Tcf7l1 prepares epiblast cells in the gastrulating mouse embryo for lineage specification

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SUMMARY
The core gene regulatory network (GRN) in embryonic stem cells (ESCs) integrates activities of the pro-self-renewal factors Oct4 (Pou5f1), Sox2 and Nanog with that of an inhibitor of self-renewal, Tcf7l1 (Tcf3). The inhibitor function of Tcf7l1 causes dependence on extracellular Wnt/β-catenin signaling activity, making its embryonic role within the ESC GRN unclear. By analyzing intact mouse embryos, we demonstrate that the function of Tcf7l1 is necessary for specification of cell lineages to occur concomitantly with the elaboration of a three-dimensional body plan during gastrulation. In Tcf7l1−/− embryos, specification of mesoderm is delayed, effectively uncoupling it from the induction of the primitive streak. Tcf7l1 repressor activity is necessary for a rapid switch in the response of pluripotent cells to Wnt/β-catenin stimulation, from one of self-renewal to a mesoderm specification response. These results identify Tcf7l1 as a unique factor that is necessary in pluripotent cells to prepare them for lineage specification. We suggest that the role of Tcf7l1 in mammals is to inhibit the GRN to ensure the coordination of lineage specification with the dynamic cellular events occurring during gastrulation.

KEY WORDS: EpiSC, Primitive streak, Tcf3, Tcf7l1, Wnt

INTRODUCTION
Mammals are unlike most other animals in that the small zygote requires substantial cell proliferation before the elaboration of a basic body plan can begin. During the growth of the early embryo, individual cells must retain the ability to make all adult cell types, i.e. pluripotency. Understanding mechanisms that control pluripotency is an important goal of stem cell research and was stimulated by the discovery of conditions enabling embryonic stem cell (ESC) cultures to be derived from outgrowths of the blastocyst inner cell mass (ICM) (Evans and Kaufman, 1981; Martin, 1981).

In vitro experiments with ESCs showed that the Oct4 (Pou5f1 – Mouse Genome Informatics), Sox2 and Nanog transcription factors constitute core components of a gene regulatory network (GRN) that stimulates self-renewal of pluripotent cells. The GRN model is supported by overlapping sites of chromatin occupancy for Oct4, Sox2 and Nanog proteins, including on another’s genes (Boyer et al., 2005; Cole et al., 2008; Loh et al., 2006; Marson et al., 2008), and extensive protein-protein interactions between the three factors (Chambers and Tomlinson, 2009; Kim et al., 2008; Lian et al., 2008; Wang et al., 2006). Tcf7l1 (formerly Tcf3) has been identified as a crucial regulator of the pluripotency GRN in ESCs by studies showing that Tcf7l1 co-occupies Oct4, Sox2 and Nanog sites in chromatin (Cole et al., 2008; Marson et al., 2008; Tam et al., 2008) and that Tcf7l1 regulates the expression of Oct4 and Nanog target genes (Cole et al., 2008; Pereira et al., 2006; Tam et al., 2008; Yi et al., 2008). Recently, the Esrrb transcription factor was identified as a direct target of Tcf7l1 regulation important for Tcf7l1-mediated effects on self-renewal in vitro (Martel et al., 2012).

Whereas Esrrb, Oct4, Sox2 and Nanog all stimulate self-renewal, genetic experiments unequivocally show that Tcf7l1 inhibits self-renewal (Guo et al., 2011; Pereira et al., 2006; Salomonis et al., 2010; Wray et al., 2011; Yi et al., 2011; Yi et al., 2008). Interestingly, Tcf7l1 is a transcriptional repressor in the Wnt/β-catenin pathway (Wu et al., 2012), and Wnt activity is necessary for mouse ESC self-renewal (ten Berge et al., 2011). Ablating Tcf7l1 is sufficient to replace a requirement for Wnt/β-catenin signaling, indicating that endogenous Tcf7l1 expression causes ESC dependence on Wnt/β-catenin (Wray et al., 2011; Yi et al., 2011). Conversely, Wnt3a treatment rescues self-renewal in ESCs inhibited by Tcf7l1 overexpression (Ye et al., 2011). Roles for Tcf7l1 in differentiation have been suggested, but in vitro differentiation assays have revealed only minor and variable lineage specification defects in Tcf7l1-deficient ESCs (Pereira et al., 2006; Salomonis et al., 2010; Tam et al., 2008). Thus, whereas the embryonic function of factors that stimulate the GRN (i.e. Oct4, Nanog and Sox2) is clearly necessary to stimulate the self-renewal of pluripotent cells as early embryos expand (Avilion et al., 2003; Mitsui et al., 2003; Nichols et al., 1998), an embryonic function for an inhibitor of GRN activity, such as Tcf7l1, has not been elucidated. As such, it is not clear why pluripotent cells express high levels of an ostensible inhibitor of their self-renewal.

With a perspective that the evolution of the pluripotency GRN in mammals included the Tcf7l1 inhibitor activity to enable some aspect of early embryogenesis, we reasoned that examining embryogenesis in Tcf7l1 mutant embryos would elucidate a role for Tcf7l1 in pluripotent cells. Building upon previous work showing that Oct4, Sox2, Nanog and Tcf7l1 are expressed during gastrulation (Avilion et al., 2003; Hart et al., 2004; Merrill et al., 2004; Morkel et al., 2003; Yamaguchi et al., 2005; Yeom et al., 1996), we define changes in their protein expression that occur in epiblast cells prior to and during cell lineage specification. Gene expression defects in Tcf7l1−/− embryos coincided with a delay in the specification of mesoderm at the primitive streak region, demonstrating that Tcf7l1 is necessary to couple lineage specification with primitive streak morphogenesis. In vitro, ESCs required Tcf7l1 to rapidly convert to a state in which they formed mesoderm in response to Wnt/β-catenin signaling. We suggest that the activity of Tcf7l1 as a negative regulator of the pluripotency GRN is closely related to its...
first embryonic function, which enables appropriate responses to lineage specification signals.

MATERIALS AND METHODS

Preparation of embryos for multi-dimensional expression analysis

Embryos were fixed within decidua for 1 hour at 4°C in 4% paraformaldehyde (PFA), washed in PBS, and cryopreserved by washing in 15% sucrose for 1 hour at room temperature and in 30% sucrose overnight at 4°C. Transverse 8 μm sections through entire embryos were taken using a Microm HM550 cryostat and collected four to a slide in groups of four slides at a time, such that the first slide contained sections #1, #5, #9 and #13, the second slide sections #2, #6, #10 and #14, and so on. This enabled tracking of the position of sections along the proximal-distal axis of each embryo, orientation of the anterior-posterior axis, and use of adjacent epiblast sections for four individual experiments. For each assay, sections throughout the entire proximal-distal axis of each embryo were used. Embryos were staged by morphological features (Downs and Davies, 1993) and expression of brachyury, an early marker of mesoderm cells at the primitive streak (PS) (supplementary material Fig. S1A) (Wilkinson et al., 1990). Unless multiple sections of single embryos are depicted, as in Fig. 4, the images show embryo sections from the proximal epiblast, as depicted in supplementary material Fig. S1A.

Immunofluorescent staining

Embryo sections for immunofluorescence staining were fixed for 8-10 minutes in cold 4% PFA, washed with PBS, blocked for 1 hour at room temperature in 1% BSA, 0.1% Triton X-100 and 5% normal donkey serum, and treated overnight at 4°C with the following antibodies diluted in blocking solution: rabbit anti-Tcf7l1 (Pereira et al., 2006) (1:500), rat anti-E-cadherin (1:100, M. Takeichi, DHSB), rabbit anti- Nanog (1:100, Abcam), goat anti-Oct4 (1:500, Santa Cruz), goat anti-Sox2 (1:500, Santa Cruz) and goat anti-brachyury (1:500, Santa Cruz). FITC-, Texas Red- and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:100. Immunofluorescence was imaged using a Zeiss LSM 700 or Zeiss LSM 5 Pascal microscope.

Quantification was performed on proximal epiblast sections of early-streak embryos. Individual detection channels were separately imported into Photoshop (Adobe) as individual RGB channels to create composite images in which each channel could be analyzed separately or together. The center of the PS in each embryo was identified by cell morphology and the presence of Prickle1 expression in nearby sections from the same embryo. Each nucleus was numbered and its distance from the center of the PS was calculated using the Ruler and Measurement tool. The Quick Selection tool was used to select the epiblast, and expression of each protein was normalized to the mean level of expression throughout the entire proximal-distal axis of each embryo. Distance measurements were normalized to the entire length of the proximal-distal axis of each embryo, orientation of the anterior-posterior axis, and use of adjacent epiblast sections for four individual experiments. For each assay, sections throughout the entire proximal-distal axis of each embryo were used. Embryos were staged by morphological features (Downs and Davies, 1993) and expression of brachyury, an early marker of mesoderm cells at the primitive streak (PS) (supplementary material Fig. S1A) (Wilkinson et al., 1990). Unless multiple sections of single embryos are depicted, as in Fig. 4, the images show embryo sections from the proximal epiblast, as depicted in supplementary material Fig. S1A.

In situ hybridization

Whole-mount in situ hybridization was performed as described (Merrill et al., 2004). Probes for brachury, Nanog, Gsc and Prickle1 were kind gifts from D. Wilkinson (Wilkinson et al., 1990), I. Chambers (Chambers et al., 2003), E. DeRobertis (Conlon et al., 1994) and T. Rodriguez (Crompton et al., 2007), respectively. All other probes were PCR cloned from cDNA or genomic DNA corresponding to the following regions of the full-length Ensembl cDNA sequence: Mixl1 (bp 994-2175), Oct4 (bp 469-935) and Sox1 (bp 2325-2681).

ESC transition assays

Mouse ESCs were maintained on gelatin-coated plates in serum-containing medium [Knockout DMEM (Gibco) supplemented with 15% fetal calf serum (Atlanta Biologicals)]. The ESC transition procedure was adapted from previous work (Gao et al., 2009). Briefly, ESCs were plated at 2x10³ cells/cm² in serum-containing ESC media on fibronectin (Millipore)-coated plates.

After 24 hours, media were replaced with N2B27 media (Gibco) containing 20 ng/ml activin A (R&D Systems) and 12 ng/ml Fgf2 (R&D Systems). Cells were given fresh medium every day, and were passaged with Dispase (Roche) every 2 days. CHIR99021 (Stemgent) was added to N2B27 media to a final concentration of 3 μM at 24-hour time points as depicted in Fig. 6A. For ESC colony formation assays, colonies were trypsinized to a single-cell suspension and plated in normal ESC culture conditions at 1000 cells per well, and scored for ESC-like colonies after 4 days.

Generation of doxycycline-inducible Nanog transgenic mice and in vivo induction of Nanog expression

Standard molecular biology techniques were used to construct a targeting vector. Briefly, Nanog cDNA was cloned from pPyCagNanogLP (a kind gift of Dr Ian Chambers) into a vector containing a Tet operator (TetO) and upstream and downstream polyadenylation sequences (Chambers et al., 2003). This TetO-Nanog cassette was then cloned into an HPRT targeting vector and electroporated into F3 ESCs (both kind gifts of Dr Stephen Duncan) (Misra et al., 2001). Four independent ESC clones were injected by the UIC Core Transgenic Facility into recipient C57BL/6 blastocysts for germline transmission. Males harboring this TetO-Nanog gene cassette were mated to females ubiquitously expressing the reverse tetracycline transactivator from the Rosa26 locus (derived from JAX stock #005670) and females were checked for vaginal plugs daily. Pregnant females were fed Chow containing 20 mg/kg doxycycline (Bioserv) at embryonic day (E) 2.5 to induce Nanog expression throughout the epiblast prior to the pre-streak stage (supplementary material Fig. S4E). Owing to the integration of the TetO-Nanog cassette into the Hprt locus on the X-chromosome and X-inactivation of the paternal X-chromosome in extra-embryonic tissues, this mating scheme restricted Nanog overexpression to the epiblast.

RESULTS

Dynamic expression of pluripotency factors during gastrulation

Previous studies showed that pluripotency factors (Tcf7l1, Oct4, Sox2 and Nanog) are expressed in the epiblast at the time of lineage specification (Avilion et al., 2003; Hart et al., 2004; Merrill et al., 2004; Morkel et al., 2003; Yamaguchi et al., 2005; Yen et al., 1996). Each of these analyses focused on a single factor or provided limited data for multiple stages of gastrulation. Comparisons of factor expression were further obscured by the combination of the speed of progression through gastrulation (Downs and Davies, 1993; Rivera-Perez et al., 2010), differences in embryo staging, and different types of expression analyses among the studies. In addition, cell-based experiments demonstrated that relatively small changes in the levels of Oct4, Sox2 and Nanog have a significant influence on the specification of lineages during differentiation in vitro (Chambers et al., 2003; Mitsui et al., 2003; Niwa et al., 2000; Thomson et al., 2011). Thus, although the previous reports combine to demonstrate the expression of pluripotency factors during gastrulation, analysis of expression was not completed with sufficient precision to compare the relative levels of factors.

We used two complementary methods to examine pluripotency factor expression during gastrulation. First, changes in the patterns of expression were determined during progression through the pre-streak, early-streak and mid-streak stages of gastrulation. Brachury protein was absent from pre-streak embryos (Fig. 1A), but appeared in early-streak embryos, where it marked the posterior epiblast prior to formation of a nascent layer of mesoderm (Fig. 1A’), which was evident later in mid-streak embryos (Fig. 1A”). Tcf7l1 was uniformly expressed throughout the epiblast of pre-streak embryos, but began to reduce near the primitive streak (PS) of early-streak embryos, and progressively diminished in the posterior epiblast through gastrulation (Fig. 1B-B”). Oct4 protein expression remained uniform throughout the epiblast of pre-, early- and mid-streak
correlation (the PS (Fig. 1F). Both Tcf7l1 and Sox2 showed a strong positive
expression in ESCs (Pereira et al., 2006; Yi et al., 2008). The opposing expression patterns of Nanog and Sox2
expression showed a strong negative correlation with
early-streak embryos were chosen for analysis because they have
the PS, a conclusion supported by double immunofluorescence
shows that Nanog is expressed prior to brachyury as cells move
towards the PS, indicating that it does not need to be lost for
district of the PS region in the early- and mid-streak
labels lateral cells of the proximal epiblast move towards and enter the PS region
the epiblast, as described in
expression in individual cells relative to their position in the epiblast, as described in 
the PS (r-axis). The number of nuclei analyzed (n) and Pearson
ration between protein expression patterns and the distance of
and Sox2 intensify during progression through the early-streak and mid-streak
increase (Fig. 1E’) and Nanog levels increase (Fig. 1E’,E”) at the PS.
To provide a complementary analysis of pluripotency factor
dynamics prior to lineage specification, we measured levels of 
protein immunoreactivity in individual epiblast cells of early-streak embryos. Labeling experiments showed that lateral cells of the proximal epiblast move towards and enter the PS region (Lawson et al., 1991) (supplementary material Fig. S1B). Thus, the correlation between protein expression patterns and the distance of a cell from the PS can be used to represent the protein expression changes that occur in epiblast cells as they move towards the PS. Early-streak embryos were chosen for analysis because they have just initiated mesoderm specification at the PS. Brachyury showed the expected strong negative correlation with distance from the PS (r=−0.74), as brachyury was expressed only in epiblast cells near the PS (Fig. 1F). Both Tcf7l1 and Sox2 showed a strong positive correlation (r=0.60 and r=0.65), whereas Oct4 expression did not appear to change near the PS (r=0.32) (Fig. 1G-I). Like brachyury, Nanog expression showed a strong negative correlation with distance from the PS (r=−0.72); however, Nanog was expressed in a broader posterior domain of epiblast cells (Fig. 1J). This indicated that Nanog is expressed prior to brachyury as cells move towards the PS, a conclusion supported by double immunofluorescence staining (supplementary material Fig. S1C). Taken together, these data indicate that, as epiblast cells move towards the PS, they undergo gene expression changes through which Nanog expression is increased and Tcf7l1 and Sox2 are downregulated.

Tcf711 accelerates the dynamics of pluripotency factor expression during gastrulation
Unlike embryos genetically lacking Oct4, Sox2 or Nanog gene products, Tcf7l1−/− embryos survive implantation and undergo gastrulation (Avilion et al., 2003; Merrill et al., 2004; Mitsui et al., 2003; Nichols et al., 1998). Ablation of Tcf7l1 on a mixed genetic background (C57BL/6/129Sv) generated a broad range of morphogenetic defects that were phenotypically classified into two groups (mildly and severely affected) occurring at roughly equal frequency (48% and 52%, respectively) (Merrill et al., 2004). In this mixed background, mesoderm markers displayed a variety of abnormal expression patterns in mutants; the earliest defects were reported for E7.0 embryos and included reduced brachyury (Merrill et al., 2004). We reasoned that the variable expressivity would complicate analysis of gene expression in Tcf7l1−/− epiblasts and sought to reduce variability. Repeated backcrosses (>20 generations) produced a Tcf7l1−/− strain congenic for C57BL/6, from which greater than 95% of Tcf7l1−/− embryos displayed the previously described severe phenotype (Merrill et al., 2004). Although the genetic determinants influencing the Tcf7l1−/− phenotype remain unknown, the uniformity of the Tcf7l1−/− phenotype permitted faithful examination of pluripotency factor expression among mutant embryos.

Analysis of pluripotency factor expression in Tcf7l1−/− blastocysts and E5.5 embryos indicated no significant differences relative to Tcf7l1+/+ (either Tcf7l1+/− or Tcf7l1+/+) embryos (supplementary material Fig. S2A,B). Differences were first detected at E6.5, as the level of Nanog mRNA was significantly increased in Tcf7l1−/− embryos (Fig. 2A). By contrast, Oct4 mRNA levels were not increased in Tcf7l1−/− embryos (Fig. 2A), which is consistent with weak direct effects of Tcf7l1 ablation on Oct4 expression in ESCs (Pereira et al., 2006; Yi et al., 2008). The domain of Nanog mRNA expression was expanded to the anterior of Tcf7l1−/− embryos (Fig. 2B).
To determine whether Tcf7l1 affected the dynamics of pluripotency protein expression during PS formation, we analyzed immunoreactivity in Tcf7l1−/− embryos as described above for wild-type embryos (Fig. 1). The most substantial effect was on Nanog protein, which was detected in every cell of Tcf7l1−/− epiblasts at pre-, early- and mid-streak stages (Fig. 2C–C’; supplementary material Fig. S3B–E) but not in E5.5 embryos (supplementary material Fig. S2B). Oct4 levels were uniform throughout the Tcf7l1−/− epiblast (Fig. 2D–D’). Sox2 protein levels remained uniform in Tcf7l1−/− pre-streak and early-streak embryos (Fig. 2E–E’), and reduction of Sox2 protein at the PS region did not occur in Tcf7l1−/− embryos until the mid-streak stage (Fig. 2E”). Comparison of relative factor levels with position in early-streak Tcf7l1−/− embryos showed a weak negative correlation for Nanog ($r=-0.36$) and no significant correlation for Oct4 ($r=0.09$) or Sox2 ($r=0.10$) (Fig. 2F–H). Comparing patterns in Tcf7l1−/− and Tcf7l1+/- epiblasts shows that Tcf7l1 is necessary for the changes to Nanog and Sox2 expression in epiblast cells as they move towards the PS to undergo lineage specification (supplementary material Fig. S1,D,E).

Given the ability of Nanog to stimulate self-renewal in vitro and the repression of Nanog promoter activity by Tcf7l1 (Chambers et al., 2003; Mitsui et al., 2003; Pereira et al., 2006), we tested whether dysregulated Nanog is sufficient to cause a Tcf7l1−/− phenotype by engineering a doxycycline-inducible Nanog transgenic mouse (supplementary material Fig. S4). Induction of Nanog overexpression in transgenic embryos generated an expansion of Nanog expression similar in timing and pattern to the dysregulated Nanog expression in Tcf7l1−/− embryos (compare Fig. 2I with 2C’; supplementary material Fig. S4E). The patterns of Oct4, Sox2 and Tcf7l1 gene expression (Fig. 2J–P) were indistinguishable from those observed in transgenic embryos lacking Nanog overexpression (supplementary material Fig. S5) and in wild-type embryos (Fig. 1B–D,G–I). Thus, the role of Tcf7l1 in the epiblast extends beyond the regulation of Nanog expression.

**Tcf7l1 is necessary for maturation of the epiblast during gastrulation**

Recent work showed that pluripotency, as assessed by the ability of cells to form EpiSCs, is lost from mouse embryos between E7.5 and E8.25 (Osorno et al., 2012). This coincides with the reduction of Oct4 and Nanog mRNA, which begin declining at E7.5 and are undetectable by E8.5 (Fig. 3A–E) (Osorno et al., 2012). In Tcf7l1−/− embryos, Nanog and Oct4 mRNAs were expressed at high levels throughout the embryonic ectoderm through E8.5 (Fig. 3A’–E’). Otx2 is another marker of epiblast progression and has recently been shown to stabilize an epiblast-like state (Acampora et al., 2013). Otx2 mRNA is uniformly expressed throughout the early epiblast and becomes progressively restricted to the anterior neuroectoderm by E7.5 in wild-type embryos (Fig. 3F). In Tcf7l1−/− embryos, Otx2...
expression persisted throughout the entire E7.5 epiblast (Fig. 3F'). Severely affected E8.5 Tcf7l1−/− embryos lacked morphological signs of neural fold formation, which is typically complete by this time (Fig. 3) (Merrill et al., 2004). The early neuroectoderm marker Sox1 is induced by E8.0 and rapidly intensifies from E8.0 to E8.5 during neuroectoderm differentiation in wild-type embryos (Fig. 3G-I). In Tcf7l1−/− mutants, Sox1 mRNA was not detectable through E8.5 (Fig. 3G-I). The abnormal morphology and altered patterns of gene expression indicated that neural specification is defective in epiblast cells lacking Tcf7l1, and that the Tcf7l1−/− epiblast retained characteristics of earlier epiblast stages for a prolonged period.

**Coupling of mesoderm specification and primitive streak induction requires Tcf7l1**

We next tested the hypothesis that effects of Tcf7l1 in the epiblast are important in preparing cells for lineage specification. In normal mouse embryos, the timing of mesoderm specification occurs concomitantly with the formation of the PS. Therefore, examining the onset of mesoderm specification in Tcf7l1−/− embryos presented the possibility of identifying a novel role in coordinating cell fates with morphogenesis. However, the rapid formation of the PS during gastrulation generates substantial variability in developmental stage between individual embryos in a single litter (Downs and Davies, 1993; Rivera-Perez et al., 2010), and distinct markers have not been identified in the mouse to separate the specification of mesoderm from the induction of morphological PS formation. In chickens, inhibition of the Wnt planar cell polarity (PCP) pathway blocked cell movement in the PS without inhibiting mesoderm gene expression (Voiculescu et al., 2007). Thus, the PCP pathway is specifically required for PS morphogenesis and not for lineage specification at the PS. The PCP pathway gene Prickle1 was previously shown to be expressed in gastrulation stage embryos (Crompton et al., 2007), indicating its potential utility as a marker of morphological PS formation. Indeed, the timing and pattern of Prickle1 mRNA expression in the epiblast are very similar to those of brachyury protein expression (Fig. 4A). Therefore, we examined the coordination of brachyury and Prickle1 expression in individual embryos by generating arrays of cryosections such that several assays could be performed on adjacent sections throughout the proximodistal length of individual embryos.

Perfect coordination of Prickle1 mRNA and brachyury protein expression at the onset of PS formation was apparent in all Tcf7l1+ embryos (Fig. 4B; n=43 of 43), and we never observed Tcf7l1+ embryos that expressed Prickle1 without coincident brachyury expression in adjacent sections. Mixl1 expression, another early marker of mesoderm lineage specification, was also perfectly coupled with Prickle1 expression in Tcf7l1−/− embryos (Fig. 4D). Thus, Prickle1 served as a valuable marker to stage embryos and determine whether mesendoderm lineage specification should be detectable. In Tcf7l1−/− embryos defined as early-streak stage based on the presence of Prickle1 expression in the posterior epiblast, the expression of brachyury, Mixl1 and Gsc was strikingly absent in adjacent sections (Fig. 4C, n=16 of 16; Fig. 4E, n=6 of 6; supplementary material Fig. S6A-A', n=3 of 3). This demonstrated that mesendoderm specification was uncoupled from PS formation in Tcf7l1−/− embryos. Brachyury and Mixl1 expression and mesenchymal mesoderm cell types were all apparent in later stage Tcf7l1−/− embryos (supplementary material Fig. S6B-C') (Merrill et al., 2004; Wu et al., 2012). Formation of mesoderm cells indicates that Tcf7l1 is not necessary for the specification of mesoderm per se, but it is specifically needed for the initial response of epiblast cells to lineage specification signals. Taken together with normal Prickle1 expression in Tcf7l1−/− embryos, these data demonstrated that, rather than an overall delay in gastrulation, Tcf7l1−/− epiblast cells exhibit delayed mesendoderm specification relative to the induction of the PS, thus uncoupling these two tightly linked processes.

Tcf/Lef-β-catenin activation of target genes, including brachyury, has been associated with mesendoderm gene expression at the PS (Arnold et al., 2000; Galceran et al., 2001). Although mesendoderm gene expression occurred in later stage Tcf7l1−/− embryos (supplementary material Fig. S6C,C') (Merrill et al., 2004; Wu et al., 2012), it was formally possible that Tcf7l1 could be required to stimulate initial lineage specification through Tcf7l1-β-catenin complexes. To test this possibility, we used the Tcf7l1ΔNΔN knock-in mouse, in which the Tcf7l1-β-catenin interaction is ablated (Wu et al., 2012). Tcf7l1ΔNΔN embryos displayed perfectly coupled brachyury and Prickle1 expression (Fig. 4F), indicating that Tcf7l1-β-catenin is not necessary for timely mesoderm specification during PS formation. Tcf7l1ΔNΔN embryos also exhibited normal expression of Nanog (supplementary material Fig. S6D,D') and proper restriction of Otx2 expression in the E7.5 epiblast (supplementary material Fig. S6D'). Finally, the expression of Wnt-β-catenin was.

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**Fig. 3. Tcf7l1 is required for silencing of pluripotency factor expression and early neuroectoderm specification.** (A-I) Whole-mount in situ hybridization for Oct4 (A-C'), Nanog (D-D'), Otx2 (F-F') and Sox1 (G-G') in Tcf7l1+ (A-I) and Tcf7l1−/− (A'-I') mouse embryos of the indicated ages. Lateral views, except for frontal views in G,G'.H,H'.I.'.
target gene Axin2 was indistinguishable between Tcf7l1+, Tcf7l1−/− and Tcf7l1ΔN/ΔN embryos (supplementary material Fig. S6E). Together, these data indicated that the requirement for Tcf7l1 in the epiblast was independent of its interaction with β-catenin.

Forced Nanog expression is sufficient to block lineage specification in ESCs (Chambers et al., 2003; Mitsui et al., 2003; Thomson et al., 2011). We used Nanog-overexpressing transgenic embryos to test whether expanded expression of Nanog was responsible for the delayed mesoderm lineage specification in Tcf7l1−/− embryos. Of 18 Nanog-overexpressing embryos examined, 17 had coupled brachyury and Prickle1 expression indistinguishable from non-expressing controls (Fig. 4G). Thus, recapitulating Nanog dysregulation was not sufficient to disturb the dynamics of pluripotency factor expression (Fig. 2I-P) or to cause a significant delay in mesoderm specification.

Tcf7l1 stimulates a switch in the response to Wnt/β-catenin signaling from self-renewal to mesoderm specification

To determine the mechanism underlying the delayed mesoderm specification in Tcf7l1−/− embryos, we examined the transition from naïve to primed states of pluripotency in vitro. When switched to EpiSC culture conditions (serum-free N2B27 media with Fgf2 and activin A), naïve ESCs rapidly adopt a primed EpiSC morphology, exhibit reduced alkaline phosphatase (AP) activity and lose the ability to self-renew as naïve ESCs (Guo and Smith, 2010; Rugg-Gunn et al., 2012). After 3 days of EpiSC conditions (Fig. 5A), nearly all Tcf7l1+/+ colonies (98%) exhibited an AP-negative EpiSC-like morphology (Fig. 5B). Conversely, less than half of Tcf7l1−/− colonies (48%) formed AP-negative EpiSC-like colonies after 3 days (Fig. 5B).

To measure the commitment to a primed state after 3 days in EpiSC conditions, colonies were dissociated into single cells, replated in ESC conditions and assayed for AP-positive ESC-like colonies after 4 days. Few (2.0±1.4) ESC colony-forming units (CFU) were recovered per 1000 Tcf7l1+/+ cells replated (Fig. 5C), consistent with previous observations that EpiSCs do not readily revert to the naïve ESC state (Bernemann et al., 2011; Guo and Smith, 2010). By contrast, the number of ESC CFU (36±2.8) was significantly greater from replated Tcf7l1−/− cells (Fig. 5C), indicating that more Tcf7l1−/− cells retained the capacity to self-renew as ESCs. This resistance to priming was temporary for Tcf7l1−/− ESCs, as nearly all Tcf7l1−/− colonies exhibited an AP-negative EpiSC-like morphology after 5 days in EpiSC conditions (supplementary material Fig. S7A).

The expression of several genes differs between naïve ESCs and primed EpiSCs. Whereas Oct4 is typically expressed at similar levels in ESCs and EpiSCs, Nanog, Sox2 and other markers of the
naïve state (Esrrb, Pecam1, Rex1 (Zfp42 – Mouse Genome Informatics)] are expressed at far lower levels in EpiSCs, and epiblast-specific genes (Fgf5, Dnmt3b) are expressed at much higher levels (Brons et al., 2007; Greber et al., 2010; Tesar et al., 2007). Indeed, after 3 days in EpiSC conditions, Fgf5 and Dnmt3b were increased whereas Nanog, Sox2, Esrrb, Pecam1 and Rex1 levels were reduced in both Tcf7l1+/+ and Tcf7l1−/− cells (Fig. 5D; supplementary material Fig. S7B). However, the reduction of the naïve markers Esrrb, Pecam1 and Rex1 was significantly less substantial in Tcf7l1−/− cells (Fig. 5D). After an additional 2 days in EpiSC conditions, Sox2, Esrrb, Pecam1 and Rex1 were further decreased in Tcf7l1−/− to levels similar to those in Tcf7l1+/+ cells after 3 days in EpiSC conditions (Fig. 5D). Somewhat surprisingly, Oct4 levels decreased in both Tcf7l1+/+ and Tcf7l1−/− cultures after 5 days in EpiSC conditions (Fig. 5D). This is likely to indicate some level of conversion to a later epiblast state and that differentiation...
is occurring in this assay, consistent with decreased Oct4 in EpiSCs derived from E6.5 epiblasts compared with E5.5 epiblasts (Bermemann et al., 2011). Together, these data indicate that, in the absence of Tcf7l1, ESCs were delayed, but not completely blocked, in transitioning to a primed, epiblast-like state when subjected to EpiSC conditions. This in vitro delay was consistent with Tcf7l1 repressing naïve ESC self-renewal and consistent with a function of Wnt/β-catenin in maintaining a naïve state (ten Berge et al., 2011).

Wnt/β-catenin clearly stimulates self-renewal of mouse ESCs, i.e. cells in a naïve state (ten Berge et al., 2011; Sato et al., 2004; Ying et al., 2008). Conversely, activation of Wnt signaling in EpiSCs by addition of the Gsk3 inhibitor CHIR99021 (CH) promotes mesendoderm and extra-embryonic differentiation (Greber et al., 2010). Furthermore, recent reports indicate that Wnt/β-catenin stimulates mesoderm specification of human ESCs, i.e. cells considered to be in a primed state (Davidson et al., 2012; Singh et al., 2012). We reasoned that the differences in these responses to Wnt/β-catenin could be dependent on the transition from naïve to primed pluripotency. To test this, we activated Wnt/β-catenin signaling at 24-hour intervals after switching from ESC to EpiSC conditions (Fig. 6A) to identify the point at which the cellular response to Wnt/β-catenin switches from self-renewal to mesoderm specification. CH was used to stimulate Wnt/β-catenin signaling because of its ability to activate the pathway regardless of changes to Wnt-receptor complexes that may occur during the transition.

When CH was added immediately upon switching cells to EpiSC conditions (CH1), Tcf7l1−/− colonies maintained an ESC-like morphology (Fig. 6B), the ability to self-renew in ESC conditions (Fig. 6C) and naïve gene expression (Fig. 6D). When CH was added 24 or 48 hours (CH2 and CH3) after switching to EpiSC conditions, its effectiveness in maintaining ESC-like characteristics progressively diminished (Fig. 6B-D). Thus, CH inhibited the transition to the primed state, but this ability was limited to a short window of time at the beginning of the transition. Like Tcf7l1−/− cells, Tcf7l1+/− cells cultured in the CH1 regimen exhibited enhanced ESC characteristics (Fig. 6B-D). However, unlike Tcf7l1−/−, the maintenance of ESC-like morphology and gene expression by CH was not attenuated in Tcf7l1−/− cells in the CH2 or CH3 regimen (Fig. 6B,C). Thus, the window during which CH could inhibit the transition to the primed state was expanded by the absence of Tcf7l1.

To determine whether Tcf7l1 regulation of the transition to the primed state affected mesendoderm specification, we measured the expression of brachyury, Mixl1, Gsc and Foxa2. Prior to transitioning to a primed state, ESCs expressed very low levels of these genes, and CH did not stimulate Mixl1, Gsc or Foxa2 expression in either Tcf7l1+/− or Tcf7l1−/− EpiSCs (Fig. 6E; supplementary material Fig. S7C; left). Brachyury expression was increased by CH in ESCs (Fig. 6E, left). Each gene was expressed at much lower levels (~100- to 1000-fold) in ESCs compared with cells subjected to 5 days of EpiSC conditions (Fig. 6E; supplementary material Fig. S7C; right), indicating that mesoderm specification is dependent on transition to the primed state. The notable point is that, both before and after the transition to a late epiblast state, Tcf7l1−/− and Tcf7l1+/− cells expressed similar levels of mesoderm marker genes and exhibited similar responses to CH. Thus, Tcf7l1 was not necessary for mesendoderm gene expression in cells that had acquired characteristics of late epiblast cells. By contrast, Tcf7l1−/− cells exhibited markedly reduced mesendoderm gene expression in response to CH during the transition between states (Fig. 6E; supplementary material Fig. S7C; middle). The effect was strongest for Mixl1 and Gsc, which effectively were not induced in Tcf7l1−/− cells until 5 days of culture in EpiSC media was complete. Taken together with the effects on naïve cell characteristics (Fig. 6B-D), these results show that Tcf7l1 stimulates a rapid transition to a primed state, which is necessary for the response to mesoderm specification signals.

DISCUSSION

The activity of Tcf7l1 as a transcriptional repressor is integrated into the Oct4/Sox2/Nanog GRN (Cole et al., 2008; Marson et al., 2008; Yi et al., 2008). Tcf7l1-repression of the Esrrb gene (Martello et al., 2012) further emphasizes that Tcf7l1 functions in ESCs as an intrinsic inhibitor of pluripotent cell self-renewal. By contrast, an in vitro function for the repressor activity of Tcf7l1 in pluripotency was not clear. In particular, it was remarkable that this inhibitor of pluripotent cell self-renewal was expressed at high levels in pluripotent cells, rendering them dependent on Wnt/β-catenin activity in vitro (ten Berge et al., 2011; Wray et al., 2011; Yi et al., 2011). The work presented here provides a new understanding of the in vitro role of Tcf7l1 in pluripotent cells by showing that Tcf7l1 is needed for pluripotent cells to be properly prepared for lineage specification in response to differentiation stimuli (Fig. 7). This activity of Tcf7l1 is needed for the specification of mesoderm from pluripotent epiblast cells to be coupled with the induction of the PS during gastrulation.

In considering the importance of the coupling function, one must distinguish the consequences of an uncoupling effect from those of simply slowing down embryogenesis. An overall slowing of embryonic events does not necessarily cause significant effects. Indeed, early mouse embryos can suspend development for a period of days in a process called diapause, and then return to normal embryogenesis (Renfree and Shaw, 2000). By contrast, uncoupling PS induction and mesoderm specification disrupts the normal sequence of events that occur during gastrulation. Given the dynamics of gastrulation, when epiblast cells divide frequently (~10-hour doubling time) (Snow, 1977) and require less than 24 hours to enter the PS, become mesoderm and migrate away from the PS (Lawson et al., 1991), it seems likely that an uncoupling event resulting in a delay of only a few hours would nonetheless have substantial downstream effects. After delayed mesoderm specification at E6.5-6.75, Tcf7l1−/− embryos display a number of patterning defects, including ectopic axial mesoderm at the expense of mesendoderm.
of paraxial and lateral mesoderm lineages, the appearance of partial duplications to the primary body axis by E7.5 (Merrill et al., 2004), and a lack of neuroectoderm specification (Fig. 3). Although not directly tested, it is likely that these patterning defects in Tcf7l1−/− embryos are secondary to the uncoupling, and that coordinating mesoderm specification with the induction of the PS is the crucial embryonic function of Tcf7l1.

To elucidate a cellular mechanism underlying the delayed mesoderm specification in Tcf7l1−/− embryos, a combination of stem cell culture and embryo experiments was conducted. These experiments provide a good opportunity to assess the similarities and differences between the two systems with respect to lineage specification. Some in vitro effects occur similarly in ESCs and embryos; reduction of Sox2 precedes mesoderm specification of ESCs in response to activation of Wnt signaling by CH (Thomson et al., 2011) as well as in the PS region of the epiblast (Fig. 1D). This role appears to be broadly conserved, as SoxB1 proteins also regulate lineage specification events in chicken and Xenopus embryos and inhibit mesendoderm differentiation in human ESCs (Acloque et al., 2011; Shih et al., 2010; Wang et al., 2012). The transcriptional repressor activity of Tcf7l1 on the Nanog promoter described in ESCs also appears to function in the epiblast during gastrulation (Pereira et al., 2006). By contrast, ESC differentiation has been shown to be sensitive to Oct4 and Nanog levels (Nichols et al., 1998; Niwa et al., 2000; Chambers et al., 2003; Mitsuji et al., 2003; Thomson et al., 2011); however, Oct4 protein immunoreactivity was uniform throughout the pre-streak to late-streak epiblast and Nanog protein levels actually increased in epiblast cells as they moved towards the PS. Moreover, forced expression of Nanog in the epiblast had no apparent effect on lineage specification. We propose that these inconsistencies are due to the different pluripotent states of ESCs and postimplantation epiblast cells. These observations highlight the need for a greater understanding of the different stages of pluripotency for broadly comparing mechanisms of in vitro and in vivo lineage specification.

At precisely what stage of development is Tcf7l1 repressor activity needed? Tcf7l1 had little effect on mesoderm gene expression in cells that were kept in a naïve state (i.e. self-renewing ESCs) and cells that had already progressed to a primed state (i.e. cells cultured for 5 days in EpiSC conditions). The primary effect of Tcf7l1 was apparent only as cells transitioned from a naïve to primed state. Analysis of heterogeneity within EpiSC cultures and differences between lines of EpiSCs shows that primed cells can exist in multiple metastable states (Bernemann et al., 2011; Han et al., 2010). Compared with earlier stages, later stages of primed cells express higher levels of brachyury and are more resistant to reversion back to a naïve state (Bernemann et al., 2011). Based on the retention of ESC colony-forming potential of Tcf7l1−/− cells in EpiSC media, we suggest that Tcf7l1 is necessary for a relatively early stage of the priming process. Thus, our results indicate that despite most of the research on Tcf7l1 and Wnt/β-catenin signaling being focused on self-renewing ESCs, Tcf7l1 is needed to function as an intrinsic inhibitor of self-renewal during the transition to a primed state. This conclusion provides an explanation for variability in assays measuring differentiation defects of Tcf7l1 mutant ESCs; since primary defects occur early, measuring lineage marker expression examines the result of secondary or tertiary events that are likely to be influenced by the context of the differentiation assay.

Interestingly, our results help explain seemingly contradictory results concerning the effects of Wnt/β-catenin signaling on pluripotent cells. Both self-renewal and mesoderm specification responses to Wnt/β-catenin have been described previously. Several studies have shown that Wnt/β-catenin signaling promotes or is required for the self-renewal of mouse ESCs (ten Berge et al., 2011; Hao et al., 2006; Ogawa et al., 2006; Sato et al., 2004; Ying et al., 2008). Although some controversy remains from contradictory reports of the effects of Wnts on human ESCs (Dravil et al., 2005; Sato et al., 2004), recent studies show that Wnt/β-catenin stimulates mesoderm differentiation of human ESCs (Davidson et al., 2012; Singh et al., 2012), and the requirement for Wnt/β-catenin signaling in mesoderm specification in mouse embryos has been demonstrated genetically (Huelsken et al., 2000; Kelly et al., 2004; Liu et al., 1999). We recapitulated these distinct responses to Wnt/β-catenin in a cell-based assay in which ESCs were subjected to EpiSC culture conditions. Cells required 2 days to convert from a self-renewal response to a mesoderm specification response to Wnt/β-catenin signaling. The timing of the switch corresponds to that of a previously described switch in cell state detected via the surface protein expression identities of ESCs and EpiSCs (Rugg-Gunn et al., 2012). Thus, Wnt/β-catenin stimulates the self-renewal of naïve cells by preventing the Tcf7l1-mediated transition to a primed state (Fig. 7). Once pluripotent cells reach the primed state, Wnt/β-catenin stimulates differentiation (Fig. 7).

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

J.A.H. and C.I.W. performed all experiments. J.A.H. and B.J.M. conceptually designed experiments, analyzed data and wrote the manuscript. B.J.M. supervised the project.

Supplementary material

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References


