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. Blastocoel 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 relocalization of proteins such as integrin $\alpha5\beta1$ and integrin $\alpha7\beta1$ 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 extra-embryonic 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, spongiotrophoblast 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; Mitsui 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 (Pesce 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 *Eco*RI fragment encompassing exons 1 and 2 was used as left homologous arm, and a 2.1 kb *Hind*III/*Xba*I fragment containing 3′ UTR as right arm (as shown in Fig. 8A). Electroporated R1 ES cell clones were doubly selected with G418 and gancylovir, 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 generate chimaeric mice, which were then mated with 129 females 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′-GAAAGCGCCTGTCTCCAGCACCC-3′; primer B, (reverse primer) 5′-AACACTCCTGCGTCCTCCAGTCAC-3′; primer C, (Neo^r forward primer) 5′-CAGCGCATCGCCTCTATCCGCC-3′. A 440 bp amplification product is generated from the mutant allele between primer C 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 whole-embryo RNA preparation cultured under the experimental conditions tested.

Primers used were as follows: *β-actin*, (forward) 5′-ggcccagagcaagagaggtatcc-3′ and (reverse) 5′-acgcacgatttccctctcagc-3′ (30 cycles; product size 460 bp); *Cdx2*, (forward) 5′-gcagtccctaggaagccaagtga-3′ and (reverse) 5′-ctctcggagagcccaagtgtg-3′ (35 cycles; product size 162 bp); *Fgfr2*, (forward) 5′-gacaagcccaccaactgcacc-3′ and (reverse) 5′-cgtcccctgaagaacaagagc-3′ (40 cycles; product size 217 bp); *Hand1*, (forward) 5′-atgaacctcgtgggcaggta-3′ and (reverse) 5′-tcactggttagctccagcg-3′ (40 cycles; product size 550 bp); *Eomes*, (forward) 5′-gtgacagagacggtgtggagg-3′ and (reverse) 5′-agaggaggccgttggtctgtgg-3′ (35 cycles; products sizes 350 bp and 304 bp); *Pl1* (Csh1 – Mouse Genome Informatics) (forward) 5′-atcttctcagaaatgcagctg-3′ and (reverse) 5′-gatcattgctttcagaaggtc-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/immunoStain.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 (Mitsui et al., 2003; Dr Yamanaka, NAIST, Japan) 1:400; monoclonal rat anti-integrin α7

(undiluted CA5; gift of Dr Ann Sutherland, University of Virginia) (Klaffky et al., 2001); rabbit anti-mouse ZO-1α+ 1:250; rabbit anti-ZO-1α- at 1:250 (both gifts of Dr Bhavwanti Sheth, UK); and rat anti-E-cadherin (Sigma) 1:500. Secondary antibodies (Cy3 or Cy5-donkey anti-mouse; biotin or Cy3-donkey anti-rabbit; Cy5-donkey anti-rat (Jackson ImmunoResearch Laboratories) were used at 1:300-1:400, and Cy5-streptavidin at 1:1000. To visualize nuclei, embryos were incubated in YOYO-1 or YOYO-3 (Molecular Probes) at 1:400-1:1000 dilution with 10-20 μg/ml RNAse A, for 15-30 minutes at room temperature.

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% CO_2 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 CO_2 controller. Temperature and CO_2 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.

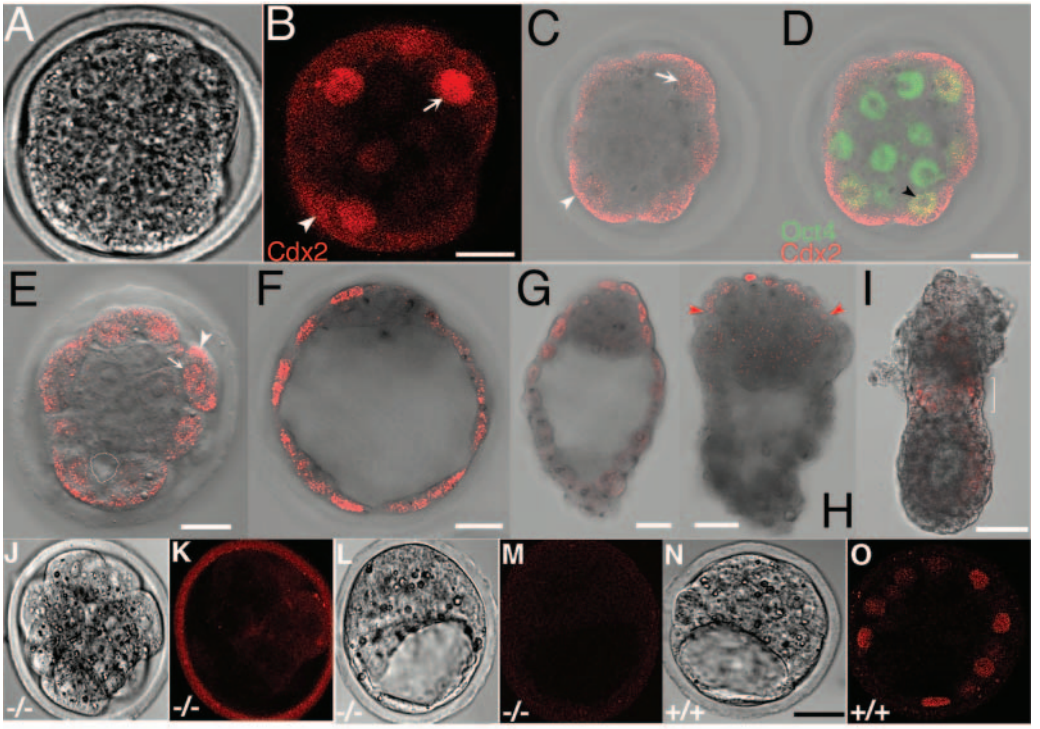

Fig. 1. Cdx2 is expressed in cells of the TE lineage. (A) DIC image of late morula stage embryo (~16 cell). (B) Immunofluorescent localization of Cdx2 in embryo shown in A. Monoclonal anti-Cdx2 antibody detects high levels of Cdx2 in nuclei of outer cells (arrow) and lower cytoplasmic levels (arrowhead). (C) Polyclonal anti-Cdx2 (CNL) antibody detects similar expression pattern, including cytoplasmic staining (arrowhead) at late morula (~25 cell) stage. (D) Oct4 and Cdx2 protein in same embryo shown in C. Cdx2 (red) is restricted to outer cells, but Oct4 (green) is still detectable in all outer cells. Oct4 and Cdx2 colocalize in outer cells (e.g. black arrowhead). (E-I) Composite DIC confocal images of polyclonal anti-Cdx2 staining of (E) 3.25 dpc early blastocyst, (F) 3.5 dpc blastocyst, (G) 4.5 dpc implanting blastocyst, (H) 4.75 dpc and (I) 5.5 dpc. Arrow indicates nuclear staining, white arrowhead indicates cytoplasmic. Red arrowheads demarcate polar TE. Bracket indicates ExE. (J) DIC image of $Cdx2^{-/-}$ morula. (K) Monoclonal anti-Cdx2 staining of embryo shown in J. (L) DIC image of $Cdx2^{-/-}$ blastocyst. (M) Monoclonal anti-Cdx2 staining of embryo shown in L. (N) DIC image of wild-type blastocyst. (O) Monoclonal anti-Cdx2 staining of embryo shown in N. Scale bar: 15 μm in A-G; 30 μm in H,I; 20 μm J-O.

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 (3.25 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 overlaying 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 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 blastocoel. 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 blastocoel of *Cdx2* mutants initially expanded, but began to collapse around the time that non-mutant littermates 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 trophectoderm 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 blastocoel, 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, five 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).

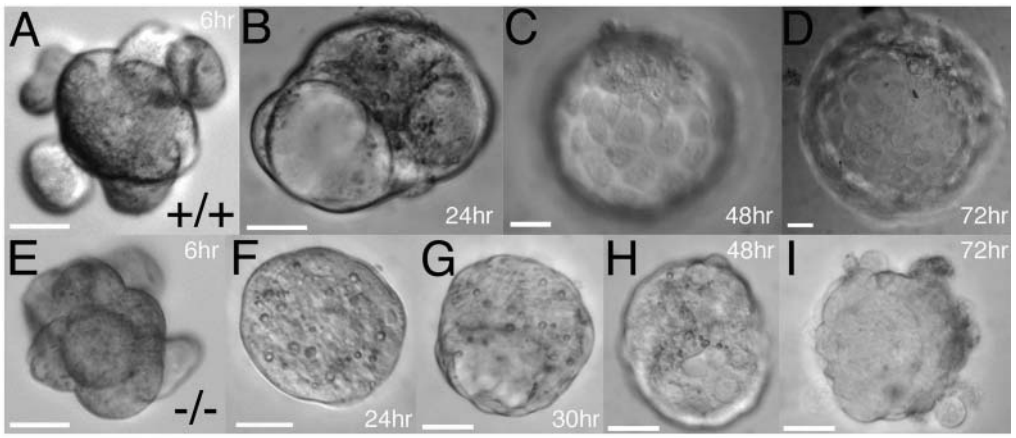

Fig. 2. Cultured *Cdx2*$^{-/-}$ embryos fail to form an expanded blastocyst. Bright-field images of *Cdx2*$^{+/+}$ embryos (A-D) and *Cdx2*$^{-/-}$ embryos (E-I). Eight-cell embryos from *Cdx2*$^{+/-}$ intercrosses were individually cultured, monitored for 72 hours, and images recorded at (A,E) 6 hours;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

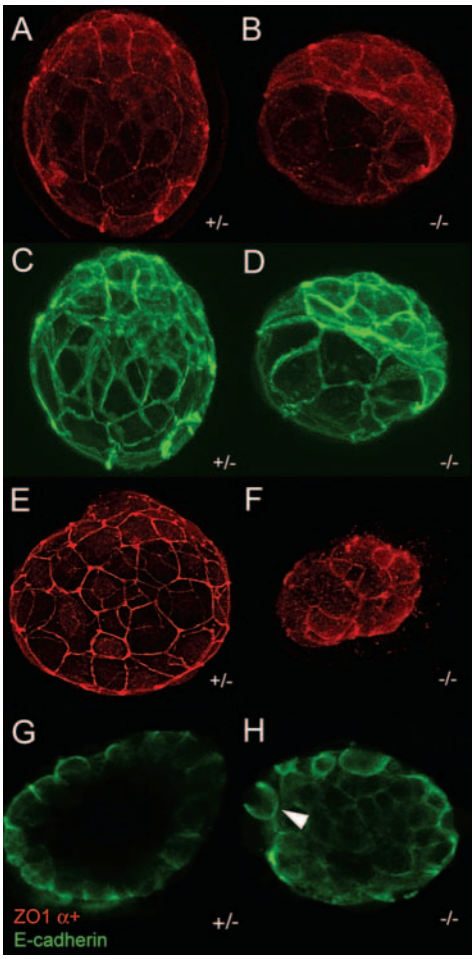

Fig. 3. Epithelial integrity of the TE is not maintained in *Cdx2* mutant embryos. (A) Immunolocalization of ZO-1α+ in $Cdx2^{+/-}$ early blastocyst. (B) ZO-1α + in $Cdx2^{-/-}$ early blastocyst. (C) Immunolocalization of E-cadherin in wild type embryo shown in A. (D) E-cadherin in $Cdx2^{-/-}$ embryo shown in B. (E) ZO-1α + in $Cdx2^{+/-}$ late blastocyst. (F) ZO-1α + in extreme example of a $Cdx2^{-/-}$ late blastocyst. (G) E-cadherin in $Cdx2^{+/-}$ late blastocyst. (H) E-cadherin in typical $Cdx2^{-/-}$ late blastocyst. (A-F) Projections of multiple optical sections. (G,H) Single sections.

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

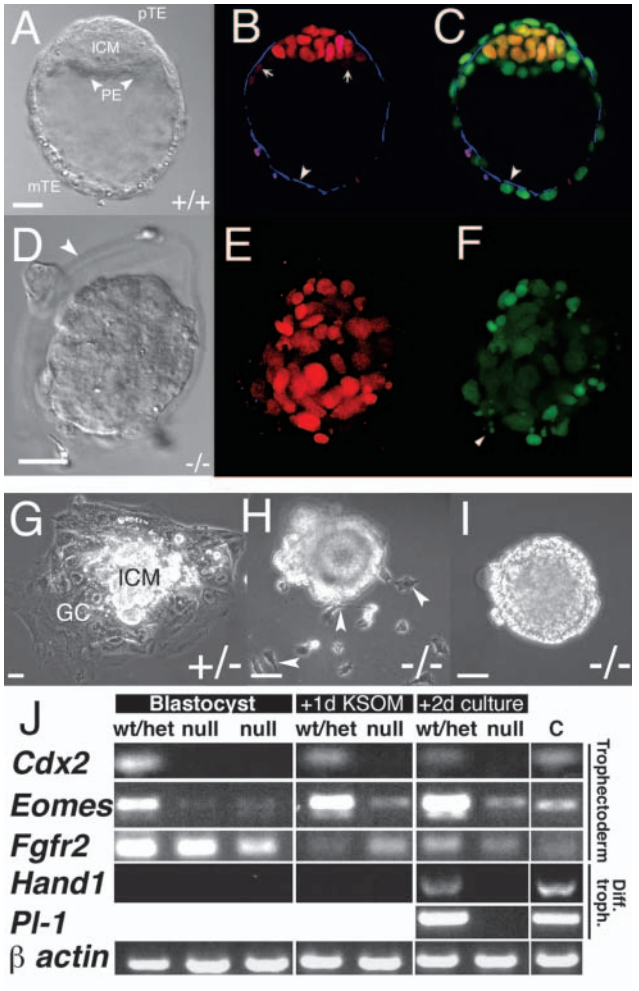

Fig. 4. Reduced expression of trophectoderm markers in $Cdx2^{-/-}$ blastocysts. (A) DIC image of $Cdx2^{+/+}$ 4.5 dpc blastocyst. Mural TE (mTE), polar TE (pTE), inner cell mass (ICM) and primitive endoderm (PE, arrowheads) indicated. (B) Immunolocalization of Oct4 (red), integrin α7 (blue) in embryo shown in A. Oct4 levels are reduced in the PE (arrows). Integrin α7 is specifically expressed in the trophectoderm (arrowheads in B,C). (C) Composite image of B and YOYO-1 (green, nuclear staining) (B and C are single optical sections). (D) DIC image of $Cdx2^{-/-}$ 4.5 dpc, a littermate of the embryo shown in A, encased in its zona pellucida (arrowhead). (E) Oct4 (red) and integrin α7 (blue) in embryo shown in D. Integrin α7 expression is undetectable, while Oct4 expression is found in almost all cells (compare E with F). (F) YOYO-1 (green) staining in embryo shown in D,E. Many nuclei are fragmented (e.g. arrowhead) (E,F are projected image composed of 10 confocal optical sections). Scale bars: 20 μm. (G-I) Trophoblast outgrowth formation assay. Blastocysts (3.5 dpc) from $Cdx2^{+/-}$ intercrosses were individually cultured in tissue culture plates uncoated or pre-coated with ECM substrate. (G) Outgrowth of a $Cdx2^{+/-}$ embryo. (H,I) $Cdx2^{-/-}$ embryos failed to attach, and formed a rounded mass of cells devoid of a typical blastocoel. Parietal endoderm cells were detected in some $Cdx2^{-/-}$ embryos cultures (K; arrowheads). Scale bars: 50μm. GC, trophoblast giant cells. (J) Semi-quantitative RT-PCR analysis for trophoblast markers in individual embryos from $Cdx2^{+/-}$ intercrosses. RNA was extracted from individually cultured blastocysts and analyzed by RT-PCR. Culture conditions are indicated (top): non-cultured 3.5 dpc blastocyst (Blastocyst), 1 day in KSOM (+1d KSOM), additional 48 hours culture in presence of serum (+2d culture). Presumptive genotype is indicated over each lane: wt/het, $Cdx2^{+/+}$ or $Cdx2^{+/-}$ embryo; null, $Cdx2^{-/-}$ embryo; C, control TS cell-derived RNA.

Table 1. *Cdx2*$^{-/-}$ blastocysts plated on different substrata fail to attach and form a trophoblast outgrowth

	Cdx2 genotype*		
ECM substrate	+/+	+/–	–/–†
Fibronectin	43/43	75/75	0/29(1)
Gelatin	5/5	10/10	0/4(1)
Laminin	12/12	13/13	0/7
No substrate	2/2	10/10	0/3

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.

differentiation is compromised beyond the expanded blastocyst stage.

C*dx2* 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*$^{+/-}$ intercrosses, 22 TS cell lines were obtained from a total of 36 blastocysts initially cultured. However, none were *Cdx2*$^{-/-}$ by genotype. Thus, *Cdx2* is required for all aspects of early TE development, both diploid proliferation and polyploid giant cell development.

The pluripotency markers Oct4 and Nanog are ectopically expressed in the putative TE of 3.5 dpc *Cdx2* mutant blastocysts

We examined the expression of the pluripotency-associated genes *Oct4* and *Nanog* to assess whether the outside cells of *Cdx2* mutants were correctly specified. At 3.5 dpc, *Oct4* was expressed in all cells of *Cdx2*$^{-/-}$ blastocysts, including the outer cells, whereas *Oct4* was restricted to the ICM in control embryos (3/3 mutants, 3/3 non-mutants; Fig. 5A,B). As Oct4 is normally expressed in all cells prior to the blastocyst stage and then downregulated in outer cells, these results suggest that its downregulation in the TE requires expression of *Cdx2*.

Nanog was also expressed in the outside cells of *Cdx2*$^{-/-}$ 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.

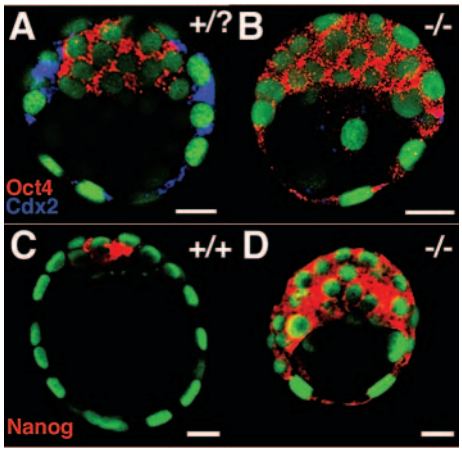

Fig. 5. *Oct4* and *Nanog* are expressed in outer cells of *Cdx2*$^{-/-}$ blastocysts. (A,B) Fluorescent in situ hybridization of *Oct4* (red) and *Cdx2* (blue), and YOYO-1 nuclear dye (green). (A) *Cdx2*$^{+/-}$ or *Cdx2*$^{+/+}$ blastocyst. (B) *Cdx2*$^{-/-}$ blastocyst. Each panel is a composite image of single confocal optical sections. (C,D) Fluorescent whole-mount in situ hybridization of *Nanog* (red) and YOYO-1 (green). Each panel is a composite projected image of two confocal optical sections. (C) *Cdx2*$^{+/+}$ blastocyst. (D) *Cdx2*$^{-/-}$ blastocyst. Scale bars: 20 μm.

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-

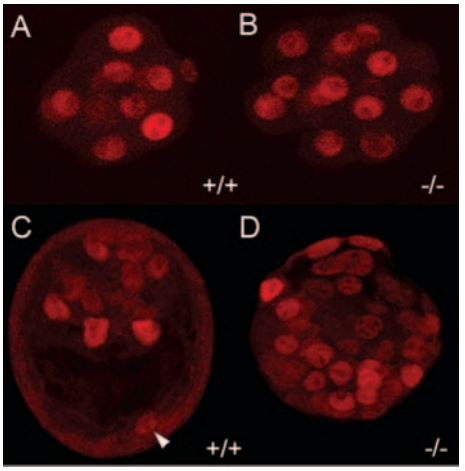

Fig. 6. *Nanog* is not downregulated in the outer cells of *Cdx2*$^{-/-}$ embryos. (A) Immunolocalization of Nanog in *Cdx2*$^{+/+}$ morula. (B) Nanog in *Cdx2*$^{-/-}$ morula, littermate of embryo shown in A. Nanog is detected in inner and outer cells in non-mutant and mutant morulae. (C) Nanog in *Cdx2*$^{+/+}$ in early blastocyst. Low levels of Nanog can be detected in occasional TE nuclei (arrowhead), but is largely downregulated in the TE by this stage. (D) Nanog in *Cdx2*$^{-/-}$ early blastocyst. Nanog expression persists in TE cells in *Cdx2* mutants. All panels are projections of multiple optical sections.

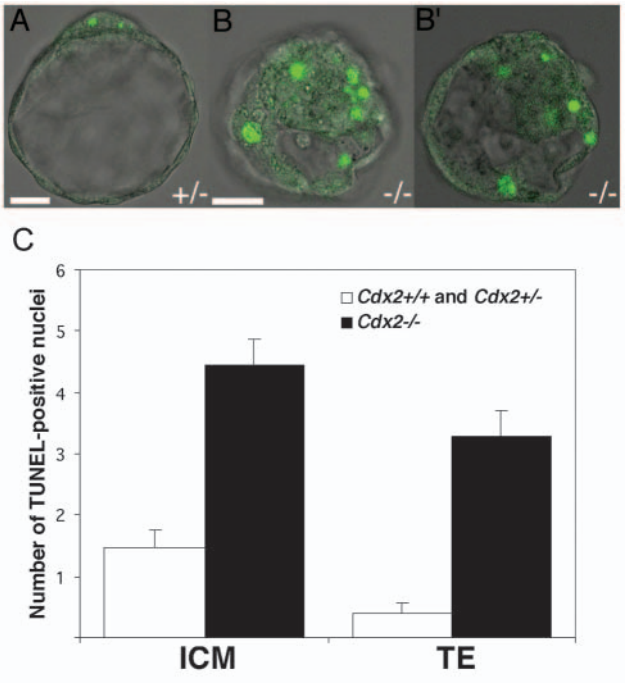

Fig. 7. Cultured *Cdx2*$^{-/-}$ embryos exhibit excessive cell death at the expanded blastocyst stage. (A-B′) Composite images of single confocal optical sections superimposed on DIC images. Zona pellucida-free eight-cell stage embryos from *Cdx2*$^{+/-}$ intercrosses were cultured for 48 hours followed by TUNEL analysis. (A) *Cdx2*$^{+/-}$; TUNEL-positive nuclei were detected in the ICM. (B,B′) *Cdx2*$^{-/-}$ blastocyst; TUNEL-positive nuclei were observed in the ICM and TE. (B′) Optical section taken at a different level of the embryo in B. Scale bars: 25 μm. (C) The number of TUNEL-positive nuclei in the ICM and TE of *Cdx2*$^{+/+}$ or *Cdx2*$^{+/-}$ embryos (n=16) and *Cdx2*$^{-/-}$ embryos (n=7) was scored from confocal optical sections of individual 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.

fold in the ICM and TE, respectively, compared with their *Cdx2*$^{+/+}$ or *Cdx2*$^{+/-}$ littermates (Fig. 7C). We thus conclude that lack of *Cdx2* leads to increased cell death at the expanded blastocyst stage, predominantly in the outer cell layer.

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. 8J). 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.

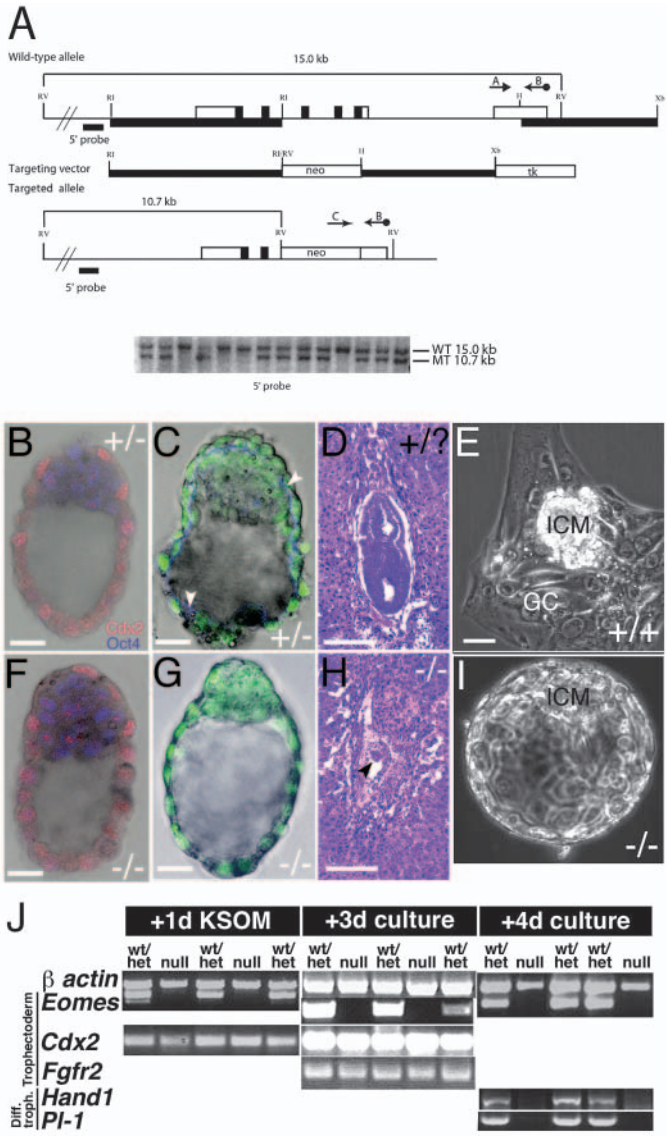

Fig. 8. *Eomesodermin* (*Eomes*) homozygous mutant embryos are viable through 6.0 dpc, but fail to differentiate the trophoblast lineage in vivo and in culture. (A) Targeted disruption of *Eomes* gene. (Top panel) Restriction maps of the mouse *Eomes* gene, the targeting construct and the predicted structure of the targeted *Eomes* allele. The six exons are shown as boxes, with the filled region indicating T-domain. In the targeting construct, a 4.3 kb segment containing two-thirds of the T-domain and the C-terminal half of *Eomes* was replaced with a PGK-neo cassette (neo), which was flanked by a 3.4 kb of *Eomes* 5′ and 2.1 kb of *Eomes* 3′ sequences (black boxes). An MC1-tk cassette (tk) was used as negative selector. Arrows indicate the position of primers for PCR-based genotyping. (Bottom panel) Representative Southern blot analysis of tail DNA isolated from pups. The 5′ probe recognizes 15.0 kb wild-type and 10.7 kb targeted *Eco*RV fragments. (B,F) Immunolocalization of Cdx2 (red) and Oct4 (blue) in 4.5 dpc embryos from $Eomes^{+/-}$ intercrosses. (B) $Eomes^{+/-}$; (F) $Eomes^{-/-}$. (C,G) Immunolocalization of integrin α7 (blue) and YOYO-1 (green) in 4.75 dpc embryos from $Eomes^{+/-}$ intercrosses. Integrin α7 is expressed in the trophectoderm of the $Eomes^{+/-}$ embryo (arrowheads in C), but is undetectable in the $Eomes^{-/-}$ embryo (G). (D,H) Morphology of embryos from $Eomes^{+/-}$ intercrosses at 6.0 dpc. (D) Presumptive $Eomes^{+/+}$ or $Eomes^{+/-}$ embryo. (H) Presumptive $Eomes^{-/-}$ embryo; arrowhead indicates an ICM-like structure. (E,I) Trophoblast outgrowth assay. Blastocysts (3.5 dpc) from $Eomes^{+/-}$ intercrosses were cultured individually in tissue culture plates uncoated or pre-coated with ECM substrate for 96 hours. (E) Outgrowth of an $Eomes^{+/+}$ embryo. (I) $Eomes^{-/-}$ embryo failed to attach, and remained as an expanded blastocyst. Scale bars: 20 μm for C,F,G; 25μm for B,I; 40 μm for D,E,H. GC, trophoblast giant cells. (J) Semi-quantitative RT-PCR analysis for trophoblast markers in individual embryos from $Eomes^{+/-}$ intercrosses. RNA was extracted from individually cultured blastocysts and analyzed by RT-PCR. Culture conditions are indicated genotype deduced from the absence or presence of an *Eomes*-specific PCR product is indicated over each lane.

Discussion

Cdx2 is required for the development of a functional trophectoderm

$Cdx2^{-/-}$ blastocysts cavitate normally, around the same time as non-mutant littermates, and are initially able to expand. However, the expanded blastocoel 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 trophectoderm 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 trophectoderm 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 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 trophectoderm 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 outer 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 *Fgfr2* 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 persistent maternal Cdx2 protein, as little or no Cdx2 protein was observed in *Cdx2*$^{-/-}$ morulae and early blastocysts.

Other factors could support the initial development of the TE epithelium in the absence of *Cdx2*. Two additional members of the Caudal-type TF family, Cdx1 and Cdx4, are found in the mouse. Previous analyses indicated that the three Cdx family members share partially overlapping expression patterns and functions along the embryonic axis, but only *Cdx2* is expressed in the postimplantation trophoblast, making redundancy with other Cdx genes unlikely (Beck et al., 1995; Gamer and Wright, 1993; Meyer and Gruss, 1993; van den Akker et al., 2002).

Eomes is expressed 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

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