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Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development

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Specification of cell lineages in mammals begins shortly after fertilization with formation of a blastocyst consisting of trophectoderm, which contributes exclusively to the placenta, and inner cell mass (ICM), from which the embryo develops. Here we report that ablation of the mouse *Tead4* gene results in a preimplantation lethal phenotype, and TEAD4 is one of two highly homologous TEAD transcription factors that are expressed during zygotic gene activation in mouse 2-cell embryos. *Tead4*$^{-/-}$ embryos do not express trophectoderm-specific genes, such as *Cdx2*, but do express ICM-specific genes, such as *Oct4* (also known as *Pou5f1*). Consequently, *Tead4*$^{-/-}$ morulae do not produce trophoblast stem cells, trophectoderm or blastocoel cavities, and therefore do not implant into the uterine endometrium. However, *Tead4*$^{-/-}$ embryos can produce embryonic stem cells, a derivative of ICM, and if the *Tead4* allele is not disrupted until after implantation, then *Tead4*$^{-/-}$ embryos complete development. Thus, *Tead4* is the earliest gene shown to be uniquely required for specification of the trophectoderm lineage.

KEY WORDS: *Cdx2*, *Oct4*, *Eomes*, Embryonic stem cell, Trophoblast stem cell, Morula, Blastocyst

INTRODUCTION

Cellular differentiation during mammalian development is first evident at the blastocyst stage with the appearance of two distinct cell types, the trophectoderm and the inner cell mass (ICM). The trophectoderm is a single layer of polarized epithelial cells that forms the outer wall of the blastocyst. This creates the blastocoel cavity in which resides the ICM, a group of pluripotent cells. The trophectoderm gives rise exclusively to cells that eventually make up the placenta, whereas the ICM gives rise to cells that will form the embryo, as well as the endoderm, mesoderm and ectoderm components of the placenta (Cross, 2005; Kunath et al., 2004). In vitro, it is the trophectoderm that gives rise to trophoblast stem (TS) cells, and the ICM that gives rise to embryonic stem (ES) cells.

Presumably, selective expression of one or more specific genes in the blastomeres of preimplantation embryos triggers their differentiation into either trophectoderm or ICM. For example, embryos deficient in the POU-domain transcription factor OCT4 survive through the morula stage, but cannot form an ICM and fail to give rise to embryonic stem cells in vitro (Nichols et al., 1998). OCT4 prevents trophectoderm and perhaps somatic-cell differentiation of the ICM in addition to being crucial for maintaining the pluripotent state during embryonic development. Moreover, in mouse embryonic stem cells, the relative amount of OCT4 ultimately determines cell fate (Boiani and Scholer, 2005). The counterpart to OCT4 is the caudal-type homeodomain transcription factor CDX2, a protein that is required for specification and differentiation of the trophectoderm (Strumpf et al., 2005). CDX2-deficient embryos form blastocysts but fail to implant, and genes such as *Oct4* (also known as *Pou5f1* – Mouse Genome Informatics) and *Nanog*, that are normally expressed only in the ICM, are ectopically expressed in the outer cells of the blastocyst, resulting in eventual death of the embryo. The cellular concentration of OCT4 relative to CDX2 appears to determine which of the totipotent blastomeres will become trophectoderm and which will become ICM (Niwa et al., 2005). This implies that OCT4 and CDX2 constitute the 'prime movers' in establishing the first cell-type-specific lineages during mammalian development, although how the fate of each blastomere is determined remains speculative. Hence, other genes involved in specifying trophectoderm and ICM may act upstream of either *Oct4*/*Pou5f1* or *Cdx2*.

Zygotic gene expression in the mouse begins at the 2-cell stage in development (Nothias et al., 1995; Schultz, 2002). Microinjection of DNA into mouse preimplantation embryos revealed that their ability to utilize enhancers to activate transcription also appeared with formation of a 2-cell embryo, and that the most effective enhancers contained one or more DNA binding sites for a TEAD transcription factor (Kaneko and DePamphilis, 1998). In placental mammals, there are four TEAD family members characterized by a highly conserved, virtually identical 72 amino acid TEA DNA binding domain. Furthermore, the amino acid sequences in the C-terminal halves of these proteins share 80% to 87% similarity. This conservation of structure reflects the ability of mammalian TEAD proteins to bind the same transcriptional co-activator proteins (Mahoney et al., 2005; Vassilev et al., 2001), and to substitute for TEA DNA binding domain proteins in other organisms (Deshpande et al., 1997).

Most, if not all, embryonic and extraembryonic tissues express at least one of the TEAD genes during mammalian development (Kaneko and DePamphilis, 1998). However, *Tead2* (also known as *Tef4* and *ETF*) (Jacquemin et al., 1996; Kaneko et al., 1997; Yasunami et al., 1995) and *Tead4* (also known as *Tef3*, *Tefr* and *Etfr2*) (Jacquemin et al., 1996; Yasunami et al., 1996; Yockey et al., 1996) are the only two Tead genes expressed at significant levels in preimplantation mouse embryos (Kaneko et al., 1997) (this report). Thus, TEAD2 and TEAD4, like OCT4 and CDX2, are among the earliest transcription factors expressed during mammalian development, suggesting that they too play critical roles in the early

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stages of development. Therefore, to investigate this further the *Tead2* (Kaneko et al., 2007) and *Tead4* (this work) genes were each inactivated by site-specific recombination, with the expectation that they would also be required for preimplantation development. Surprisingly, inactivation of the *Tead2* gene significantly increased the risk of exencephaly (a defect in neural tube closure), but did not otherwise prevent development of viable adults. By contrast, inactivation of *Tead4* resulted in a preimplantation lethal phenotype.

Failure of *Tead4*$^{-/-}$ embryos to implant results from a requirement for TEAD4 to activate genes required for establishment of the trophectoderm lineage. This requirement was unique to the trophectoderm lineage, because *Tead4*$^{-/-}$ embryos were still able to produce ES cells, and if the *Tead4* gene was not disrupted until after implantation occurred, then *Tead4*$^{-/-}$ embryos were able to develop into viable adults. These results suggest that expression of the *Tead4* gene during activation of zygotic gene expression in 2-cell embryos eventually triggers differentiation of totipotent blastomeres into trophectoderm.

MATERIALS AND METHODS

Generation of conditional *Tead4* knockout mice

A 129Sv/J mouse genomic BAC clone [clone 440(L02), GS control number 26941] containing exon 2 of *Tead4* was from Incyte Genomics. A 13 kb *Eco*RI fragment containing exon 2 was used to insert LoxP sites into the flanking introns (Fig. 1). The targeting vector contained a pGK-neo cassette for positive selection, and a pGK-tk cassette for negative selection against non-homologous recombinants. Linearized plasmid DNA was used to electroporate 129Sv/J embryonic stem (ES) cells. 280 G418-resistant clones were screened by Southern blotting using *Pst*I digests of genomic DNA and a 1.8 kb *Eco*RI-*Eco*RV probe corresponding to a sequence immediately upstream of left homology arm ('LAP' in Fig. 1A). The resulting fragments were 4.9 kb for the wild-type and 6.3 kb for the conditional (cond.) allele. Positive clones were further verified by Southern blotting for recombination of the downstream homology arm using *Eco*RV-digested ES cell DNA. Confirmed ES clone 271 harboring the conditional *Tead4* allele was microinjected into C57Bl/6 blastocysts, and blastocysts were implanted into FVBN foster mothers. Chimeric founder males were crossed with C57Bl/6 females and the resulting F1 generation was screened by Southern blotting using the LAP. An unconditional knockout (KO) line was generated by crossing homozygous *TEAD4*lox/lox mice with transgenic mice expressing *Cre* recombinase under control of the EIIa promoter (Lakso et al., 1996). Mice were screened by PCR to verify germline transmission, using primers TEAD4.20, TEAD4.27, and TEAD4.28 (Fig. 1E) or P1, P2 and P4 (Fig. 1D). Size of PCR products were as follows: primers TEAD4.20, TEAD4.28 and TEAD4.27, 294 bp for the wild-type and 480 bp for KO allele; P1/P2, 550 bp for the wild-type allele; P2/P4, 650 bp for the KO allele; P1/P3, 400 bp for the conditional allele. PCR genotyping of the Meox2-Cre transgene was performed as described previously (Tallquist and Soriano, 2000).

Adult animals were genotyped from tail clip DNA. Cell lines were genotyped after multiple passages in the absence of primary MEF feeder cells. Blastocysts were genotyped after isolation, outgrowth assays, or immunostaining using DNA lysis buffer containing 50 mM Tris-HCl pH 8.0, 0.5% Triton X-100, 200 μg/ml proteinase K. The embryos were digested at 55°C for 2 hours and the proteinase K was inactivated at 95°C for 5 minutes.

In situ hybridization

Uteri from wild-type embryonic day 6.5 (E6.5) pregnant mice were collected, separated into individual placentas and fixed for 24 hours in freshly prepared 4% paraformaldehyde in PBS (pH 7.4). Tissues were then dehydrated, embedded in paraffin, and 10 μm thick sections cut. Consecutive sections were hybridized to an in vitro-transcribed ^{33}P-labeled cRNA probe for TEAD4, as described previously (Wilkinson and Nieto, 1993), but with greater stringency by hybridizing overnight at 54°C. Probe templates were made by RT-PCR from E15 mouse hind limb total RNA and cloned into pGEM-T (Promega). Primers used for amplification were TEAD4.1 and TEAD4.2 (Table 1).

Embryos

Preimplantation embryos were collected by flushing uteri from CD1 females with M2 medium (Sigma) as described previously (Nagy et al., 2003). For blastocyst outgrowth experiments, E3.5 embryos from *Tead4* heterozygous matings were cultured on 0.1% gelatin-treated plates in RPMI1640 containing 20% ES-qualified FBS (Chemicon) and standard supplements for up to 5 days.

RNA preparation, real-time PCR and RT-PCR

Total RNA was isolated from preimplantation embryos using RNeasy micro columns (Qiagen). For real-time PCR, RNA was reverse-transcribed using TaqMan reagents (Applied Biosystems). *Tead4* and *Gapdh* probes were obtained from Applied Biosystems. Quantification of gene expression was

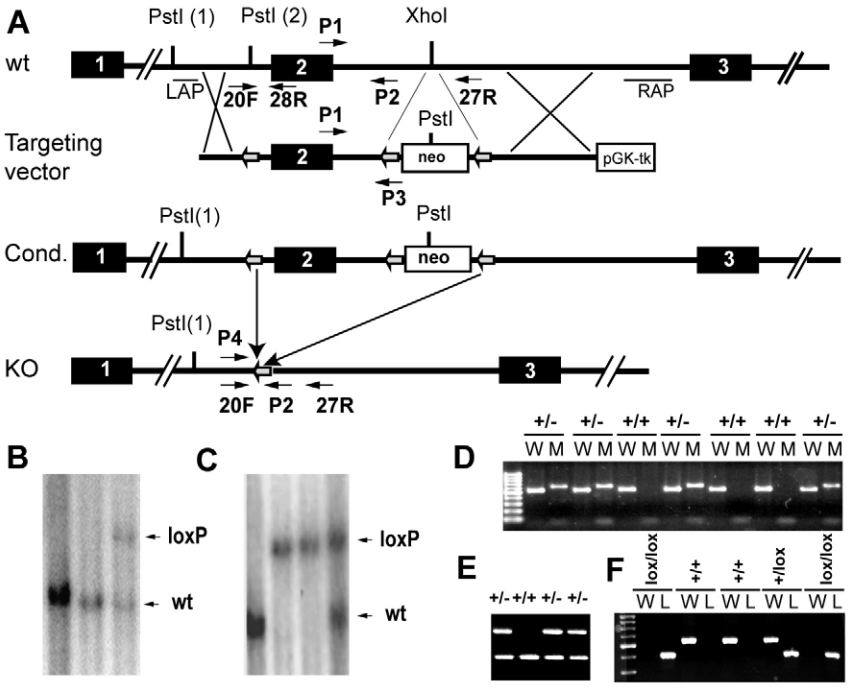

Fig. 1. Generation of conditional and full *Tead4* knockout mice. (**A**) Exon 2 of the mouse *Tead4* locus (black boxes) was targeted for homologous recombination. Indicated are the positions of the left arm probe (LAP), right-arm probe (RAP), and binding sites for PCR primers used for genotyping (Table 1), and restriction sites used for ES cell screening and for the insertion of loxP sites (open arrows in targeting vector). After homologous recombination, exon 2 is flanked by loxP sites in the conditional *Tead4loxP* allele (Cond.). Following Cre-mediated recombination, the *Tead4* knockout (KO) allele lacks exon 2. Genomic DNA from ES cells (**B**) and tail snips (**C**) was subjected to Southern blotting-hybridization with the LAP. (**D**,**E**) Genotypes were routinely confirmed by PCR analysis of tail DNA obtained from the progeny of matings between *Tead4*$^{+/-}$ mice showing the absence of *Tead4*$^{-/-}$ offspring. Primers: P1, P2, P4 in D and TEAD4.20 (20F), TEAD4.27 (27R), TEAD4.28 (28R) in E. (**F**) PCR-based genotyping, using primers P1, P2, and P3, of F2 animals produced from mating heterozygotes conditional (*Tead4*+/lox) animals shows recovery of wild-type, heterozygous and homozygous *Tead4* conditional offspring . W, wild type; L, *loxP*; M, mutant.

Table 1. PCR primers

Marker	Forward primer (5′-3′)	Reverse primer (5′-3′)
Tead4.1	ATTACCTCCAACGAGTGGAGCTCT	
Tead4.2		TCATTCTTTCACAAGTCGGT
Tead4.20	GATTAAAGGCTCACTCAGAGG	
Tead4.27		CTCAACATACAGTTTGAAGCAC
Tead4.28		AGCTCCACTCGTTGGAGGTAAT
P1	CTAGCATTAAGGAATGTCCCGA	
P2		CTCAACATACAGTTTGAAGCAC
P3		CGTATAGCATACATTATACGAAG
P4	GTGTTCTTAGAGGTACAGTCA	
RT-PCR primers		
Tead4	GCACCATTACCTCCAACGAG	GATCAGCTCATTCCGACCAT
Fgf4	TCTACTGCAACGTGGGCATC	CTTCATGGTAGGCGACACTC
Nanog	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG
Sox2	GGCAGCTACAGCATGATGCAGGAGC	CTGGTCATGGAGTTGTACTGCAGG
Rex1	GACTAAGAGCTGGGACAC	TTCTGGCCACTTGTCTTTGC
T	CCGGTGCTGAAGGTAAATGT	TGACCGGTGGTTCCTTAGAG
Hnf4α	AAATGTGCAGGTGTTGACCA	AGGAGCAGCACGTCCTTAAA
Gapdh	CCAAGTAGTAGTACATC	TTCTTACTCCTTGGAGGC

Specific primers for *β-actin*, *Oct4* (Nichols et al., 1998) and *Cdx2*, *Fgfr2* and *Eomes* (Strumpf et al., 2005) have been published.

performed using an ABI Prism 7000 sequence detection system. For RT-PCR, RNA was collected from individual embryos and reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen). Primers are given in Table 1.

Immunofluorescent staining

Immunofluorescent staining was performed as described previously (Strumpf et al., 2005) using primary antibodies against CDX2 (Biogenex), OCT4 (Santa Cruz Biotechnologies) and CDH1 (Sigma), and visualized with Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen) or FITC goat anti-rat secondary antibody (Zymed). Embryos were then examined using a Zeiss LSM510 confocal microscope.

Cell lines and embryoid bodies

TS cell lines and trophoblast giant (TG) cells were generated from E3.5 embryos of *Tead4* heterozygous matings as described previously (Tanaka et al., 1998). After several passages, the TS cells were removed from MEFs and genotyped for *Tead4*. TS cells lines were similarly generated from E2.5 embryos, except that the embryos were treated with acidic Tyrode's solution to remove their zonae pellucidae, placed in wells containing a small droplet of calcium- and magnesium-free PBS, allowed to adhere to the surface of the dish, and then gently seeded into the wells in culture medium. ES cells lines were generated from E2.5 morulae as described previously (Tesar, 2005). Embryoid bodies were produced as described Kaneko et al. (Kaneko et al., 2004).

RESULTS

Impaired implantation and lethality in TEAD4 knockout mice

TEAD factors have been studied extensively in cultured cells, particularly in cardiac and skeletal muscles where they regulate differentiation. However, with the exception of TEAD1, the functions of these proteins in vivo have remained elusive. To address this problem, separate conditional knockouts were constructed for the genes *Tead2* (Kaneko et al., 2007) and *Tead4* (this report) by targeting their TEA DNA-binding domains.

The murine *Tead4* gene encompasses 42.7 kb on chromosome 6, and exon 2 harbors almost half of the TEA DNA-binding domain. The conditional mouse line *Tead4*lox/lox was obtained by inserting loxP sites into the introns flanking exon 2 (Fig. 1A). Heterozygous mice were established by crossing male chimeras with C57Bl/6 females. Founders were bred among themselves to establish a homozygous *Tead4*lox/lox line (Fig. 1F). These mice were viable and fertile, and exhibited no obvious morphological malformations or abnormal behavior.

To address the role of TEAD4 in early mouse development, an unconditional knockout line was generated by crossing lox/lox mice with EIIa-Cre mice that ubiquitously express *Cre* recombinase (Lakso et al., 1996). This resulted in germline transmission of the recombined allele. Heterozygous offspring from this cross were also viable, fertile and apparently normal in morphology and behavior. Next, *Tead4* heterozygotes were mated and their progeny genotyped. Out of a total of 367 pups, only wild-type and heterozygous offspring, but no homozygotes were identified (Table 2).

To determine whether the lethality resulted from a preimplantation or a postimplantation defect, *Tead4* heterozygous mice were mated and then screened between E6.5 and E15.5 for the presence of *Tead4*$^{-/-}$ embryos. Out of a total of 71 embryos, no homozygous embryos were recovered and no signs of resorbed placentas were detected (Table 2). Thus, TEAD4 is required prior to E6.5, suggesting a role for TEAD4 in preimplantation development.

Table 2. Genotypes of progeny from Tead heterozygous matings

	Genotype			
Progeny	+/+	+/–	–/–	Total
♂ ***Tead4***$^{+/-}$ × ♀ ***Tead4***$^{+/-}$				
Viable adults	120	247	0	367
Implanted embryos (E6.5-15.5)	24	47	0	71
E3.5 embryos	20	42	(16)*	78
TS cell lines from E3.5 blastocysts	14	22	0	36
TS cell lines from E2.5 embryos	7	5	0	12
ES cell lines from 19 E2.5 embryos	5	2	1	8
♂ ***Tead2***$^{+/-}$ × ♀ ***Tead2***$^{+/-}$				
Viable adults	48	99	35	182
E3.5 blastocysts	6	16	5	27

Either *Tead4*$^{+/-}$ mice or *Tead2*$^{+/-}$ mice were mated. Embryos were recovered either at E2.5 or E3.5 days post-fertilization and tested for their ability to yield either TS or ES cells. Genotyping was performed either on the entire embryo, or on the embryo-derived cell line.
*All 16 of the *Tead4*$^{-/-}$ embryos at E3.5 were abnormal morulae.

Tead4 is first expressed during preimplantation development and trophectoderm differentiation

Previous studies did not detect *Tead4* mRNA in preimplantation mouse embryos (Hamatani et al., 2004; Kaneko et al., 1997). To understand why TEAD4-deficient embryos arrest prior to implantation, the relative amount of *Tead4* mRNA was quantified by real-time RT-PCR during early development. The results revealed that *Tead4* mRNA was barely detectable in unfertilized and fertilized eggs, but increased from the 2-cell embryo through blastocyst stages with the maximum level observed in 8-cell embryos and morulae (Fig. 2A).

To determine if *Tead4* expression was specific to the trophectoderm or ICM cell lineages, trophoblast stem (TS) cells, embryonic stem (ES) cells and their derivatives were analyzed. TS cells are derived from the polar trophectoderm of blastocysts and can differentiate into several trophoblast cell types (Cross, 2005; Kunath et al., 2004). In vitro, TS cells proliferate in response to fibroblast growth factor 4 (FGF4), and in the absence of FGF4, they differentiate into invasive trophoblast giant (TG) cells (Cross, 2005) that mediate the process of implantation and invasion of the embryo into the uterine endometrium and deciduum. In contrast to TS cells, embryonic stem (ES) cells are derived from the ICM and contribute exclusively to embryonic cell types and to nontrophoblast-derived extra-embryonic tissues (Smith et al., 1988; Williams et al., 1988). In vitro, ES cells can be induced to differentiate into embryoid bodies that contain cells derived from all three embryonic layers.

Tead4 expression was 27-fold greater in TS cells than in ES cells, and this high level of expression was maintained as TS cells differentiated into TG cells in vitro (Fig. 2B). Similarly, the relatively low level of *Tead4* expression in ES cells was maintained upon their differentiation into embryoid bodies. This result was confirmed and extended using in situ hybridization technology. *Tead4* mRNA was found predominantly in the extraembryonic portion of the conceptus, in particular in the dividing trophoblast cells of the ectoplacental cone (EPC) (Fig. 2C,D). Expression was also observed in extraembryonic layers of the embryo, including the chorion and giant trophoblast cells underlying the maternal part of the placenta, as well as in the maternal deciduum. *Tead4* expression was not detected in the embryo proper. Taken together, these data demonstrate that *Tead4* is expressed primarily in the trophectoderm-derived cell lineage during early and mid-embryonic development.

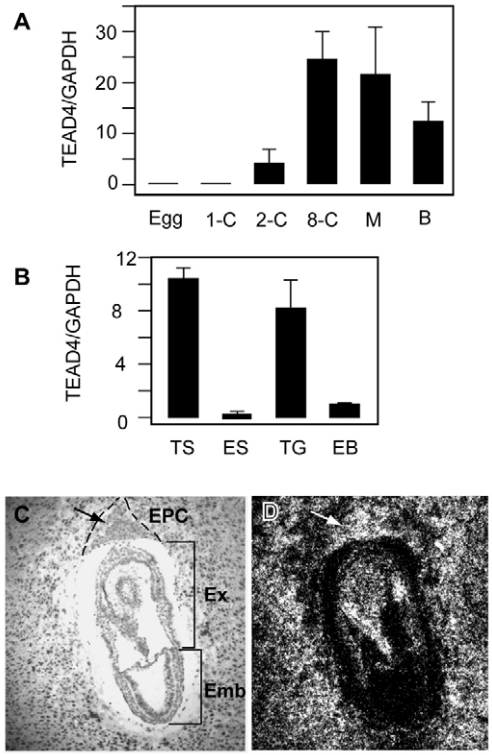

Fig. 2. *Tead4* is expressed in preimplantation embryos and trophoblast cell lines. (**A**,**B**) Real-time RT-PCR was used to quantify the levels of *Tead4* mRNA in unfertilized eggs, 1-cell embryos (1-C), 2-cell embryos (2-C), 8-cell embryos (8-C), morulae (M) and blastocysts (B) (A), as well as in trophoblast stem (TS) cells, trophoblast giant (TG) cells, embryonic stem (ES) cells and embryoid bodies (EB) (B). Each sample was internally normalized to *Gapdh* mRNA. *Gapdh* mRNA level per egg or embryo in A and *Gapdh* mRNA level per cell in B were essentially constant. Error bars represent the s.e.m. of three independent assays. (**C**,**D**) In situ hybridization was used to detect *Tead4* transcripts at E6.5. Bright-field (C) and dark-field (D) images of an embryo in utero. *Tead4* is broadly expressed in all extraembryonic layers and deciduum, but highest levels are in extra-embryonic (Ex) trophoblast cells of the ectoplacental cone (EPC, outlined by a dashed line). No expression was observed in the embryonic germ layers (Emb).

TEAD4 is required for expression of trophectoderm-specific genes

Cdx2 expression is required for the establishment of TS cells, and CDX2-deficient embryos develop to the blastocyst stage but fail to implant due to loss of trophectoderm cell integrity (Strumpf et al., 2005). By contrast, the *Oct4/Pou5f1* gene is required for establishment of ES cells, and OCT4-deficient embryos develop a blastocoel cavity but not an ICM (Nichols et al., 1998). Moreover, at the blastocyst stage, *Cdx2* is expressed only in trophectoderm cells, and *Oct4* is expressed only in the ICM (Niwa et al., 2005; Strumpf et al., 2005).

To determine if TEAD4 is required for *Cdx2* expression, embryos obtained from heterozygous matings were stained with antibodies against CDX2 and then genotyped. As expected, CDX2 protein was present in the trophectodermal cells of $Tead4^{+/+}$ and $Tead4^{+/-}$ blastocysts at E3.5 (Fig. 3A). In contrast, CDX2 was not detected at E3.5 in $Tead4^{-/-}$ embryos, all of which exhibited an abnormal morphology characterized by poorly formed blastomeres unevenly distributed within the zona pellucida, and by the absence of a blastocoel cavity. These embryos were henceforth referred to as 'abnormal morulae'. CDX2 also was absent from $Tead4^{-/-}$ morulae at E2.5, although these embryos were morphologically indistinguishable from $Tead4^{+/+}$ and $Tead4^{+/-}$ embryos of the same age. In fact, CDX2 was present in only a few cells of normal 8-cell embryos or morulae recovered at E2.5 with genotypes of either $Tead4^{+/+}$ or $Tead4^{+/-}$, and was absent in 4-cell embryos (data not shown). Presumably, these CDX2-positive cells were destined to become trophectoderm.

Consistent with previous studies (Strumpf et al., 2005), OCT4 protein was present only in the ICM of $Tead4^{+/+}$ or $Tead4^{+/-}$ blastocysts at E3.5, all of which appeared morphologically normal (Fig. 3B). However, the E3.5 abnormal morulae, all of which were $Tead4^{-/-}$ (Table 2), produced OCT4 protein in all of their blastomeres. Thus, TEAD4 was required for *Cdx2*, but not *Oct4* gene expression. Since $Cdx2^{-/-}$ embryos also express OCT4 in all of their blastomeres (Strumpf et al., 2005), these results further reveal that TEAD4 is required for *Cdx2* expression which normally suppresses *Oct4* expression in those cells destined to become trophectoderm (Niwa et al., 2005).

RT-PCR was used to determine if TEAD4 was required for expression of a battery of genes associated with preimplantation development (Fig. 3C). Consistent with the immunofluorescence analysis, *Cdx2* mRNA was present in E3.5 blastocysts that were either *Tead4*$^{+/+}$ or *Tead4*$^{+/-}$, but absent from E3.5 *Tead4*$^{-/-}$ abnormal morulae, whereas *Oct4* mRNA was present in all embryos. Moreover, E3.5 *Tead4*$^{-/-}$ abnormal morulae expressed eomesodermin (*Eomes*) at a reduced level, consistent with a requirement for TEAD4 to specify the trophectoderm lineage. *Eomes* is expressed in the trophectoderm layer of blastocysts and postimplantation extraembryonic tissue, where it acts downstream of CDX2 and is required for trophoblast development (Russ et al., 2000; Strumpf et al., 2005). In the absence of CDX2, *Eomes* expression is low but detectable (Strumpf et al., 2005), demonstrating that CDX2 is partially responsible for activating *Eomes*, and consistent with the effects of TEAD4 deficiency presented here. By contrast, expression of *Fgfr2* was not affected. *Fgfr2* is normally restricted to the outer cells of compacted morulae and highly expressed in the trophectoderm layer of blastocysts (Haffner-Krausz et al., 1999). Thus, TEAD4 was required for expression of some, but not all trophectoderm-specific genes.

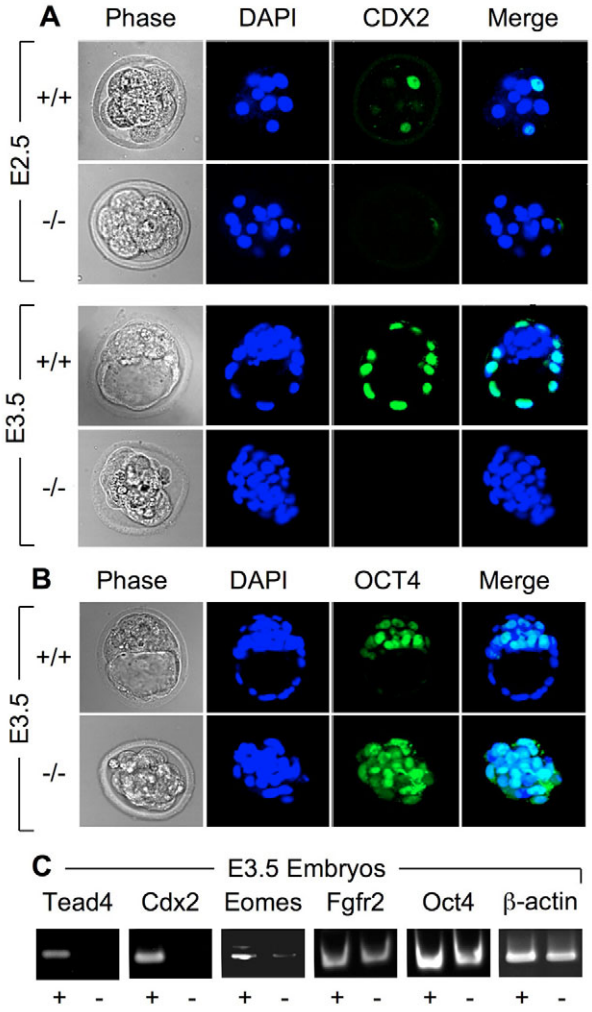

Fig. 3. TEAD4 is required for expression of some genes during preimplantation development, but not others. (**A**) Embryos from *Tead4*$^{+/-}$ heterozygous intercrosses were collected either at E2.5 or E3.5 and immunostained with anti-CDX2 antibody (green). DNA was labeled with DAPI (blue), and embryos were photographed under phase contrast optics (Phase). Confocal images of DNA and CDX2 stains were merged. Although the top panel appears to show an 8-cell embryo, there are four more nuclei present outside the focal planes shown. Embryos were genotyped after imaging. Wild-type and *Tead4* heterozyotes were morphologically indistinguishable, so only wild-type and *Tead4* knockout embryos are shown (total number of embryos examined: *Tead4*$^{+/+}$, *Tead4*$^{+/-}$: 13 for E.2.5 and 14 for E3.5; *Tead4*$^{-/-}$: 3 at E2.5 and 4 at E3.5). (**B**) Embryos from *Tead4*$^{+/-}$ heterozygous intercrosses were collected at E3.5 and stained for OCT4 protein (green) as described in A. Typical examples are shown of *Tead4*$^{+/+}$ blastocysts (n=16), and of *Tead4*$^{-/-}$ embryos (n=4). (**C**) Total RNA was isolated from a single E3.5 blastocyst and a single E3.5 abnormal morula, and RT-PCR was used to detect expression of the *Tead4*, *Cdx2*, *Eomes*, *Fgfr2* and *Oct4* genes. *β-actin* RT-PCR products are shown as a reference.

Formation of a blastocoel cavity requires TEAD4

TEAD4 deficiency prevented formation of a blastocoel cavity, resulting in the appearance of abnormal morulae at E3.5 (Figs 3, 4, 5 and Table 2). The fact that all E2.5 embryos, including knockouts, appeared as normal morulae strongly suggested that *Tead4*-deficient embryos failed to develop into blastocysts in vivo.

Compaction of 8-cell embryos into morulae, and the subsequent formation of trophectoderm requires E-cadherin [renamed cadherin 1 (*Cdh1*)], a calcium-dependent cell adhesion molecule (Kan et al., 2007). However, *Cdh1*$^{-/-}$ mouse embryos can develop normally until E2.5 because of maternally inherited CDH1. Thereafter, in the absence of zygotic *Cdh1* expression, defects in the embryos are detected at E3.5 and E4.5 when morulae appear with an abnormal morphology similar to those described here for *Tead4*$^{-/-}$ embryos (Ohsugi et al., 1997). Therefore, to determine if TEAD4 was required for *Cdh1* expression, E3.5 *embryos* were stained with antibodies specific to CDH1 protein and then genotyped. The results

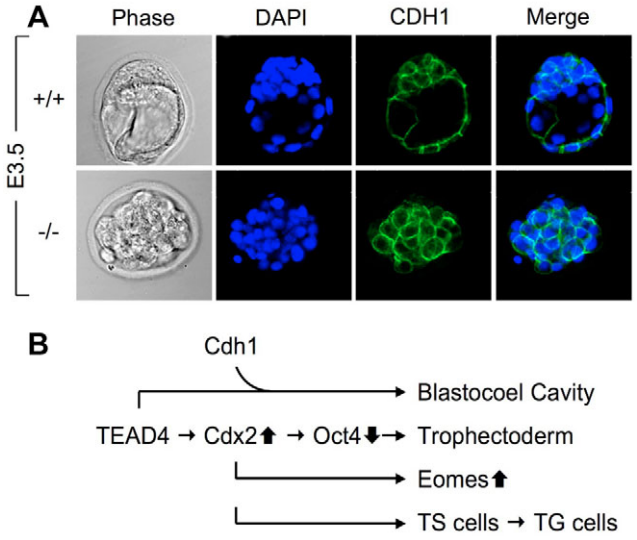

Fig. 4. TEAD4 is required for the formation of a blastocoel cavity, but not through regulation of cadherin 1 (*Cdh1*). (**A**) E3.5 embryos were treated as in Fig. 3, except that they were immunostained with anti-CDH1 antibody (green). (**B**) Schematic representation of the relationship between transcription factor TEAD4 and other genes and events in the establishment of the trophectoderm lineage. Upward and downward pointing arrows indicate increased and decreased expression, respectively.

revealed that *Tead4*$^{-/-}$ blastomere membranes contained similar amounts of CDH1 protein as wild-type embryos (Fig. 4A). Nevertheless, blastocoel cavities were clearly absent in E3.5 *Tead4*$^{-/-}$ embryos, and the well-defined adhesion boundaries characteristic of trophectodermal polar epithelium were absent. Thus, TEAD4 was required for the formation of a blastocoel cavity, but not through regulation of *Cdh1*.

TEAD4 is required for establishment of trophoblast stem cells

The studies described above revealed that TEAD4 was required for *Cdx2* expression. CDX2, in turn, was reported to be required for establishment of TS cells (Strumpf et al., 2005). To test if TS cells can be derived from *Tead4*$^{-/-}$ embryos, E3.5 embryos were isolated from heterozygote matings, and then cultured under conditions permissive to TS cell proliferation (Fig. 5). After 1-2 days, E3.5 blastocysts shed their zona pellucida and attached to the dish. After 5 days, they had produced characteristic cellular outgrowths in which TG cells were clearly evident. No difference was detected between *Tead4*$^{+/+}$ and *Tead4*$^{+/-}$ blastocysts. By contrast, the abnormal morulae shed their zonae but never attached to the dish. Neither did they produce cellular outgrowths or form blastocoel cavities. Thus, all TS cell lines were either *Tead4*$^{+/+}$ or *Tead4*$^{+/-}$; none of them were *Tead4*$^{-/-}$ (Table 2).

The ability to derive TS cell lines has been demonstrated for early blastocyst stage to 10-somite pair stage (E8) embryos (Kunath et al., 2004). Since E3.5 *Tead4*$^{-/-}$ embryos arrested development prior to blastocoel formation, they might not have been capable of generating TS cells. Therefore, an attempt was made to isolate TS cells from E2.5 embryos, some of which developed blastocoel cavities and yielded TS cell outgrowths, and some of which yielded outgrowths without forming blastocoel cavities. However, none of the TS cell lines derived from E2.5 embryos were *Tead4*$^{-/-}$ (Table 2). We conclude that at least one *Tead4* allele is required for establishment of TS cells.

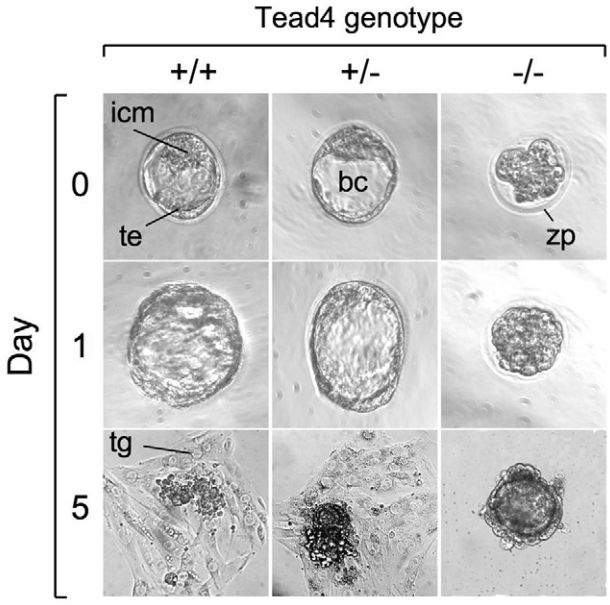

Fig. 5. *Tead4*$^{-/-}$ embryos appear abnormal at E3.5 and fail to form blastocyst outgrowths in vitro. E3.5 embryos from heterozygous matings were collected and cultured in gelatin-treated tissue culture dishes. *Tead4*$^{+/+}$ and *Tead4*$^{+/-}$ embryos appear as normal blastocysts with inner cell masses (icm), trophectoderm (te), blastocoel cavities (bc) and zonae pellucidae (zp), whereas *Tead4*$^{-/-}$ embryos appear as abnormal morulae. After 5 days in culture, the embryos and their outgrowths (tg, trophoblast giant cells) were collected for genotyping.

TEAD4 is not required for establishment and differentiation of embryonic stem cells

The fact that *Tead4*$^{-/-}$ embryos failed to develop into blastocysts, the stage from which ES cells are usually derived, together with the fact that E3.5 *Tead4*$^{-/-}$ embryos failed to attach to the surface of culture dishes, suggested that TEAD4 may also be required to derive ES cells. To test this hypothesis, ES cells were derived from E2.5 embryos using a method that allows ES cell lines to be isolated from morulae with high efficiency (Tesar, 2005). Our results revealed that *Tead4* was not required for establishment of ES cells (Fig. 6B; Table 2), since *Tead4*$^{-/-}$ and *Tead4*$^{+/+}$ ES cells were morphologically indistinguishable (Fig. 6A). Moreover, *Tead4*$^{-/-}$ ES cells expressed genes specific for pluripotent ES cells at levels comparable to those in *Tead4*$^{+/+}$ ES cells (Fig. 6C), including *Oct4/Pou5f1*, *Fgf4*, *Nanog*, *Sox2* and *Rex1* (also known as *Zfp42* – Mouse Genome Informatics). Additionally, neither *Tead4*$^{-/-}$ nor wild-type cells expressed the mesoderm marker brachyury (*T*), whereas it was clearly detected in control samples (data not shown).

To test the developmental potential of *Tead4*$^{-/-}$ ES cells in vitro, embryoid bodies were derived from both *Tead4*$^{-/-}$ and *Tead4*$^{+/+}$ ES cells and examined by semi-quantitative RT-PCR for expression of differentiation markers. Embryoid bodies are aggregates of cells derived from ES cells and propagated under conditions that prevent them from adhering to a surface. Upon aggregation, differentiation is initiated and the cells begin to recapitulate embryonic development (Keller, 2005). Embryoid bodies could be generated from both wild-type and *Tead4*$^{-/-}$ ES cells with similar ease. During differentiation, both wild-type and mutant ES cell lines rapidly repressed expression of pluripotency markers *Rex1* (Fig. 6D) and *Sox2* (data not shown) and increased expression of genes specific for endoderm (*Hnf4a*, *Gata6*), ectoderm (*Fgf5*) and mesoderm (brachyury, *T*) (Fig. 6D). Taken together, these results revealed that TEAD4 was not required for ES cell differentiation into the three primary tissue lineages.

TEAD4 is not required during postimplantation development

Tead4 is expressed in several embryonic tissues between E10.5 and E15.5 (Jacquemin et al., 1996). Therefore, to determine if TEAD4 is required during postimplantation development, the conditional *Tead4* allele (see Fig. 1) was disrupted in the embryo after implantation had occurred. *Tead4* heterozygotes were mated with *Meox2-Cre*$^{+}$ mice that express Cre recombinase exclusively in the epiblast beginning at ~E5.5 (Tallquist and Soriano, 2000). The epiblast originates from the ICM and gives rise to the three germ layers of the embryo. The *Tead4*$^{+/-}$;*Meox2-Cre*$^{+}$ mice were then mated to mice carrying the *Tead4* conditional allele ('lox'), producing *Tead4*$^{lox/-}$;*Meox2-Cre*$^{+}$ embryos. Their offspring were genotyped and found to include *Tead4* knockouts (Fig. 7A). These conditional *Tead4*$^{-/-}$ mice exhibited no obvious morphological abnormalities.

Tissue from each major organ system of the *Tead4*$^{-/-}$;*Meox2-Cre*$^{+}$ mice was assayed by RT-PCR for the presence of *Tead4* mRNA. *Tead4* mRNA was detected at varying levels in all tissues from wild-type mice except for liver and spleen (Fig. 7B). Controls included *Tead4*$^{+/+}$ and *Tead4*$^{-/-}$ ES cells. In contrast to *Tead4*$^{+/+}$, *Tead4*$^{-/-}$;*Meox2-Cre*$^{+}$ tissues did not express *Tead4*

mRNA. These results revealed that TEAD4 is required for establishment of the trophectoderm lineage during preimplantation development, whereas is it dispensable for survival later in development.

TEAD2 is not required during preimplantation development

The deletion of exons 2 and 3 in the *Tead2* gene (Kaneko et al., 2007) was similar to the deletion that eliminated exon 2 in the *Tead4* gene. In each case, the knockout allele lacked the translational start codon and a large portion of the DNA binding domains, respectively. The next in-frame start codon occurs in the fourth exon of each gene, downstream of the TEAD domain. Thus, even if truncated mRNAs were translated adventitiously, the resulting proteins would not bind DNA (Kaneko and DePamphilis, 1998). However, whereas *Tead4*$^{-/-}$ embryos failed to develop into blastocysts, *Tead2*$^{-/-}$ embryos formed blastocysts at normal Mendelian frequency and were capable of developing into viable and fertile adults (Table 2). Of the two highly homologous TEAD transcription factors expressed during preimplantation development, only TEAD4 was required for mouse development prior to implantation.

DISCUSSION

TEAD4 triggers specification of trophectoderm in preimplantation embryos

Results presented here reveal *Tead4* as the earliest gene identified to date that is required for specification of the trophectoderm cell lineage (summarized in Fig. 4B). *Tead4* expression commenced at the 2-cell stage when zygotic transcription begins, and then increased to a peak between the 8-cell and morula stages, before declining in blastocysts. Concurrent with this peak, *Tead4*-deficient embryos arrested their development after 8-cell embryos underwent compaction but before the appearance of a blastocoel cavity. Compaction between blastomeres occurred normally in 8-cell *Tead4*$^{-/-}$ embryos, as evidenced by the fact that *Tead4*$^{+/+}$, *Tead4*$^{+/-}$ and *Tead4*$^{-/-}$ E2.5 embryos were morphologically indistinguishable, and their blastomeres did not disaggregate after their zonae were dissolved with Tyrode's acid (data not shown). Moreover, the outer membrane of each blastomere contained CDH1, a protein specifically required for cell fusion in trophectoderm, at concentrations equivalent to wild-type embryos. However, instead of forming a blastocoel cavity, the blastomeres became irregular in shape and unevenly distributed. Furthermore, they failed to generate TS cells in vitro, and to express *Cdx2*. In *Cdx2*$^{-/-}$ blastocyts, the blastocoel cavity eventually collapses into a ball of cells resembling the *Tead4*$^{-/-}$ abnormal morulae described here. This could account for the inability of *Cdx2*$^{-/-}$ blastocysts to implant. *Cdx2* protein first appears in 8-cell embryos (Niwa et al., 2005) (this work). One report suggests that maternally inherited CDX2 protein is present as early as the two-cell stage (Deb et al., 2006), but the efficacy of this study has been questioned (Vogel, 2006). Thus, TEAD4 clearly acts upstream of CDX2, and given the fact that *Tead4* mRNA appears as early as the 2-cell stage, it may be directly responsible for activating *Cdx2*. There are several TEAD consensus-binding sites within the *Cdx2* promoter region, including intron sequences, but it is as yet unclear whether TEAD4 binds to them.

In contrast to *Cdx2*$^{-/-}$, *Tead4*$^{-/-}$ embryos did not form blastocoel cavities either in vivo or in vitro. Therefore, TEAD4 must activate other trophectoderm-specific genes in addition to *Cdx2*. Since *Eomes* is down regulated in both *Cdx2*$^{-/-}$ (Strumpf et al., 2005) and *Tead4*$^{-/-}$ embryos, *Eomes* is either directly or indirectly regulated by

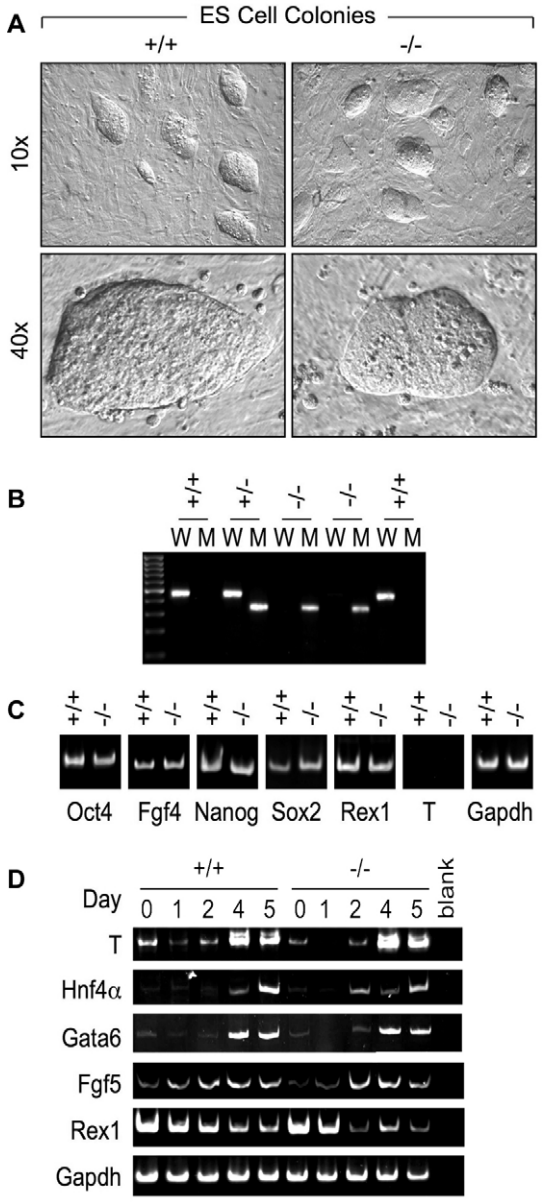

Fig. 6. *Tead4*$^{-/-}$ ES cells appear similar to and express the same markers as wild-type cells. (**A**) *Tead4*$^{+/+}$ and *Tead4*$^{-/-}$ ES cells generated from E2.5 embryos at 10× and 40× magnification. (**B**) ES cells derived from embryos produced from *Tead4* heterozygous matings were genotyped for *Tead4*. (**C**) Semi-quantitative RT-PCR of RNA from *Tead4*$^{+/+}$ and *Tead4*$^{-/-}$ ES cells for markers of pluripotentcy including *Oct4*, *Fgf4*, *Nanog*, *Sox2* and *Rex1*, and differentiation [brachyury (*T*) at 30 cycles], with *Gapdh* as a reference. (**D**) Semi-quantitative RT-PCR analysis of embryoid bodies generated from *Tead4*$^{+/+}$ and *Tead4*$^{-/-}$ ES cells at days 0, 1, 2, 4 and 5. Differentiation markers include *Rex1* for undifferentiated ES cells, brachyury (*T*) (at 35 cycles) for mesoderm, *Hnf4a* and *Gata6* for endoderm, and *Fgf5* for ectoderm, with *Gapdh* as a reference control. W, wild type; M, mutant.

CDX2, which in turn, is regulated by TEAD4. Conversely, *Fgfr2*, which encodes the trophectoderm receptor for FGF4 (Arman et al., 1998; Haffner-Krausz et al., 1999), and *Cdh1*, which encodes a cell adhesion molecule required for trophectoderm formation (Kan et al., 2007), are fully expressed in both *Cdx2*$^{-/-}$ (Strumpf et al., 2005) and *Tead4*$^{-/-}$ embryos. Thus, TEAD4 does not regulate expression of all

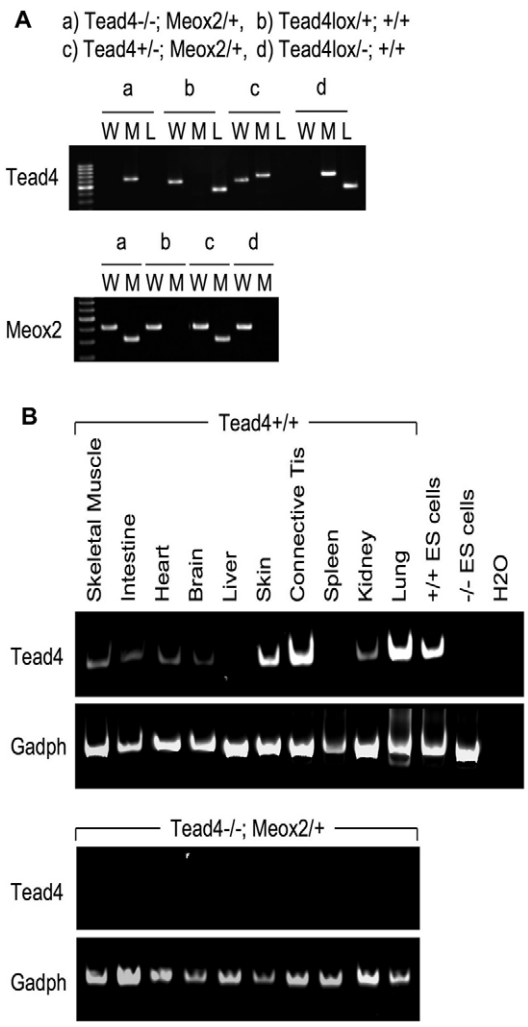

Fig. 7. *Tead4*$^{-/-}$;*Meox2-Cre*/+ mice were viable despite the absence of *Tead4* mRNA. (**A**) PCR genotyping of mouse tail samples from matings of *Tead4*$^{lox/lox}$ and *Tead4*$^{+/-}$;*Meox2-Cre*$^{+}$ animals. (**B**) Analysis of *Tead4* mRNA expression in major tissues isolated from wild-type and *Tead4*$^{-/-}$;*Meox2-Cre*$^{+}$ mice by RT-PCR. The *Tead4* PCR was performed using 37 cycles with *Gapdh* as a reference (at 35 cycles). W, wild type; L, *loxP*; M, mutant.

genes involved in formation of trophectoderm but appears to trigger a critical event early in the establishment of trophectoderm, sometime during the 8-cell to morula transition.

TEAD4 ⇒ *Cdx2* ⇑ ⇒ *Oct4* ⇓ ⇒ trophectoderm

Previous studies have shown that *Oct4* is expressed in all blastomeres of early morulae, but as the outer layer of cells differentiates into the trophectoderm during the transition from morula to blastocyst, *Oct4* expression is suppressed as *Cdx2* expression increases (Strumpf et al., 2005). Moreover, changing the ratio of OCT4 to CDX2 in ES cells can determine their fate. A high OCT4:CDX2 ratio promotes the maintenance of the totipotent ICM, whereas a low ratio promotes differentiation into trophectoderm (Niwa et al., 2005). Thus, trophectoderm differentiation appears to occur in response to an increase in the ratio of CDX2 to OCT4.

We show here that TEAD4 deficiency prevented the expression of CDX2, but not OCT4. Whereas OCT4 was restricted to the ICM of wild-type and heterozygous blastocysts, it appeared in every blastomere of mutant E3.5 embryos. This is consistent with TEAD4 regulating *Cdx2* by stimulating its expression in the outer blastomeres of morulae. Thus, it may be that OCT4:TEAD4 is actually the critical ratio that specifies the trophectoderm lineage. *Tead4* mRNA levels are high in TS and TG cells relative to ES cells and embryoid bodies, but it remains to be determined if increasing TEAD4 levels in ES cells can mimic the ability of CDX2 to induce ES cells to behave like trophectoderm.

OCT4 drives expression of FGF4 from the ICM, and FGF4 maintains the pluripotency of cells in the polar trophectoderm. The FGF receptor protein FGFR2 is required for trophectoderm to respond to FGF4. Both *Oct4* and *Fgfr2* expression occur in the absence of TEAD4. Therefore, failure of *Tead4*$^{-/-}$ embryos to establish the trophectoderm layer in blastocysts and TS cells could result from the failure to express one or more genes that respond to the FGFR2 signal. Alternatively, TEAD4-CDX2 and FGF4-FGFR2 could control separate pathways that cooperate to control proper development of the trophectoderm.

During development, TEAD4 is required only for trophectoderm specification

Given the expression of *Tead4* in 2-cell embryos, the possibility was considered that *Tead4* functioned as a master switch for differentiation of all blastomeres into either trophectoderm or ICM. Our data demonstrate that TEAD4 was required exclusively for trophectoderm specification. Only wild-type or heterozygous TS cells could be isolated either from E2.5 or E3.5 embryos (Table 2). By contrast, *Tead4*$^{-/-}$ ES cells could be isolated from E2.5 morulae. These cells did not express *Tead4* mRNA, but they did differentiate into ectoderm, mesoderm and endoderm in vitro. Wild-type ES cells isolated from E2.5 morulae have been reported to make chimeric mice (Tesar, 2005), and similar experiments are in progress with *Tead4*$^{-/-}$ ES cells.

The ES cells derived from *Tead4*$^{-/-}$ embryos were similar in appearance to wild-type ES cells and expressed similar levels of pluripotency markers. Moreover, they differentiated normally in vitro, as embryoid bodies derived from wild-type and *Tead4*$^{-/-}$ cells downregulated pluripotency markers and upregulated markers of the three primary cell types with similar kinetics.

Conditional ablation of *Tead4* in the postimplantation epiblast by intercrossing *Tead4*$^{lox/-}$;*Meox2-Cre*$^{+}$ mice confirmed these results in vivo. Unlike the full knockout, specific *Tead4* inactivation in the epiblast of 5- to 7-day-old embryos resulted in viable *Tead4*$^{-/-}$ offspring despite the lack of *Tead4* mRNA in all major tissues. Moreover, these mice had no obvious defects at the level of gross morphology. The fact that *Tead4* is expressed in many tissues of wild-type mice certainly suggests that it plays a role either in the maintenance or in regeneration of tissues, but whatever those roles may be, they are not crucial to embryonic development. Thus, it appears *Tead4* is indispensable only at the earliest stages of development and only for trophectoderm specification.

Trophectoderm specification requires functional TEAD4 protein

One concern in the analysis of any genetic mutation is that the observed phenotype resulted from the absence of the mutated gene's function or from the unexpected production of a dominant negative inhibitor from the remaining gene fragment. The C-terminal half of all four mammalian TEAD proteins contains a highly conserved transcriptional co-activator binding site for YAP65 (Vassilev et al., 2001) and TAZ (Mahoney et al., 2005). Translational start codons that could potentially translate this protein-binding domain from a

truncated mRNA exist at identical sites in both the *Tead4* and *Tead2* knockout alleles. Therefore, one would expect the C terminal fragment in *Tead2*$^{-/-}$ embryos to have the same potential toxicity as the C-terminal fragment in *Tead4*$^{-/-}$ embryos.

Three lines of evidence suggest the early preimplantation arrest of *Tead4* nullizygous embryos did not result from production of a toxic C-terminal polypeptide. First, *Tead4*$^{+/-}$ mice were viable and fertile, indicating that any toxic effects that might arise from the C-terminal fragment of the deleted allele were negligible. Second, very similar deletions in two highly homologous genes, *Tead2* and *Tead4*, did not produce the same phenotype; *Tead4*$^{-/-}$ embryos arrested development prior to formation of blastocysts, but *Tead2*$^{-/-}$ embryos did not (Table 2). In fact, most *Tead2*$^{-/-}$ embryos developed into adult mice. Finally, deletion of *Tead4* in postimplantation embryos using the *Meox2-Cre* strategy resulted in viable *Tead4*$^{-/-}$ adults in which the *Tead4* gene was ablated in all of the tissues examined, despite expression of *Tead4* in postimplantation tissues. Taken together, these results strongly suggest any adventitious expression of the C-terminal protein fragment was not toxic during postimplantation development.

TEAD transcription factors are not functionally redundant

The role of TEAD4 in specifying the trophectoderm lineage appears to be unique among TEAD family members. A very similar deletion in the closely related *Tead2* gene did not interfere with preimplantation development or implantation, even though both genes are expressed concurrently during preimplantation development (Kaneko et al., 2007). This was surprising since their DNA binding and transactivation domains are highly similar and because all four TEAD proteins appear to bind the same transcriptional co-activators (Mahoney et al., 2005; Vassilev et al., 2001). Therefore, either TEAD2 is not expressed in the same blastomeres as TEAD4, or the two genes bind to different DNA sequences in the presence of their transcriptional co-activator (Halder and Carroll, 2001), or they bind to the same DNA sequence but to different co-activators (but see above). Evidence argues against the first possibility since *Tead2* is expressed in both ICM and trophectoderm of blastocysts at relatively equivalent levels (Kaneko et al., 2004). What is clear is that inactivation of the *Tead2* alleles markedly increased the risk of exencephaly, a defect in neural tube closure that occurs during postimplantation development as early as E11.5 (Kaneko et al., 2007). As for the remaining Tead genes, mouse embryos lacking TEAD1 fail to develop a proper heart and die between E11 and E12 (Chen et al., 1994), and TEAD3-deficient mice have not yet been described. Thus, most, if not all, of the TEAD transcription factors serve at least one nonredundant function in mammalian development. Additional roles may be revealed in the future that are currently masked by the ability of other members to substitute for the ablated TEAD protein. Other roles for TEAD proteins may also exist in adult animals during regeneration of adult neural stem cells (Ramalho-Santos et al., 2002) or muscle (Zhao et al., 2006). Furthermore, other defects in adult *Tead2* and *Tead4* nullizygous mice may manifest themselves with age.

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