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Author for correspondence:
Patrick P. L. Tam
e-mail: ptam@cmri.org.au

Differential response of epiblast stem cells to Nodal and Activin signalling: a paradigm of early endoderm development in the embryo

Keren Kaufman-Francis[1,†], Hwee Ngee Goh[1,†], Yoji Kojima[1,3], Joshua B. Studdert[1], Vanessa Jones[1], Melinda D. Power[1], Emilie Wilkie[1,2], Erdahl Teber[2], David A. F. Loebel[1,4] and Patrick P. L. Tam[1,4]

[1]Embryology Unit, and [2]Bioinformatics Group, Children's Medical Research Institute, Westmead, New South Wales 2145, Australia
[3]Institute of Integrated Cell-Material Science, Kyoto University, Kyoto 606-8501, Japan
[4]Sydney Medical School, University of Sydney, Sydney, New South Wales 2008, Australia

[†]These authors contributed equally to this study.

Mouse epiblast stem cells (EpiSCs) display temporal differences in the upregulation of *Mixl1* expression during the initial steps of *in vitro* differentiation, which can be correlated with their propensity for endoderm differentiation. EpiSCs that upregulated *Mixl1* rapidly during differentiation responded robustly to both Activin A and Nodal in generating foregut endoderm and precursors of pancreatic and hepatic tissues. By contrast, EpiSCs that delayed Mixl1 upregulation responded less effectively to Nodal and showed an overall suboptimal outcome of directed differentiation. The enhancement in endoderm potency in Mixl1-early cells may be accounted for by a rapid exit from the progenitor state and the efficient response to the induction of differentiation by Nodal. EpiSCs that readily differentiate into the endoderm cells are marked by a distinctive expression fingerprint of transforming growth factor (TGF)-β signalling pathway genes and genes related to the endoderm lineage. Nodal appears to elicit responses that are associated with transition to a mesenchymal phenotype, whereas Activin A promotes gene expression associated with maintenance of an epithelial phenotype. We postulate that the formation of definitive endoderm (DE) in embryoid bodies follows a similar process to germ layer formation from the epiblast, requiring an initial de-epithelialization event and subsequent re-epithelialization. Our results show that priming EpiSCs with the appropriate form of TGF-β signalling at the formative phase of endoderm differentiation impacts on the further progression into mature DE-derived lineages, and that this is influenced by the initial characteristics of the cell population. Our study also highlights that Activin A, which is commonly used as an *in vitro* surrogate for Nodal in differentiation protocols, does not elicit the same downstream effects as Nodal, and therefore may not effectively mimic events that take place in the mouse embryo.

1. Introduction

In the postimplantation mouse embryo, the formation of the primitive streak (PS) heralds the beginning of gastrulation. Cells in the epiblast are recruited to the PS and, as they disengage from the neighbouring epithelial cells and ingress through the PS, they acquire a mesenchymal morphology [1]. Cells emerging from the PS are either incorporated into an expanding layer of mesenchymal cells (the mesoderm) or become integrated into the pre-existing visceral endoderm layer to form the definitive endoderm (DE) [2]. The DE, together with a subset of visceral endoderm cells, constitutes the precursors of the epithelial tissues of the fetal digestive tract and its associated organs [3]. Fate mapping studies have revealed that progenitors of the DE and mesoderm are localized to the anterior segment of the

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PS [4–6]. It is unclear, however, if the anterior primitive streak (APS) cells have a dual potential to contribute to mesoderm and endoderm (i.e. mesendoderm progenitors) [7], or are a mixed population of two types of germ layer progenitors.

In the gastrula embryo, cells in different segments of the PS may be subjected to graded levels of signalling activity, based on the expression pattern of the pathway genes and the loss-of-function phenotypes [8,9]. Nodal (a transforming growth factor (TGF)-β-related factor) activity is high in the APS, bone morphogenetic protein (BMP) activity peaks in the posterior PS and fibroblast growth factor (FGF) activity is higher in the middle region than other parts of the streak. High Nodal activity is required for the induction of the putative mesendoderm progenitors [10,11] and mutant embryos with enhanced Nodal activity gain more endoderm cells, whereas those with reduced or absent Nodal function are deficient in DE [8–10,12,13].

Epiblast stem cells (EpiSCs) are self-renewing multipotent cells that are derived from the epiblast and ectoderm of post-implantation mouse embryos at the pre-gastrulation to the late gastrulation stages [14–17]. These EpiSCs are maintained *in vitro* by culturing them in the presence of Activin A (another TGF-β-related factor) and FGF2 [18], reminiscent of the provision of Nodal and FGF signals at the APS of the embryo [9,19,20]. Irrespective of the developmental stage of origin, the established EpiSC lines are developmentally comparable to the ectoderm of the late-gastrula-stage mouse embryo with regard to their transcriptome. Furthermore, EpiSCs are enriched with gene transcripts that are expressed by APS cells [17], and when transplanted into the PS of a host embryo they display the range of cell fates and express the lineage markers that are characteristic of the descendants of APS cells [17,21]. These functional and genetic attributes of the EpiSCs point to the possibility that they are the *in vitro* counterpart of the APS cells and, therefore, would be an informative experimental model for studying lineage differentiation of the mouse epiblast and, in particular, the PS.

In this study, we investigated endoderm development in the context of the propensity of EpiSCs to differentiate to endodermal lineages, in response to TGF-β signalling induced by Nodal and Activin A. Our findings provide new insights into the role of Nodal signalling in the formation of the DE during mouse gastrulation.

2. Endoderm lineage propensity of the epiblast stem cells

Analysis of the transcriptome of EpiSCs revealed that while the gene expression profiles are globally similar among the established lines, they can be clustered into distinct subgroups according to the expression profile of genes that are characteristic of embryonic germ layers (endoderm, mesoderm and neurectoderm) [17]. By assaying the temporal pattern of expression of genes associated with germ layer formation in embryoid bodies (EBs) over a 4-day period, EpiSC lines were found to respond differently to the induction of differentiation. In particular, the temporal expression profile of *Mixl1*, a gene that is expressed in the PS and required for DE formation [22], varied across the set of EpiSCs analysed. Prior to differentiation, *Mixl1* expression was comparable across all EpiSC lines analysed [17]. Upon differentiation, EpiSCs could be classified into three groups according to the pace at which *Mixl1* expression is upregulated. A subset of EpiSC lines showed rapid upregulation of *Mixl1* (termed Mixl1-early); a second group showed a much delayed upregulation of *Mixl1* (Mixl1-late) and a third group (Mixl1-intermediate) showed peak expression of *Mixl1* at a time point in between.

Our previous work has shown that cell lines in these three categories can be distinguished by the expression profiles of selected genes prior to differentiation [17], suggesting that the readiness to differentiate is influenced by their intrinsic molecular characteristics. Re-analysing the transcriptome of the undifferentiated EpiSCs with reference to their Mixl1-category revealed that the Mixl1-early EpiSCs showed higher expression of pluripotency and endoderm-related genes, whereas the Mixl1-late EpiSCs show higher expression of mesenchyme and neural-related genes [17]. EpiSCs of the three categories of *Mixl1* expression pattern consistently showed different outcomes of differentiation. Mixl1-early EpiSCs express endoderm lineage markers at a higher level during *in vitro* differentiation within EBs and they generate teratomas with more abundant endoderm derivatives than Mixl1-intermediate and Mixl1-late EpiSCs [17].

In the embryo, *Mixl1* is expressed in the PS and downregulated in DE cells [23,24]. The rapid changes in *Mixl1* expression in differentiating Mixl1-early EpiSCs are therefore reminiscent of the *in vivo* situation where *Mixl1* expression mirrors the specification of the endoderm progenitors, and the transition to DE is accompanied by the cessation of expression. The findings of the transcriptome analysis outlined above suggest that endoderm differentiation propensity of a cell line may be negatively correlated with its ability to undergo neural and mesoderm differentiation [21,25]. Mixl1-early EpiSCs may therefore be entrained with a molecular signature that primes them to differentiate into DE and this is reflected in the rapid switch between upregulation and downregulation of *Mixl1*, which is similar to the changes in *Mixl1* expression during DE formation *in vivo*.

3. Impact of TGF-β activity on endoderm differentiation

EpiSCs from different Mixl1-response groups respond differently to conditions that direct the differentiation of the stem cells to endoderm. Mixl1-early EpiSCs expressed higher levels of genes that signify the presence of gut endoderm cells, liver and pancreas than cells of Mixl1-intermediate or Mixl1-late categories [17]. The induction of differentiation was achieved by treating the EpiSCs with Activin A at a concentration higher than that for maintenance of the cell line. Activin A acts as a mesoderm inducer in *Xenopus* embryos [26–28] and as an inducer of endoderm differentiation of mouse and human pluripotent stem cells [29–34]. However, although genes encoding TGF-β receptors such as *Acvr2a*, *Acvr2b* and *Acvr1b* are expressed in the gastrula-stage mouse embryo, *Inhba* (for Activin A) is not expressed. It is generally considered that Activin receptors *in vivo* mediate the signalling activity of other TGF-β proteins, with Nodal being the most probable signalling factor. Consistent with this postulation, mutations of the receptors, co-receptor (Cripto), intracellular transducers (Smad2, -3 and -4) and *Nodal* proximal enhancer that impact adversely on the Nodal/Smad/Foxh1 pathway lead to defects in PS function and endoderm formation [10,13,35–38]. Furthermore, a comparison of the effect of Nodal and Activin A on the differentiation of mouse embryonic stem cells shows that

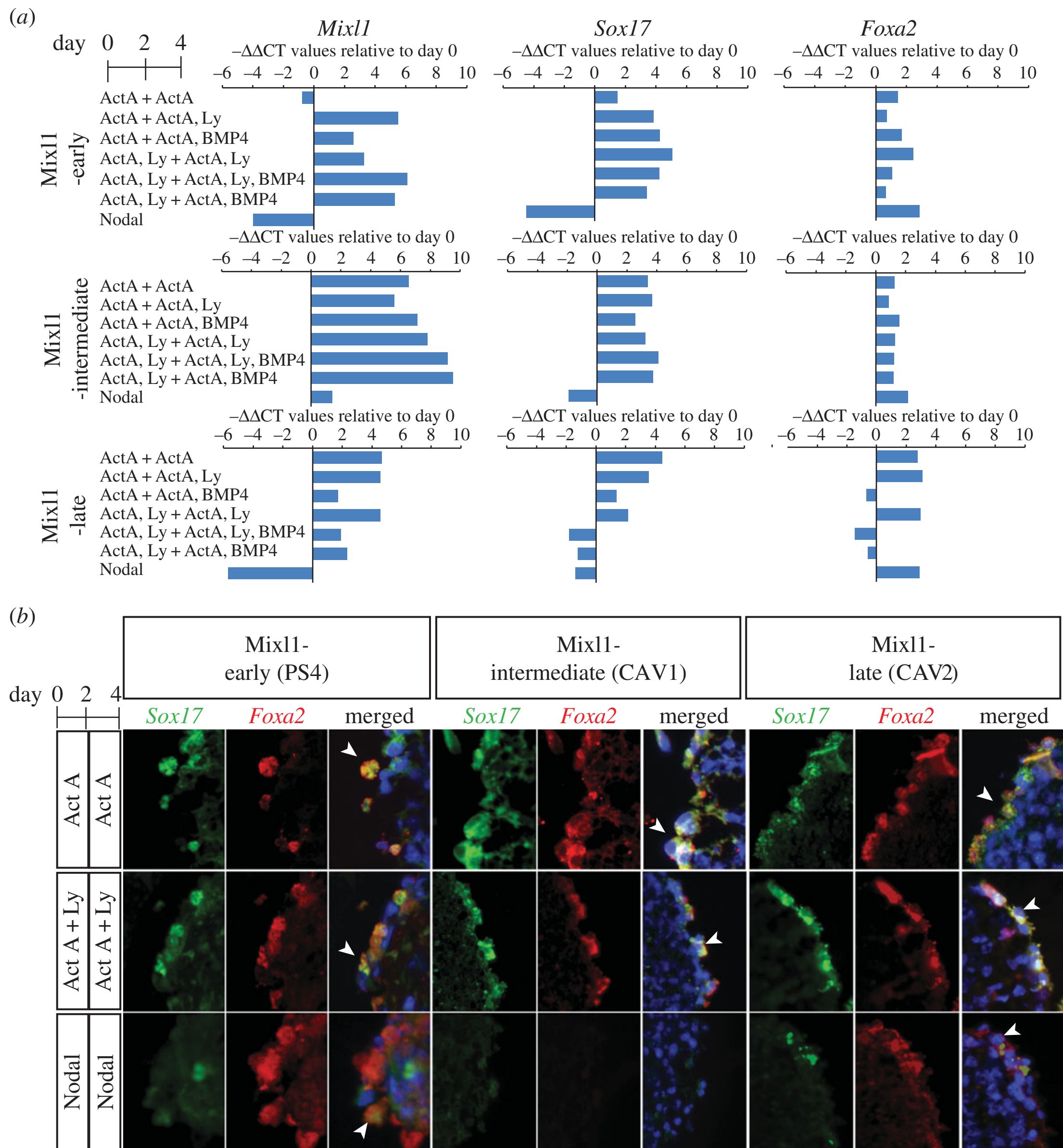

Figure 1. Differentiation of Mixl1-early, Mixl1-intermediate and Mixl1-late EpiSCs. (*a*) Level of expression of *Mixl1* (PS marker), *Sox17* and *Foxa2* (definitive endoderm marker) in day-4 EBs cultured under seven culture protocols. Gene expression level is presented as the negative value of the relative difference in threshold cycles ($-\Delta\Delta$CT; normalized against *Actb*) relative to day 0 value and is the mean of triplicate cultures. Positive value, upregulation; negative value, downregulation. (*b*) Endoderm cells co-expressing Foxa2 and Sox17 (arrowheads) in day-4 EBs, visualized by immunofluorescence of Foxa2 (red) and Sox17 (green). (Online version in colour.)

the DE generated by Nodal activity is better able to colonize the embryonic foregut and differentiate into pancreatic cells *in vivo* [30]. This suggests that while Activin A signals through a similar molecular pathway to Nodal, it might not fully substitute the function of Nodal in endoderm differentiation.

In view of the developmental similarities between EpiSCs and PS cells, we tested whether Nodal and Activin A may have different effects on endoderm differentiation and if the response may also be correlated with the endoderm lineage propensity of the EpiSCs.

4. EpiSCs of different lineage propensity respond differently to Nodal and Activin A

EpiSC lines of Mixl1-early (PS4), Mixl1-intermediate (CAV1) and Mixl1-late (CAV2, CAV4) groups were studied for differentiation to DE. EpiSC-derived EBs were cultured in medium supplemented with Nodal for 4 days, or with Activin A or Activin A + phosphoinositide 3-kinase (PI3K) inhibitor (Ly294002; Ly) for day 0–2 and then in various combinations of BMP4 and PI3K inhibitor for day 2–4 (figure 1*a*). Endoderm formation was assessed by qPCR analysis of the expression of mesendoderm progenitors (*Mixl1*) and DE (*Sox17* and *Foxa2*) markers (figure 1*a*). In Mixl1-early EpiSCs, *Mixl1* expression was low by day 4 when cultured in either Activin A or Nodal-supplemented medium, but *Mixl1* expression remained high in all other conditions (Activin A + other factors). *Sox17* was expressed in all Activin A cultures, but was reduced in Nodal culture. By contrast, *Foxa2* expression was detected under all conditions and most strongly with Nodal. Mixl1-intermediate EpiSCs had elevated expression of all three markers in all Activin A conditions. However, in Nodal cultures, expression of *Mixl1* was only increased slightly, *Sox17* was reduced and *Foxa2* was increased compared with day 0. Mixl1-late EpiSCs displayed variable responses to most

Activin A + BMP4 conditions, though upregulated *Mixl1*, *Sox17* and *Foxa2* in Activin A and Activin A + Ly cultures. The expression pattern of these genes in response to Nodal was similar to that of Mixl1-early EpiSCs.

The EBs were further analysed by immunofluorescence to visualize cells that co-expressed Sox17 and Foxa2 (figure 1*b*, electronic supplementary material, figure S1), which are likely to be equivalent to endoderm cells. $Foxa2^{+}$;$Sox17^{+}$ cells were detected in the outer cell layers of EBs of Mixl1-early EpiSCs in all culture conditions. For Mixl1-intermediate EpiSCs, $Foxa2^{+}$;$Sox17^{+}$ cells could be detected in EBs differentiated under most combinations of Activin A and PI3K inhibitor but not in Nodal-supplemented culture. For Mixl1-late EBs, $Foxa2^{+}$;$Sox17^{+}$ cells were present in Activin A, Activin A + Ly (without BMP4) and Nodal-treated conditions. Endoderm-like cells were, therefore, induced by Activin A in EpiSCs of all three categories but only in Mixl1-early cells and, at a much lower abundance, in Mixl1-late cells following Nodal induction. To assess the efficiency of Activin A and Nodal induction of DE versus visceral endoderm-like cells, the enrichment of the CXCR4-positive population [39,40] in the EBs was quantified by FACS analysis (figure 2*a*, electronic supplementary material, figure S2). CXCR4 is expressed in embryonic germ layers, but not extraembryonic visceral endoderm. Mixl1-early EpiSCs showed the greatest enrichment of CXCR4-positive cells, whereas the Mixl1-intermediate and Mixl1-late EpiSCs responded modestly to induction by Activin and Activin + Ly, and weakly to Nodal (figure 2*a*).

The expression of other lineage markers in response to the various growth factors was also examined (electronic supplementary material, figure S3). Mixl1-late and Mixl1-intermediate lines showed high levels of *Sox1* expression in response to Activin A treatments, indicative of the presence of neuroectoderm progenitors in EBs derived from these lines. Conversely, the Mixl1-early line showed either a relatively weak induction or downregulation of *Sox1* in response to all the test conditions. Lines from all groups showed low-level expression of *Pax6* suggesting the lack of differentiation of more advanced ectoderm derivatives. Expression of markers for pluripotency (*Oct4* and *Nanog*) was reduced in all EpiSC lines but stayed relatively higher in Mixl1-late cells cultured under Activin A-containing conditions, which might indicate the persistence of undifferentiated stem cells. Other non-endoderm lineage markers, *Meox1*, *T* and *Sox7* were generally expressed at similar levels among the cell lines, irrespective of culture conditions (electronic supplementary material, figure S3).

Overall, these results show that Mixl1-early EpiSCs respond effectively to both Nodal and Activin A by expressing endoderm markers, generating *Sox17* and *Foxa2*-positive cells, and showing enrichment of CXCR4-expressing cells. Compared with Mixl1-early EpiSCs, Mixl1-intermediate and Mixl1-late EpiSCs respond poorly to Nodal and are less efficient in endoderm differentiation.

5. Nodal promotes differentiation of epiblast stem cells with enhanced endoderm propensity

The ability of the EpiSCs to differentiate into cells with foregut endoderm properties in day 4 EBs was assessed by the expression of seven validated markers (*1810019J16Rik*, *B4galt6*, *Capn6*, *Cldn4 Igfbp5*, *Rbm47* and *Rhou*; figure 2*b*) that were identified as transcripts enriched in the foregut endoderm of early-somite-stage mouse embryos (electronic supplementary material, figure S4, and tables S1 and S2). In Mixl1-early EBs, various combinations of foregut endoderm markers could be detected in all culture conditions. In Mixl1-intermediate EpiSCs, foregut endoderm markers were expressed when only Activin A was present (i.e. without Ly) in the first 2 days of culture. Compared to the Mixl1-early EpiSCs, only a few foregut endoderm markers could be detected in the Mixl1-late EpiSC culture. Both Mixl1-intermediate and Mixl1-late EpiSCs responded poorly to Nodal in expressing foregut endoderm markers.

To test the differentiation potential of cells with DE and foregut endoderm characteristics, we developed a protocol in which EpiSCs were subjected to extended differentiation culture for a further 2 days in media supplemented with various combinations of FGF10, BMP4, retinoic acid (RA) and an inhibitor of Hedgehog signalling (cyclopamine, cyc), following 4 days with Activin A and Ly (electronic supplementary material, figure S5a). Cells were grown as EBs for the first 2 days, dissociated and subsequently grown in an adherent culture for the next 4 days. As an example, in the presence of FGF10 and cyc, Mixl1-intermediate EpiSCs expressed foregut markers (*Tbx1*, *Pyy*, *Hnf1b*, *Hnf4a*) and the lung and thyroid marker Nkx2.1, weakly expressed the intestine marker *Cdx2* and downregulated the foregut progenitor marker *Sox2* (electronic supplementary material, figure S5b). Further extending the culture (day 6–9, electronic supplementary material, figure S5a) in medium containing FGF10, RA and cyclopamine enhanced the expression of pancreatic endoderm and endocrine precursors markers *Pdx1*, *Prox1* and *Ngn3* (electronic supplementary material, figure S5c, and data not shown).

EpiSCs of the three Mixl1 categories were then compared after a 9-day culture period for the expression of markers of pancreatic cells (*Pdx1*, *Prox1*), hepatocytes (*Afp*, *Hhex*) and cholangiocytes (*Krt19*, *Itgb4*; figure 2*c*). Mixl1-early EpiSCs expressed pancreatic and hepatocyte markers more robustly when cultured in Nodal for the first 4 days of differentiation than in Activin A + Ly, but the two cholangiocyte markers responded differently to Nodal versus Activin A. Mixl1-intermediate EpiSCs cultured in Nodal did not show any consistent pattern of marker expression for the three endoderm lineages, except that the Activin A + Ly treated cells upregulated markers of pancreatic and liver cells. Only *Itgb4* was upregulated in Mixl1-late EpiSC in either Activin A or Nodal conditions. All other markers were downregulated in Activin A + Ly culture (figure 2*c*), suggesting these EpiSCs may be predisposed for the cholangiocyte lineage.

In summary, the outcome of the directed differentiation of the EpiSCs shows that the immediacy in the activation of *Mixl1* is associated with an enhanced propensity for endoderm differentiation and receptivity to TGF-β signalling. The Mixl1-early EpiSCs respond effectively to the inductive activity of both Nodal and Activin A and generate foregut endoderm-like cells that are competent to differentiate into more advanced cell types. Induction by Nodal or Activin A at the formative phase of the endoderm lineage has a critical influence on the outcome of differentiation: Nodal promotes the expression of pancreas and liver cell markers,

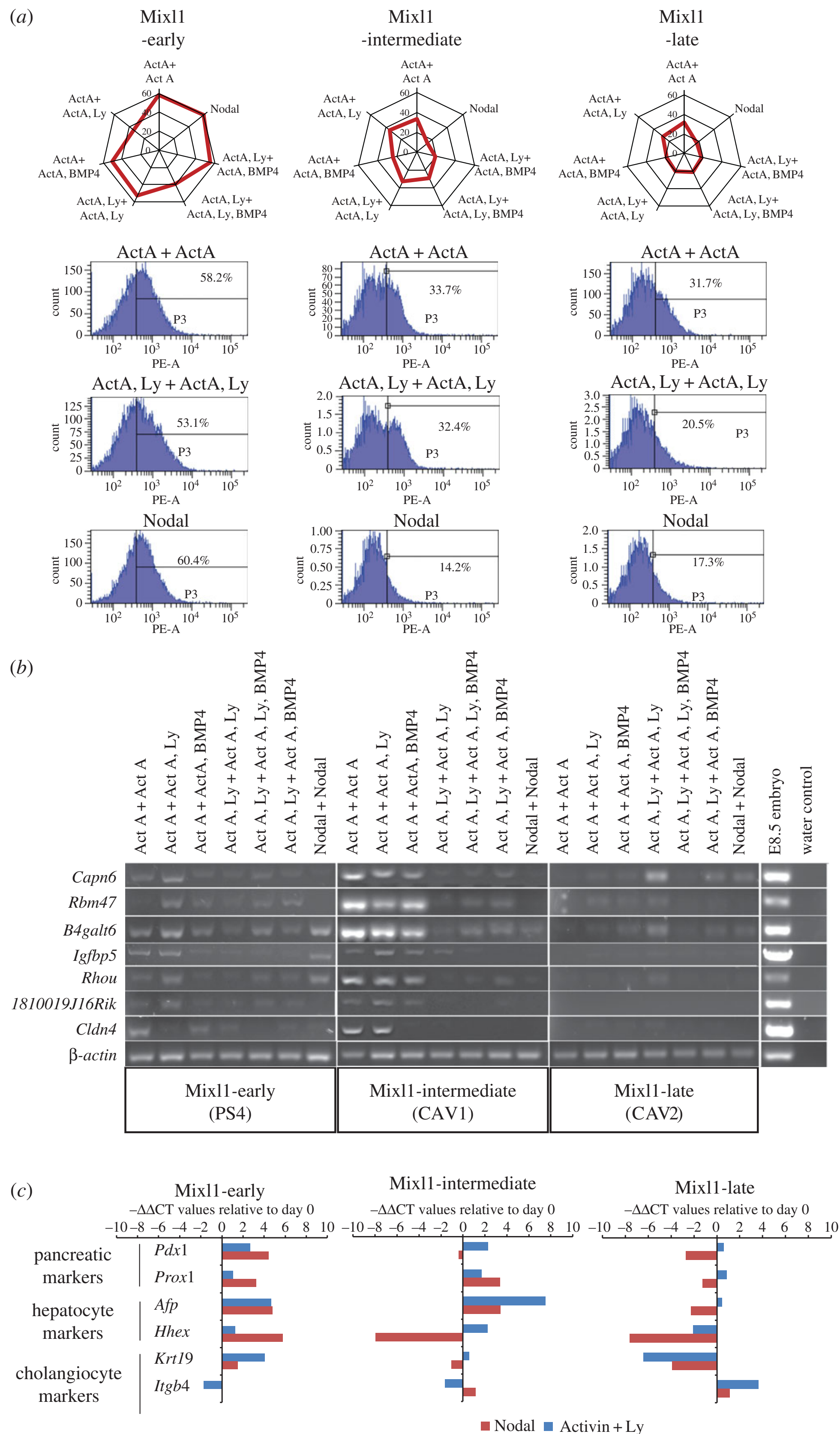

Figure 2. Differentiation of EpiSCs to endoderm lineages. (*a*) The level of enrichment of CXCR4-positive cells in day-4 EBs generated from EpiSCs of the three Mixl1 categories. Radar graphs (top row) show the data of all seven culture conditions (radial axis: level (%) of enrichment). Flow cytometry results and the % CXCR4-positive cells in the samples are shown for three conditions (rows 2–3) for each Mixl1-group. (b) PCR analysis of the expression of foregut endoderm genes in day-4 EBs cultured in seven conditions. Integrity of the cDNA sample was ascertained by PCR analysis of a housekeeping gene, β-*actin*. (c) Expression of *Pdx1*, *Prox1* (pancreatic markers), *Afp*, *Hhex* (hepatocyte markers) and *Krt19*, *Itgb4* (cholangiocyte markers) after 9-day directed differentiation of EpiSCs of the three Mixl1 categories. EpiSCs were cultured in either Activin A + Ly or Nodal at day 1–4. Gene expression level is presented as the negative value of the relative difference in threshold cycles (−ΔΔCT; normalized against *Actb*) relative to day 0 value. Positive value, upregulation; negative value, downregulation. (Online version in colour.)

whereas Activin A promotes the expression of the cholangiocyte marker *Krt19*, indicating that cells have acquired characteristics of the bile duct epithelium. The Mixl1-intermediate EpiSCs respond to Activin A more effectively than to Nodal, demonstrated by the generation of cells displaying foregut endoderm properties. These cells, however, are not competent to differentiate into cells with consistent endoderm characteristics. Mixl1-late EpiSCs do not respond effectively to either TGF-β factors to form DE.

6. Innate TGF-β signalling activity in epiblast stem cells

(a) Implications for directed differentiation of pluripotent stem cells

For directed differentiation of pluripotent cells to endoderm tissues such as hepatocytes and pancreatic endocrine cells, the road-tested strategy has been to drive differentiation first to DE-like cells, followed by enrichment of cells that display properties of the posterior foregut endoderm. This intermediate cell type is then subjected to culture conditions that promote the differentiation towards the hepatic or pancreatic tissue lineages [33]. A variety of *in vitro* culture protocols have been devised for the directed differentiation of pluripotent stem cells to endoderm derivatives. These protocols vary in parameters such as the formulation of the basic culture medium, the combination of supplements used and the dosage and timing at which they are delivered to the differentiating cells. Of particular interest is that Activin A and Nodal, two TGF-β superfamily factors, have been reported to elicit similar signalling responses and can induce differentiation of embryonic stem cells to DE-like cells with very similar molecular phenotypes [30,34], but Nodal-treated cells display greater functional capacity than their Activin A counterpart to differentiate into functionally competent pancreatic endoderm cells [30]. In our study, we observed different responses of EpiSCs to Nodal and Activin A during differentiation in culture. There is mounting evidence that in pluripotent stem cells, lineage-specific genes are co-expressed with genes of the genetic network that maintain the cells at the pluripotent state [41], which could signify that the cells are already poised to undergo lineage-specific differentiation. Some lineage-specific genes are known to play a critical role in specifying the tissue lineage. The expression of some of these lineage specifiers is regulated by pluripotency-related genes such as *Oct4* and *Sox2*, and enforced expression of these genes can substitute for *Oct4* and *Sox2* in reprogramming somatic cells to pluripotency [42]. For example, *Gata3* can efficiently substitute for *Oct4* and *Gmnn* can substitute for *Sox2* in the generation of induced pluripotent stem cells (iPSCs) [42]. The authors proposed a 'seesaw' model whereby mesendoderm lineage specifiers dampen the upregulation of ectoderm genes induced by *Sox2* while ectoderm specifiers attenuate the elevation of mesendoderm genes induced by *Oct4*. It is postulated that a balance in the counteracting effects of lineage specifiers contributes to both the induction and maintenance of pluripotency. It is also possible that slight deviations from balanced activity of lineage-specific genes might not affect pluripotency, but could confer a lineage bias in the undifferentiated cells that is revealed upon differentiation. Recent studies of iPSCs have revealed that these stem cells harbour genetic and epigenetic variations which may impact on their differentiation potential and the phenotype of the differentiated cells [43]. Collectively, these findings highlight that the efficacy of directed differentiation could be influenced by the individual characteristics of the pluripotent stem cells, which determine their response to the induction of differentiation.

Our results show that the EpiSC lines are inherently different in their endoderm differentiation potential as revealed by the immediacy of *Mixl1* activation and the enhanced expression of endoderm lineage-specific genes in the parental EpiSCs. Activin/Nodal signalling activates different transcriptional responses in human embryonic stem cells or endoderm progenitors that either promote the maintenance of pluripotency or drive cell differentiation [32]. The enhanced capacity of Mixl1-early EpiSCs for endoderm differentiation may be underpinned by the rapid emergence of *Mixl1*-expressing progenitors of the endoderm lineage and this may be the population that responds effectively to the induction of differentiation by the Activin/Nodal signals.

(b) Different downstream activity of Nodal and Activin A in epiblast stem cells

Transcriptome analysis has revealed that undifferentiated EpiSCs express genes that are characteristic of the APS [17]. In view of the contrast in endoderm differentiation propensity among EpiSCs, we examined the pattern of expression of PS genes in the three Mixl1 categories. Mixl1-early EpiSCs expressed a larger set of PS genes at a higher level than EpiSCs of the other two Mixl1 categories (electronic supplementary material, figure S6a). The Mixl1-early EpiSCs expressed higher levels of transcripts encoding members of embryonic patterning, morphogenesis and Wnt signalling pathways when compared with Mixl1-intermediate EpiSCs. They were enriched for genes related to cell–extracellular matrix interaction, cell proliferation and metabolism and growth factor activity when compared with Mixl1-late EpiSCs (electronic supplementary material, figure S6b). Mixl1-early EpiSCs therefore display more robust gene expression indicative of the propensity for germ layer differentiation.

The differences in the outcome of Activin A and Nodal induced differentiation among the EpiSCs point to the inherent differences in their receptivity to TGF-β signalling. A comparison of the expression profiles of genes encoding TGF-β signalling pathway components (ligands, receptors and transducers) reveals that EpiSCs of different *Mixl1* expression categories expressed different sets of genes before differentiation (figure 3*a*). The expression of different sets of TGF-β response genes (figure 3*d*) further showed that EpiSCs of different lineage propensity might be primed differentially to activate different signalling pathways during differentiation. Mixl1-early and Mixl1-intemediate EpiSCs activated different pathways in response to Nodal and Activin during differentiation, but Mixl1-late EpiSCs downregulated most TGF-β-associated genes (figure 3*b*). The expression levels of selected pathway genes (electronic supplementary material, figure S7a) also varied among the three groups of EpiSCs. In the Mixl1-early EpiSCs, Nodal activity engaged the activation of pathway genes of TGF-β signalling. Somewhat surprisingly, Activin A treatment elicited higher levels of expression of genes that mediate or are targets of BMP signalling than Nodal treatment (figure 3*c*).

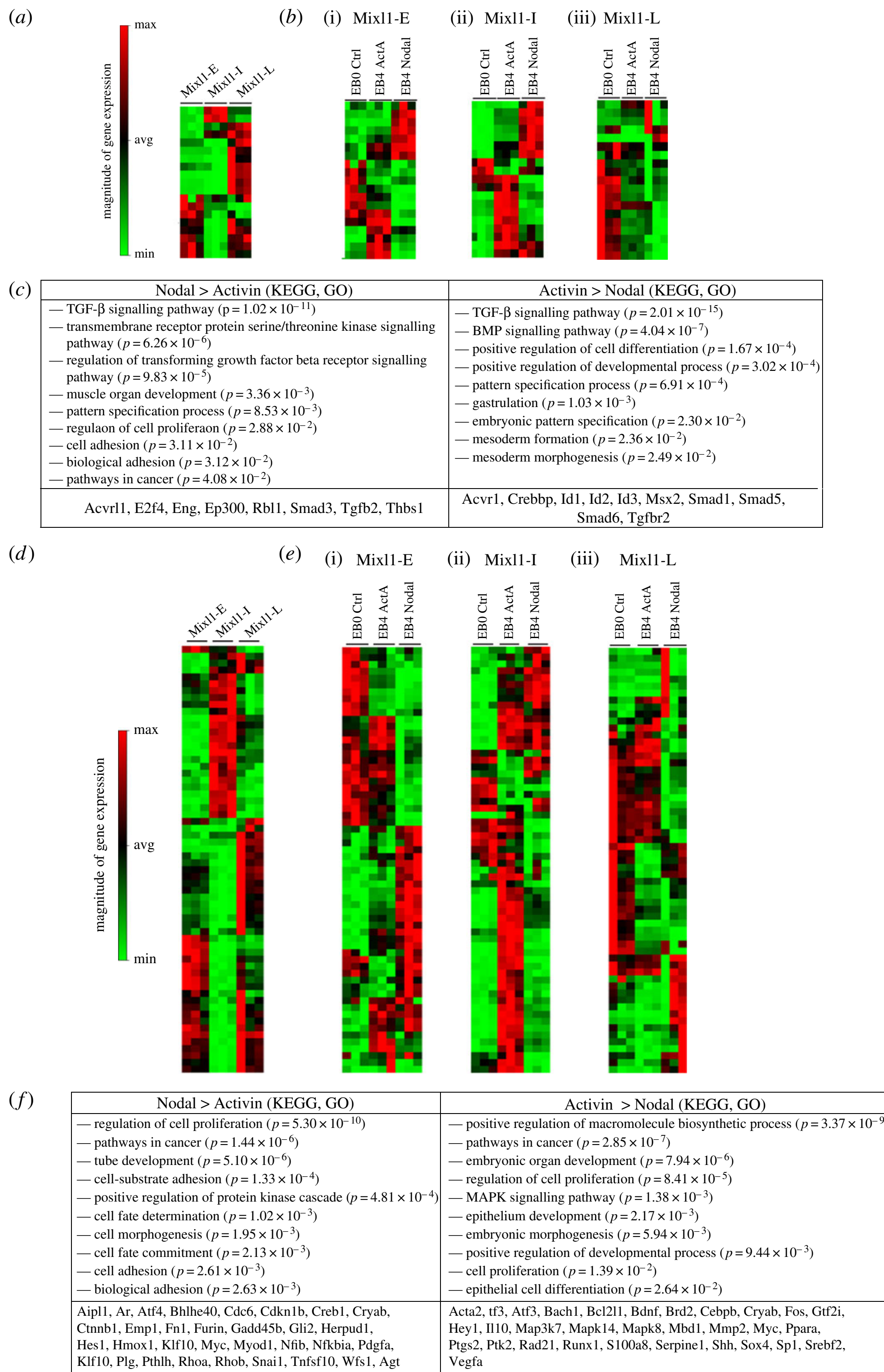

Figure 3. The expression profile of TGF-β signalling pathway components and response genes in the EpiSCs. (*a*) Heat map showing different expression patterns of pathway components in undifferentiated (day 0) Mixl1-early, Mixl1-intermediate and Mixl1-late EpiSCs. (*b*)(i)–(iii) Heat maps showing the different response of (i) Mixl1-early, (ii) Mixl1-intermediate and (iii) Mixl1-late EpiSC-derived embryonic bodies cultured for 4 days in Activin A- or Nodal-supplemented medium, compared with the expression profile at day 0 of culture. (For gene lists of the heat maps, see electronic supplementary material, table S4). (*c*) Functional annotation of genes that are significantly upregulated in cells of Nodal or Activin A cultures based on clustered GO terms using DAVID. Examples of upregulated genes are listed separately for Nodal and Activin A treatment in Mixl1-early EpiSCs. (*d*) Heat map showing different expression patterns of response genes in undifferentiated (day 0) Mixl1-early, Mixl1-intermediate and Mixl1-late EpiSCs. (*e*)(i)–(iii) Heat maps showing the different expression patterns of downstream genes of (i) Mixl1-early, (ii) Mixl1-intermediate and (iii) Mixl1-late EpiSC-derived embryonic bodies cultured for 4 days in Activin A- or Nodal-supplemented medium, compared with the expression profile at day 0 of culture. (For gene lists of the heat maps, see electronic supplementary material, table S4). (*f*) Functional annotation of genes that are differentially upregulated in Mixl1-early EpiSCs of Nodal or Activin A cultures. Examples of upregulated genes are listed separately for Nodal and Activin A treatment. The order that genes are listed on the heat map is determined by the clustering of the expression data, which were normalized against that of housekeeping genes: *Actb*, *B2M*, *Gapdh*, *Hsp90ab1* and *Gusb*. Mixl1-E, Mixl1-early; Mixl1-I, Mixl1-intermediate; Mixl1-L, Mixl1-late; EB0, day-0 embryoid bodies; EB4, day-4 embryoid bodies; ctrl, control; ActA, Activin A. (Online version in colour.)

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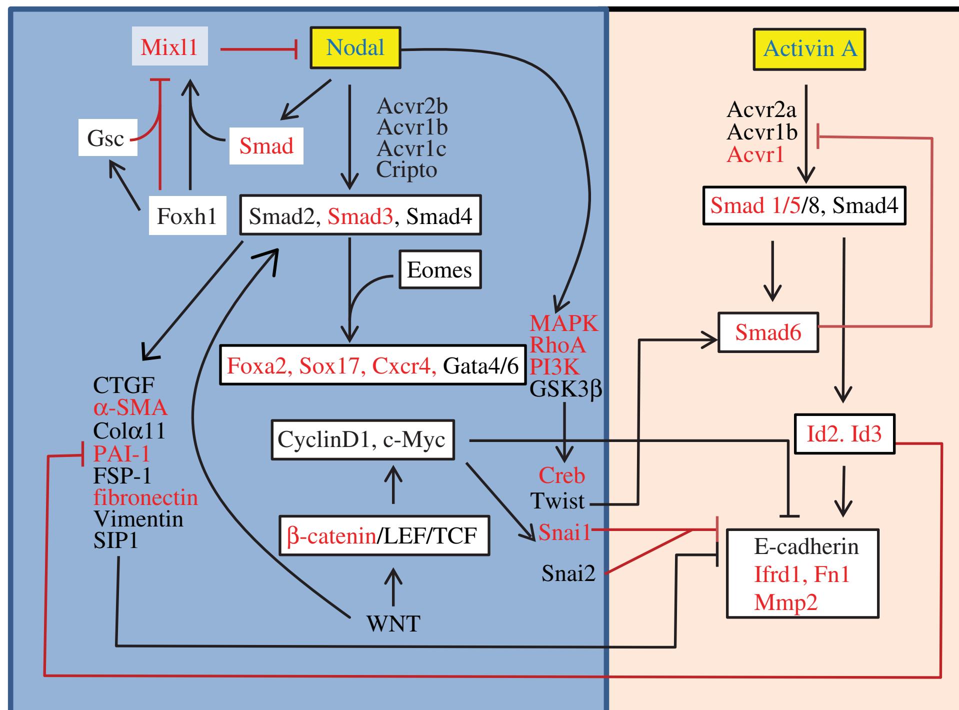

Figure 4. A model of the molecular cascade downstream of Nodal and Activin A signalling in EpiSCs. Nodal activates *Mixl1* activity via Smad and Foxh1 trans-activation to induce mesendoderm progenitors in the PS. Mixl1 in turns represses Nodal expression. Foxh1 and Gsc cooperatively repress *Mixl1* expression. Nodal signals via the TGF-β–Smad2/3/4 pathway and activates MAPK/RhoA/PI3K signalling. The downstream activity of these pathways dismantles the epithelial phenotype of the epiblast cells, and promotes the acquisition of a transitory mesenchymal cell state during gastrulation. Smad factors, along with Eomes, promote expression of endoderm genes (*Sox17*, *Foxa2*, *Cxcr4*, *Gata*). It is postulated that the acquisition of a mesenchymal phenotype is a pre-requisite step of the allocation of cells to the endoderm lineage in the embryo, which can be enhanced by Wnt/β-catenin signalling activity. Activin A, in contrast to Nodal, upregulates some components of the Smad-mediated cascade that promote via Id proteins the maintenance of an epithelial cell state. The omission of the mesenchymal state during Activin A induced differentiation may hamper the generation of competent endoderm cells that can differentiate into more advanced cell types. Genes identified as differentially expressed by microfluidic qPCR are shown in red. (Online version in colour.)

To examine whether the ability to respond to TGF-β signalling in undifferentiated EpiSCs could have entrained the cells in their response to TGF-β signalling during differentiation, the expression of responding genes was examined in cells after 4 days of differentiation as EBs. Our data (Heat maps: figure 3*e*(i)–(iii); selected gene set: electronic supplementary material, figure S7b) clearly show that all three groups of EpiSCs responded differently to Nodal and Activin A. Activin A treatment resulted in the upregulation of genes associated with the BMP pathway, most notably in Mixl1-early cells (e.g. *Crebbp*, *Msx2*, *Smad6*, *Id1*, *Id2* and *Acvr1*), and the MAPK pathway (e.g. *Mapka and Map3k7*), and genes associated with cell fate decision (e.g. *Pdgfa*, *Ctnnb1* and *Hes1*) (figure 3*f*). In addition, Activin A promoted higher expression of epithelial markers (and their upstream genes, e.g. *Id2*, *Id3*, *Fn1*, *Mmp3*, *Rhob and Ifrd1*) than Nodal and weaker expression of mesenchymal markers (e.g. *Snai1*, *Rhoa* and *Fn1*). KEGG and GO analysis of the tested TGF-β targets also shows that Activin A engaged the pathways associated with epithelial cell differentiation and development, as well as proliferation and embryonic organ development (31 genes, see figure 3*f*). By contrast, Nodal elicited the activity of a different set of pathway genes (TGF-β: *E2f4*, *Eng*, *Rbl1*, *Ep300*, *Tgfb2*, *Smad3*, *Acvrl1*; Nodal subtilisin-like convertase: *Furin*; Wnt: *Ctnnb1*) [44]. Nodal also upregulated genes that promote cell–substrate interaction, cell adhesion (*Rhoa*, *Rhob*, *Snai1*, *Ctnnb1*, *Myc*, *Cdc6*), the acquisition of mesenchymal phenotype (*Rhoa*, *Snai1*, *Myod1*, *Aipl1*, *Hes1*) and cell motility [13,45], but downregulated genes associated with an epithelial phenotype (e.g. *Id2*, *Id3* (this study); *Cdh1*) [46]).

7. The role of Nodal in gene regulation and cell behaviour during endoderm formation

The association between rapid upregulation of *Mixl1* expression and enhanced endoderm lineage propensity, and the differential response of the lineage competent EpiSCs to induction of differentiation by Nodal and Activin A have highlighted a molecular paradigm of Mixl1 and TGF-β function in endoderm formation. The initial activation of *Mixl1* in differentiating EpiSCs is reminiscent of the expression of *Mixl1* first in the PS, and subsequent deactivation mimics the loss of *Mixl1* expression in the DE *in vivo*. Mixl1 is reputed to act as a transcriptional regulator in the Nodal downstream pathway via Gata and SoxF factors to regulate endoderm formation [47–52]. That the loss of *Mixl1* activity leads to a failure to generate DE in the embryo suggests that activation of *Mixl1* in the progenitor population is critical for the formation of the endoderm. In addition, constitutive expression of *Mixl1* in differentiating mouse embryonic stem cells promotes endoderm differentiation at the expense of haematopoietic mesoderm [53]. It is therefore plausible that *Mixl1* activity is required for the cell fate decision process for the specification of the endoderm lineage. *Mixl1* expression can be activated by TGF-β/Nodal activity [54] through the interaction of

Smad and Foxh1 with the proximal response element in the *Mixl1* promoter [55]. Loss of *Foxh1* results in expanded *Mixl1* expression in the mouse gastrula [55] while loss of *Mixl1* leads to expansion of *Nodal-lacZ* expression [23]. Nodal signalling therefore is involved in both the feedforward and feedback regulatory loop that regulates *Mixl1* activity (figure 4). The timely response to Nodal-dependent regulation of *Mixl1* expression in EpiSCs ([46] and data not shown) may be the critical factor of the lineage differentiation propensity of these stem cells. Delayed activation of *Mixl1* expression in the Mixl1-intermediate and Mixl1-late EpiSCs may underpin the reduced efficiency of DE differentiation compared with Mixl1-early cells. This is reflected in the weaker activation of pathway components and targets following Nodal stimulation, and the comparatively reduced efficiency of directed differentiation to advanced endoderm cell types.

A comparison of the expression of pathway components and TGF-β response genes in EpiSCs reveals that Activin A results in greater expression of some components and targets of the BMP–Smad1/5/6/8 pathway [56] than Nodal signalling (figure 4). Of interest is that the molecular cascade activated by Activin A may be involved with maintenance of an epithelial cell phenotype, whereas Nodal seems to promote the transition to the mesenchyme phenotype. The Nodal cascade may receive input from the Wnt signalling pathway, like in human embryonic stem cells [57], to initiate Smad2/3 activation, which together with PI3K activity counteracts Activin A/Smad1/5 downstream activity. This scenario of signalling activity may point to a potential role of Nodal in the transition of cellular state during the ingression of the epiblast cells in the PS and the emergence of the nascent mesenchyme in which the endoderm progenitors reside transiently. Cells in the APS experience strong Nodal activity [56], which potentially promotes cell ingression movement and activates downstream genes that influence cell fate decisions [58–60]. Nodal negatively regulates *Mixl1* in the APS by the Foxh1–Gsc complex that recruits the repressive histone deacetylase, and enforced *Gsc* expression in EBs suppresses Foxh1-dependent *Mixl1* expression [61] The repression of Nodal-dependent *Mixl1* activity may coincide with the onset of *Gsc* expression at mid-gastrulation and the allocation of Gsc^{+}/$Foxa2^{+}$/$Sox17^{+}$ DE. Downstream of Nodal, Smad2/3 interacts with Eomes to activate the transcriptional network for endoderm formation ([46], fig. 4). It is therefore likely that Nodal plays separate roles in regulating the *Mixl1*-dependent transcriptional regulatory activity for the specification of the endoderm lineage and the transition of cell state (epithelial to mesenchymal and back to epithelial) to facilitate the generation of the DE.

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