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Maternal-zygotic knockout reveals a critical role of Cdx2 in the morula to blastocyst transition

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ABSTRACT

The first lineage segregation in the mouse embryo generates the inner cell mass (ICM), which gives rise to the pluripotent epiblast and therefore the future embryo, and the trophectoderm (TE), which will build the placenta. The TE lineage depends on the transcription factor Cdx2. However, when Cdx2 first starts to act remains unclear. Embryos with zygotic deletion of *Cdx2* develop normally until the late blastocyst stage leading to the conclusion that *Cdx2* is important for the maintenance but not specification of the TE. In contrast, down-regulation of *Cdx2* transcripts from the early embryo stage results in defects in TE specification before the blastocyst stage. Here, to unambiguously address at which developmental stage Cdx2 becomes first required, we genetically deleted *Cdx2* from the oocyte stage using a Zp3-Cre/loxP strategy. Careful assessment of a large cohort of *Cdx2* maternal-zygotic null embryos, all individually filmed, examined and genotyped, reveals an earlier lethal phenotype than observed in *Cdx2* zygotic null embryos that develop until the late blastocyst stage. The developmental failure of *Cdx2* maternal-zygotic null embryos is associated with cell death and failure of TE specification, starting at the morula stage. These results indicate that Cdx2 is important for the correct specification of TE from the morula stage onwards and that both maternal and zygotic pools of Cdx2 are required for correct pre-implantation embryogenesis.

Introduction

Prior to zygotic genome activation (ZGA), embryonic development is dependent on maternally inherited RNAs and proteins (Bachvarova and De Leon, 1980; Braude et al., 1979; Cascio and Wassarman, 1982; Van Blerkom, 1981). In the mouse, there are two waves of ZGA: a minor one at the late zygote stage and a major one at the 2-cell stage (Flach et al., 1982; Latham and Schultz, 2001). Despite this early ZGA, maternal factors are recognised as essential for embryo viability and also lineage segregation (Keramari et al., 2010; Zuccotti et al., 2011). Maternal factors can either rescue or significantly delay development of the mutant phenotype in zygotic knockout embryos (Avilion et al., 2003; Larue et al., 1994; Reithmacher et al., 1995). It is therefore important to consider the contribution of both maternal and zygotic gene expression to embryo development, particularly at the pre-implantation stages.

The first lineage segregation in the mouse embryo leads to separation of inside and outside cells that occurs in two cell division waves: at the 8–16 cell and 16–32 cell transition (Bischoff et al., 2008; Graham and Deussen, 1978; Johnson and Ziomek, 1982; Pedersen et al., 1986). Inside and outside cells will develop their unique identity due to their differential position and due to asymmetric partitioning of cell polarity proteins, such as Par1, Par3, aPKC, Jam1, Ezrin and transcripts, such as *Cdx2* (Louvet et al., 1996; Nishioka et al., 2009; Skamagki et al., 2013; Tarkowski and Wroblewska, 1967; Thomas et al., 2004; Vinot et al., 2005). Together differential partitioning of key cellular components and differential cell positioning establish the inside–outside asymmetry within the embryo that leads to development of the ICM and TE lineages.

Cdx2 is an essential transcription factor for the development of the mouse embryo at many developmental stages (Gao et al., 2009; Grainger et al., 2010; Morris et al., 2014; Stringer et al., 2012; van Rooijen et al., 2012; Zhao et al., 2014). During pre-implantation development, Cdx2 is essential for the TE lineage, but the stage of development at which Cdx2 plays a role and the processes it controls both remain unclear. Embryos with zygotic deletion of *Cdx2* develop normally until the late blastocyst stage leading to the suggestion that

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Cdx2 is involved only in maintenance of the TE lineage (Ralston and Rossant, 2008; Strumpf et al., 2005). However, down-regulation of both maternal and zygotic *Cdx2* expression by RNAi or morpholino treatments results in a much earlier phenotype that includes defects in cell polarisation, developmental arrest (Jedrusik et al., 2010) and the abnormal activity of mitochondria (Wu et al., 2010). These studies led to the suggestion that Cdx2 might have two roles in pre-implantation development: first, to ensure appropriate cell polarisation that is critical for TE formation and second, the subsequent maintenance of the TE lineage.

Functionality of maternal Cdx2 was recently questioned as embryos in which maternal *Cdx2* expression was genetically eliminated developed normally (Blij et al., 2012). Here, to address this discrepancy we have genetically ablated both maternal and zygotic *Cdx2* and filmed development of *Cdx2* maternal-zygotic and *Cdx2* maternal and *Cdx2* zygotic knockout embryos side-by-side to compare development to the blastocyst stage. This revealed that embryos deficient for both maternal and zygotic *Cdx2* show significantly reduced developmental potential and increased cell death from the morula stage onwards. The developmental lethality is significantly stronger following depletion of both maternal and zygotic pools of Cdx2, rather than when only maternal or only zygotic Cdx2 are eliminated. Together, these results lead us to conclude that both maternal and zygotic Cdx2 are important for the development of the mouse embryo and that the first stage of development at which Cdx2 plays a role is at the morula stage when specification of the TE first starts.

Materials and methods

Mouse strains

To obtain oocytes depleted of maternal *Cdx2*, we used females heterozygous for a *floxed Cdx2* gene (Gao et al., 2009) and a *Cdx2* deletion (Cdx2Δ), and carrying a *Zp3-Cre* transgene (de Vries et al., 2000) ($Cdx2^{loxP}/Cdx2\Delta$; *Zp3-Cre* females). These females were mated with $Cdx2^{loxP}/Cdx2\Delta$ males (Fig. 1). The $Cdx2^{loxP}$ line (Gao et al., 2009) was a kind gift from Klaus H Kaestner. Complete cleavage of the $Cdx2^{loxP}$ allele in the female germline by Cre recombinase results in 100% of mature oocytes carrying the Cdx2Δ. 50% of the resulting embryos that are $Cdx2\Delta/Cdx2\Delta$ are maternal-zygotic knockouts (MZ-KO) and the 50% $Cdx2\Delta/Cdx2^{loxP}$ embryos that have a wild-type (but floxed) paternal *Cdx2* allele are heterozygous maternal knockouts (M-KO). Control females were either $Cdx2^{loxP}$ homozygotes or $Cdx2^{loxP}/Cdx2\Delta$ heterozygotes and did not carry the *Zp3-Cre* transgene. To obtain embryos depleted of zygotic Cdx2 (Z-KO), mice heterozygous for the *Cdx2* targeted mutation $Cdx2^{tm1Fbe}$ that we refer to as *Cdx2-* were intercrossed (MGI:1857928) (Chawengsaksophak et al., 1997).

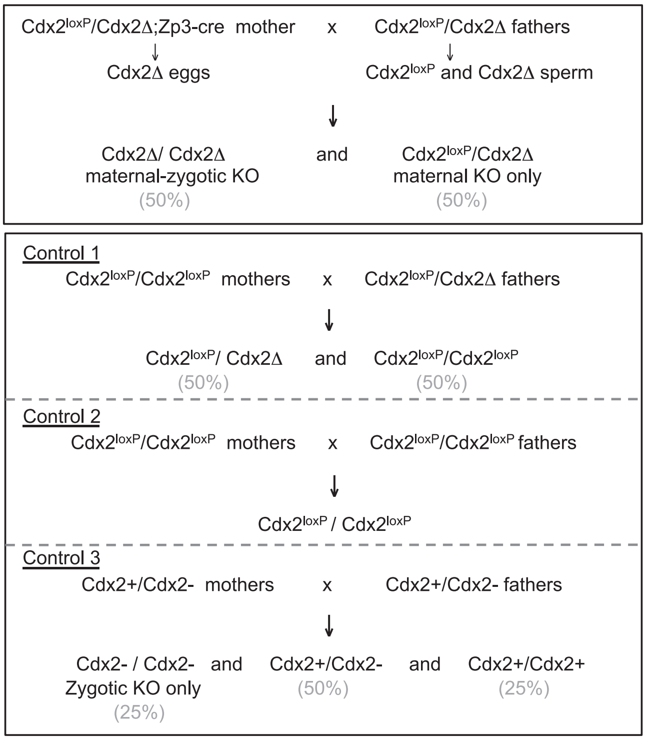

Fig. 1. Breeding schemes used to generate *Cdx2* maternal-zygotic knockout and maternal knockout embryos.

Embryo recovery, culture and time-lapse microscopy

Mouse embryos were recovered from oviducts of superovulated females (10 IU PMSG, 10 IU hCG; Intervet), collected into M2 medium with 4 mg/ml BSA, and cultured in KSOM supplemented with 4 mg/ml BSA as described before (Jedrusik et al., 2008). To record the development of each individual embryo, embryos were cultured on gridded dishes and filmed by time-lapse microscopy. Imaging was non-invasive and carried out using a wide-field Zeiss microscope with a Hamamatsu Orca ER digital camera and DIC *Z*-stacks were collected at 15 min intervals for 58 h.

To analyse any occurrence of cell death during embryo development, SYTOX fluorescent green nucleic acid stain (Life technologies) was added to the culture medium as previously described (Bedzhov and Zernicka-Goetz, 2014).

Individual embryo immunofluorescence and genotyping

Individual embryos were removed from the gridded dish upon completion of time-lapse imaging, fixed individually in 4% PFA (4 °C, 12 h) and processed for immunofluorescence as previously described (Plusa et al., 2005). Primary antibodies: mouse anti-Cdx2 (BioGenex; 1:200), rat TROMA-1 anti-cytokeratin-8 (DSHB, Iowa; 1:100); and rabbit anti-Nanog (R&D Systems; 1:200). DNA was stained with Hoechst (Sigma). Images were recorded using a Leica SP5 confocal microscope with a 40 × oil objective.

Following immunofluorescence, the genotype of each embryo was determined by PCR. To this end, genomic DNA was extracted from individual embryos in 10 μl extraction/neutralization buffers (Truett et al., 2000). 4.5–5 μl of the lysate was used in PCR reactions using the Fast Cycling PCR Kit (Qiagen) and primers: 5′-GACCGAAGTCTGCAGAACCT and 5′-GGCTCAGGACTTGCTCCTTCA to detect Cdx2 WT and Cdx2 KO alleles; and 5′-AGCCCATTGCTG-GACGGAGG and 5′-CCGCTTACCTTGACGCCACA to detect cleaved KO (null) allele.

Results

Cdx2 is required for correct development to the blastocyst stage in vivo

The stage at which Cdx2 is first required for development of the mouse embryo remains unclear because Cdx2 is expressed both maternally and zygotically (Jedrusik et al., 2010; Wu et al., 2010). To address this, we used a genetic approach to deplete maternal and zygotic pools of Cdx2 from the oocyte stage onwards by crossing females heterozygous for a *floxed Cdx2* gene and a *Cdx2* deletion and carrying the *Cre-recombinase* gene under control of the *ZP3 promotor* ($Cdx2^{loxP}/Cdx2\Delta$; *Zp3-Cre*) with $Cdx2^{loxP}/Cdx2\Delta$