL-positive nuclei in the ICM and TE in Cdx2–/– embryos compared with Cdx2+/– or Cdx2+/+ embryos was significant at P≤0.001 using Student’s t-test analysis.

**Eomes is required later than Cdx2 in the trophoblast lineage**

**Eomes** encodes a T-box TF and is expressed early in the trophoblast lineage (Ciruna and Rossant, 1999; Russ et al., 2000), including the TE of the blastocyst stage (Hancock et al., 1999). Previous analysis of Eomes knockout embryos has shown that Eomes−/− embryos survive to 6.5-7.5 dpc in utero, but display a blastocyst-like morphology and fail to form trophoblast outgrowths in vitro (Russ et al., 2000). However, the exact stage of trophoblast development affected by loss of Eomes was not clear. We generated a targeted allele of Eomes (Fig. 8A) and confirmed that blastocyst-like embryos could be identified in a quarter of the deciduae from Eomes−/+ intercrosses (Fig. 8D-H). Thus, Eomes mutants, unlike Cdx2 mutants, can induce a decidual response. To evaluate the degree of TE differentiation in Eomes−/− embryos we examined the expression of TE-specific markers (Fig. 8). Embryos from Eomes−/+ intercrosses were dissected at 4.5-4.75 dpc and Oct4 and Cdx2 expression analyzed. TE-specific expression of Cdx2 and ICM-restricted expression of Oct4 were observed in Eomes−/+ embryos (n=2), comparable with the expression pattern in Eomes−/+ and Eomes−/+ littermates (n=3; Fig. 8B,F). However, despite the relatively normal appearance of the Eomes mutant blastocysts, TE differentiation at the time of implantation was compromised, as measured by the expression of integrin α7, which was undetectable in the Eomes−/− blastocysts (two mutants, three non-mutants; Fig. 8C,G).

To determine whether the TE of Eomes−/− embryos can differentiate in vitro, blastocysts from Eomes−/+ intercrosses were assayed for trophoblast outgrowth formation (Fig. 8E,I). As previously reported (Russ et al., 2000), Eomes−/+ blastocysts failed to attach and form a TE outgrowth (Fig. 8I). Morphologically we observed that the Eomes−/− blastocysts remained as fully expanded blastocysts after 96 hours of culture, unlike Cdx2+/− embryos, which eventually collapse. We examined the expression of additional TE markers by a semi-quantitative RT-PCR analysis from individual in vitro cultured blastocysts from Eomes−/+ intercrosses, as described above for Cdx2 analysis. Analysis was performed on embryos at 24 hours, 96 hours and 120 hours of culture (Fig. 8I). The analysis indicated that in the absence of Eomes, Cdx2 and Fgfr2 are still expressed (Beck et al., 1995; Haffner-Krausz et al., 1999). However, the expression of Hand1 and Pl1 (Cross et al., 1995; Faria et al., 1991), markers of differentiated trophoblast giant cells, was undetectable in Eomes presumptive null embryos (Fig. 8J). We also attempted to derive TS cell lines from blastocysts obtained from Eomes−/+ intercrosses (Tanaka et al., 1998). Twenty-one TS cell lines were derived from a total of 36 blastocysts initially cultured. However, none was Eomes−/+ by genotype.
Cdx2 is required for the development of a functional trophoderm

Cdx2−/− blastocysts cavitate normally, around the same time as non-mutant littermates, and are initially able to expand. However, the expanded blastocoele is not maintained, embryos fail to hatch from the zona pellucida and mutants eventually collapse into a ball of cells. Cdx2 mutants fail to implant in vivo or form a trophoblast giant cell outgrowth when cultured without their zona pellucida. Analysis of epithelial markers, such as tight and adherens junction components, suggests that polarized epithelial integrity of the outer cells is initially established, but fails to be maintained by the late blastocyst stage in Cdx2−/− mutants, suggestive of a block in further TE differentiation and maintenance.

Examination of markers of TE differentiation in non-attached mutant embryos revealed no expression of Hand1 and Pl1, markers of trophoblast giant cells. The trophoderm differentiation marker Pl1 can be detected in cultured wild-type blastocysts, even if they do not form outgrowths (Nieder and Nagy, 1991). Thus, the failure of cultured Cdx2−/− blastocysts to express Pl1 is not secondary to outgrowth failure but represents a block in trophoderm differentiation. Expression of the trophoblast stem cell marker, Eomes, was also strongly reduced and TS cell lines could not be derived from Cdx2−/− embryos, suggesting a block in TS cell formation or self-renewal. As both stem cell and giant cell fate are also strongly reduced and TS cell lines could not be derived from Cdx2−/− embryos, suggesting a block in TS cell formation or self-renewal. As both stem cell and giant cell fate are blocked by absence of Cdx2, this TF must play a key role in early maintenance of the integrity and function of the blastocyst TE.

Cdx2 is required for lineage-restricted expression of Oct4 and Nanog

In Cdx2−/− embryos, Oct4 and Nanog are not downregulated in outer cells, and persist in these cells even by 4.5 dpc. Thus, it appears that Cdx2 plays a primary role in specifying the fate of the trophoderm cells by restricting the expression of Oct4 and Nanog to the ICM. One explanation for the failure of TE development in Cdx2−/− mutants could then be that the outside cells initiate blastocyst epithelium formation but do not properly specify TE fate. Retention of pluripotency-associated markers in the outer cells might indicate transformation of outder cells to a more ICM-like phenotype. However, it is not clear whether, in the absence of Cdx2, outside cells are actually converted to ICM cells. Rather, increased levels of TUNEL staining in the outer cells of Cdx2 mutants suggest that the misexpression of Oct4 and Nanog is incompatible with
maintenance of the TE phenotype, leading to subsequent cell death. The remaining ICM cells in 4.5 dpc Cdx2−/− blastocysts can continue to develop into embryoid body type structures with distinct epiblast and primitive endoderm layers. Moreover, it is possible to generate Cdx2−/− ES cells and to derive early somite embryos from them (Chawengsaksophak et al., 2004).

Cdx2 expression marks TE precursors prior to blastocyst formation

Cdx2 expression is restricted to prospective TE cells prior to restriction of Oct4/Nanog to the ICM, consistent with a role in downregulation of Oct4/Nanog in the TE. The timing and expression domains of Cdx2 and Oct4/Nanog in normal embryos, and the upregulation of Oct4/Nanog in Cdx2 mutants might suggest that Cdx2 restriction to outer cells is the primary driver of the divergence of ICM and TE lineages. Consistent with this, Niwa et al. show that overexpression of Cdx2 is sufficient to drive differentiation of ES cells into trophoblast cells, but Cdx2 is not necessary for trophoblast differentiation if Oct4 is directly downregulated (H. Niwa, unpublished).

Early segregation of Cdx2 to the outer cells of the morula and early blastocyst may thus be key for initiating TE/ICM specification. Whether Cdx2 can directly regulate Oct4/Nanog at the transcriptional level or acts post-transcriptionally is currently unknown. What drives the segregation of Cdx2 to the outside cells also remains to be determined, although the similar restricted expression pattern of Fgf2 in the outside cells (Haffner-Krausz et al., 1999) and the known role for this signalling pathway in promoting TE development (Chai et al., 1998) suggests that FGF signalling may be important upstream of Cdx2.

Why does blastocoel initiate in the absence of Cdx2?

Although Cdx2 has a pivotal role in the development of a fully functional TE and downregulation of the pluripotency-associated genes, blastocoel initiation does not require zygotic Cdx2. Nor does it seem that TE initiation can be explained by associated genes, blastocoel initiation does not require zygotic Cdx2. If this is the case, is not necessary for trophoblast differentiation if Oct4 is directly downregulated (H. Niwa, unpublished).

Eomes expression in the TE of the blastocyst, and persists in the proximal region of the ExE in early post-implantation stages. Similarity of expression with Cdx2 might suggest overlapping functions. However, we show that both phenotypic and expression analysis place Cdx2 upstream of zygotic Eomes in TE development and make it unlikely that the two genes are acting redundantly in the initial specification of the TE epithelium. Unlike Cdx2 mutant embryos, Eomes−/− blastocysts cause a decidual reaction and show full expansion and maintenance of the blastocoel in vitro, indicative of a functional TE (Russ et al., 2000) (this study). Cdx2 is expressed normally and Oct4 expression is segregated to the ICM, indicating that Eomes is not required for initial ICM/TE separation. However, neither trophoblast giant cells nor TS cells can develop from Eomes mutants and markers of TE differentiation are lost. This does not exclude overlapping functions for the two genes later in TS cell development, as may be indicated by the failure to obtain TS cells in both cases. Elucidation of their later roles will require timed conditional inactivation studies.

In conclusion, our studies have uncovered that Cdx2 and Eomes are key TFs required at distinct stages during early TE lineage development. Eomes is required for TE differentiation and proliferation beyond the expanded blastocyst stage, while Cdx2 is the earliest TE-specific TF essential for TE function and establishment of the trophoblast lineage, as well as for the lineage restricted expression of the pluripotency markers, Oct4 and Nanog. Therefore, cell fate specification in the preimplantation embryo relies on positive acting TFs in both the ICM and TE lineages. Elucidation of the regulatory mechanisms that underlie the restricted expression and feedback loops between these TFs during morula stages should shed light on how cell fate specification is initiated in the preimplantation embryo.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/9/2093/DC1

References


