Dissecting signaling hierarchies in the patterning of the mouse primitive streak using micropatterned EpiLC colonies

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SUMMARY

Embryo studies have established that the patterning of the mouse gastrula depends on a regulatory network in which the WNT, BMP, and NODAL signaling pathways cooperate, but aspects of their respective contributions remain unclear. Studying their impact on the spatial organization and developmental trajectories of micropatterned epiblast-like cell (EpiLC) colonies, we show that NODAL is required prior to BMP action to establish the mesoderm and endoderm lineages. The presence of BMP then forces NODAL and WNT to support the formation of posterior primitive streak (PS) derivatives, while its absence allows them to promote that of anterior PS derivatives. Also, a Nodal mutation elicits more severe patterning defects in vitro than in the embryo, suggesting that ligands of extra-embryonic origin can rescue them. These results support the implication of a combinatorial process in PS patterning and illustrate how the study of micropatterned EpiLC colonies can complement that of embryos.

INTRODUCTION

In the mouse embryo, the patterning of the pluripotent epiblast culminates at embryonic day (E) 6.5 with the formation of the primitive streak (PS), where posterior epiblast cells undergo the epithelial-mesenchymal transition (EMT) associated with the adoption of mesendodermal cell identities. The PS itself is patterned, as cells that emerge at different levels of the PS have distinct fates: extra-embryonic mesoderm and embryonic mesoderm when emerging posteriorly and axial mesoderm and definitive endoderm when emerging anteriorly (Kinder et al., 1999).

The signaling molecules WNT3, bone morphogenetic protein 4 (BMP4), and NODAL, are essential to form the PS, a loss of function of any one of them being sufficient to cause its absence (Brennan et al., 2001; Conlon et al., 1994; Liu et al., 1999; Winnier et al., 1995; Zhou et al., 1993). Analyses of mutant phenotypes, as well as studies performed in other model vertebrates, have shown that BMP4 promotes posterior mesoderm differentiation and counteracts the effect of NODAL, which acts in concert with the WNT/β-catenin pathway to promote anterior mesendodermal fates (Ben-Haim et al., 2006; Conlon et al., 1994; Harland, 1994; Vincent et al., 2003; Zorn et al., 1999). These studies led to a model of the mouse PS, where its anterior-posterior (A/P) patterning is governed by opposing gradients of NODAL and BMP4 signaling activities (Morgani and Hadjantonakis, 2020).

Although cell-fate allocation in the mouse PS appears to depend on the level of NODAL signaling, there is little evidence of a NODAL signaling gradient along the A/P axis of the PS (Norris et al., 2002; Peng et al., 2016). Investigating this issue in the developing mouse embryo is challenging. One difficulty stems from the demonstrated interdependence of Wnt3, Bmp4, and Nodal and another from their earlier roles in embryo patterning (Camacho-Aguilar and Warmflash, 2020; Morgani and Hadjantonakis, 2020).

An in vitro approach, allowing better control of the conditions under which epiblast patterning takes place, now provides an alternative to conduct these investigations. Human embryonic stem cells (hESCs), cultured as a monolayer on embryo-size circular adhesive micropatterns, self-organize when exposed to BMP4 and give rise to the three embryonic germ layers, arranged in concentric rings, in an ordered and reproducible sequence, forming so-called human 2D-gastruloids (h2Dgas) (Warmflash et al., 2014). A recent study showed that mouse epiblast-like cells (EpiLCs), pluripotent cells derived from mouse ESCs (mESCs), can similarly be used to generate m2Dgas (Morgani et al., 2018).

Here we describe our use of m2Dgas to study in vitro the role of WNT3, BMP4, and NODAL during gastrulation. Consistent with previous studies (Morgani and Hadjantonakis, 2021; Morgani et al., 2018), our results validate the ability of m2Dgas to recapitulate key aspects of gastrulation when stimulated by BMP or WNT signals. The effects of two Nodal mutations on m2Dgas patterning then provide new insights into the role of Nodal in cell-fate specification during gastrulation.

RESULTS

BMP4 triggers EpiLC colony patterning

Unlike mESCs, EpiLCs have the ability to respond to germ cell and PS-inductive cues and are, therefore, considered to
A

Seeding on μ-patterns

| 2i+LIF | ACTIVIN+FGF2 | ACTIVIN+FGF2+BMP4 (BAF) | Ø |
|--------|--------------|--------------------------|---|
| -48h   | EpiLC induction | 0h | EpiLC differentiation | 48h | 72h |

B

EpiLC (BMP4+Activin+FGF2)

24h  48h  72h

POU5F1

NANOG

BRACHYURY

SOX17

FOXA2

CDX2

(legend on next page)
be in a state of formative pluripotency (Hayashi et al., 2011; Kinoshiba et al., 2021), which is well suited to recapitulate epiblast patterning in vitro.

To initiate their conversion into EpiLCs, mESCs were seeded on fibronectin-coated Petri dishes in N2B27 + ACTIVIN + FGF medium \((t = -48 \text{ h}, \text{Figure 1A})\). After 24 h, they were transferred on adhesive micropatterned substrates (700μm in diameter) obtained by microcontact printing of fibronectin onto PDMS-coated glass slides and left for another 24 h. This two-step protocol ensures homogeneous seeding of the adhesive micropatterns, which is key to patterning reproducibility. At 48 h after the start of the culture \((t = 0, \text{Figure 1A})\), cells in the colonies showed an expression profile consistent with the acquisition of an EpiLC identity \((\text{Figures S1A and S1B})\) (Hayashi et al., 2011). BMP4 was then added to the EpiLC differentiation medium \((t = 0, 50 \text{ ng/mL})\) to trigger PS formation. The differentiation of the colonies was characterized by immunofluorescence (IF) after 24, 48, and 72 h of culture.

Pluripotency in the post-implantation embryo tracks the expression of Pou5f1 (OCT4), which is initially present throughout the epiblast but is lost from mesendodermal cells as they emerge from the PS (Osorno et al., 2012). NANOG expression at these stages begins in the proximal epiblast but rapidly expands to the posterior epiblast, where it persists on either side of the emerging PS. At 24 h after the addition of BMP4, these two factors were expressed throughout the colonies, with levels increasing from the colony center to its outer region \((\text{Figure 1B})\). IF at 48 and 72 h showed that the expression of OCT4 and NANOG then progressively decreased and was restricted to the center of the colonies, suggesting that epiblast cells persisted there.

The pan-mesodermal marker Brachyury (BRA, also known as T), which begins to be expressed in the posterior epiblast at E6.0–E6.25, shortly before PS formation (Perea-Gomez et al., 2004; Rivera-Perez and Magnuson, 2005), was detected in the colonies 24 h after induction, in a large outer ring of cells \((\text{Figure 1B})\). This expression strengthened after 48 h and moved inward to a more central position at 72 h. SOX17 expression was detected in a thin ring of cells within the BRA expression domain at 48 h. Both factors are expressed in the embryo in extra-embryonic mesoderm cells, which emerge from the posterior PS, and in definitive endoderm (DE) cells emerging from the anterior PS \((\text{Bultscher and Lickert, 2009})\). To determine which of these two possibilities fits the pattern we obtained, we examined CDX2, which is co-expressed with SOX17 in some posterior mesoderm derivatives, and FOXA2, which is present in posterior epiblast, anterior PS, and axial mesodermal cells and co-expressed with SOX17 in DE cells \((\text{Bultscher and Lickert, 2009})\). CDX2 was detected at the colony periphery at 48 and 72 h. In contrast, FOXA2 expression was detected in a ring of cells closer to the center, overlapping with NANOG-positive cells but not SOX17-positive cells. FOXA2 expression in BMP4-stimulated colonies is thus associated with a posterior epiblast identity, whereas SOX17 expression is associated with an extra-embryonic mesoderm identity. The fact that SOX17 and FOXA2 were not co-expressed and that FOXA2 expression was no longer detected 72 h after BMP4 addition strongly suggests that DE and axial mesoderm do not form on BMP4-stimulated colonies.

These results, in agreement with a previous report \((\text{Morgan et al., 2018})\), thus showed that when exposed to BMP4, EpiLC colonies form a specific differentiation pattern, with a ring of mesoderm surrounding a core of epiblast, both biased toward posterior identities, as embryological studies led us to expect \((\text{Kinder et al., 1999})\).

### Sustained BMP exposure prevents the establishment of distal cell identities

Post-implantation epiblast cells express NODAL and fibroblast growth factor (FGF), but the BMP4 and WNT3 they detect are initially produced by adjacent extra-embryonic cells \((\text{Rivera-Perez and Magnuson, 2005; Winnier et al., 1995})\). Only once patterning has begun do proximal and posterior embryonic cells start to express Bmp4 and Wnt3 themselves. In line with these facts, EpiLCs express Nodal, Fgf4, Fgf5, and Fgf15, but none of the relevant Bmp or Wnt genes \((\text{Du et al., 2018})\). This suggests that the addition of recombinant WNT or BMP is required to initiate EpiLC colony patterning, whereas the addition of recombinant NODAL and FGF is not, as their endogenous production may suffice.

To test these hypotheses, and to investigate the respective roles of these morphogens, we compared the developmental trajectories of colonies stimulated with different combinations of ACTIVIN(A, a proxy for NODAL), BMP4 (B), FGF2 (F), and WNT3A (W, a proxy for WNT3). As previously reported, the absence of ACTIVIN had little impact
on the conversion of ESCs into EpiLCs, presumably because of endogenous NODAL production (Figure S1B) (Buecker et al., 2014). Endogenous production of FGF probably explains that its addition was likewise unnecessary (Figure S1B). Some EpiLC colonies obtained without the addition of AF were thus left to differentiate on their own (C) or were treated for 48 h with B or W. Other EpiLC colonies, obtained as before in the presence of AF, were then treated for 48 h with BAF, WAF, or BWAF. All colonies were then left to differentiate autonomously for a further 24 h, without morphogen added, as by that stage they themselves produce the signaling molecules necessary to sustain their differentiation.

IF detection of cell-type specific markers has so far been the main approach used to characterize the differentiation of 2Dgas (Deglincerti et al., 2016; Etoc et al., 2016; Martyn et al., 2019; Morgani et al., 2018; Warmflash et al., 2014). It provides a spatial record of the result but the number of markers that can be monitored at the same time is limited and the availability of antibodies constrains their choice. Furthermore, the resulting data are not easily exploitable for principal-component analysis (PCA), a statistical tool commonly used to distinguish cell identities based on gene expression. The quantification of gene expression in pooled colonies thus emerged as a complementary approach to track and compare developmental trajectories.

The first experiment was thus performed as follows. We collected samples at $t = -48$, $-24$, 0, 8, 24, 48, and 72 h and quantified by RT-qPCR the expression of 31 markers, specifically selected to follow post-implantation embryo patterning (Peng et al., 2016; Pijuan-Sala et al., 2019) (Table S1). They included known targets of signaling pathways, such as Id1 (BMP target), Axin2 (WNT/b-catenin target), and Lefty2 (ACTIVIN/NODAL target); genes encoding secreted antagonists, such as Noggin, Chordin, and Lefty2; and several lineage markers, such as Noto (axial mesoderm) and Sox1b (neurectoderm). The genes were clustered according to the similarity of their expression dynamics (Figures 2A and 2B): cluster 1 markers were found to track the disappearance of the epiblast identity (in all colonies regardless of the treatment); cluster 2 markers, the emergence of an ectodermal/neural identity (in C, W, and WAF colonies); cluster 3 markers, the emergence of both anterior (in W and WAF colonies) and posterior (in B, BAF, and BWAF colonies) embryonic derivatives of the PS; and cluster 4 markers, the emergence of extra-embryonic derivatives of the posterior PS (in B, BAF, and BWAF colonies). To visualize the developmental trajectories, we projected the gene expression data in the plane formed by the first two principal components of the dataset, which together capture about 60% of the variance (Figure 2B). This analysis suggested that the different treatments led to only three types of trajectories: toward neurectoderm (C); toward distal identities (W and WAF); and toward proximal identities (any treatment containing B).

A second experiment, analyzed using a slightly different set of markers, gave similar results (Figures S2A and S2B). To assess the statistical relevance of the data and define averaged trajectories, a third replicate was generated. To prevent batch effects and be able to normalize gene expression to a common reference for all replicates, gene expression levels must be quantified in the same RT-qPCR experiment. To carry out the experiment efficiently, we selected the two or three genes in each cluster that in our previous analysis commanded the highest share of the variance, measured their expression in all three replicates, and performed a PCA on the resulting dataset (Figure S2C). The mean trajectories thus obtained confirmed that all BMP4-stimulated samples (B, BAF, and BWAF) followed a similar path and reached endpoints that were not statistically distinguishable, as defined by the overlap of their respective 95% confidence ellipses (Figure 2D). The results also showed that AF did not alter the developmental trajectory of W-induced colonies either, even though it had the potential to increase the activity of the ACTIVIN/NODAL and FGF signaling pathways beyond what endogenous ligands normally produce, as evidenced by endogenous NODAL production (Figure S1B) (Buecker et al., 2014).

**Figure 2. BMP prevents the establishment of distal cell identities**

(A) Timelines of the different protocols whose effects on EpiLC colony differentiation were compared. Ø, no morphogens; A, ACTIVIN, 20 ng/mL; B, BMP4, 50 ng/mL; F, FGF2, 12 ng/mL; W, WNT3A, 200 ng/mL. (B) Gene expression matrix obtained via RT-qPCR of pooled colonies at similar time points for each of the six treatments shown in (A). The comparison of gene expression dynamics led to the grouping of markers into four distinct clusters (see main text for details). (C) Projection of the RT-qPCR data shown in (B) in the space defined by the first two principal components (PCs) of the dataset allows a comparison of the developmental trajectories obtained for each treatment. (D) Average developmental trajectories obtained from three independent experiments using a selection of 12 markers. The statistical proximity of the endpoints is defined by the overlap of their 95% confidence ellipses. (E) (Left) Maximum intensity projections (MIPs) of representative immunostained m2Dgas (700 um) 48 h after the start of WNT3A (top) or BMP4 (bottom) stimulation. (Right) Quantification of FOXA2 and SOX17 expression in several m2Dgas; each dot represents a single cell. The upper right quadrant shows the ratio of double-positive cells (yellow). (F and G) MIPs of representative m2Dgas immunostained 72 h after the start of stimulation with the treatments indicated, and corresponding average fluorescence intensity radial profiles (n = 4). Scale bar, 100 µm.
achieve. However, in the absence of WNT or BMP stimulation, as in the \( \emptyset \) condition, Nodal expression was not maintained in EpiLCs, confirming the essential requirement for these signaling activities upstream of Nodal, and the colonies differentiated toward anterior ectodermal and neural identities, exhibiting a complete absence of PS and PS derivative markers.

Immunostaining of the colonies obtained in these experiments confirmed the cell identities formed in response to each treatment and revealed their position. In W-treated colonies, co-expression of FOXA2 and SOX17 identified the presence of DE cells (Figure 2E). The fact that the expression of these two markers remained separate in BMP-treated colonies marked, as before, the formation of posterior and extra-embryonic mesodermal derivatives. The homogeneous expression of SOX1 and SOX2 in untreated (\( \emptyset \)) colonies confirmed they formed neurectoderm (Figure 2F). In W-treated colonies, this neurectodermal identity was restricted to the center, whereas it was completely absent in BAF-treated colonies. Staining for OTX2, TFAP2A, GATA6, and HAND2 nevertheless confirmed the presence of an inner core of non-neural ectoderm and an outer ring of posterior mesoderm in BAF-treated colonies (Figure 2G).

These analyses show that, unlike BMP4, the W and WAF treatments promote the formation of anterior epiblast and anterior PS derivatives. The addition of BMP4, however, largely prevented the establishment of these identities, while promoting proximal fates. Interestingly, this did not appear to involve blocking WNT or ACTIVIN/NODAL signaling because the expression of \( \text{Wnt3} \) and Nodal as well as that of their respective feedback inhibitors, \( \text{Axin2} \) and \( \text{Lefty2} \), was more strongly induced in the presence of BMP4. We focused our attention on the role of endogenously produced NODAL.

**m2Dgas recapitulate Nodal regulation in the epiblast**

First, using a Nodal\(^{+/yfp}\) reporter line—where one copy of the gene expresses yellow fluorescent protein (YFP) instead of the ligand (Figure 3A [Papanayotou et al., 2014])—we recorded the spatiotemporal dynamics of Nodal expression in differentiating colonies by time-lapse imaging. Stimulation with B or W both resulted within a few hours in a strong and homogeneous induction of Nodal expression in the entire colony (Figures 3B and 3C). This expression peaked at \( t = 24 \) h and then decreased, disappearing more rapidly in the center of the colony than in its periphery (Figures 3B and 3C), Video S1. The ring of cells where Nodal expression persisted longest was also positive for phospho-SMAD2 (pSMAD2) and was part of the BRA expression domain (Figures 1B, 3B, 3C, S3A, and S3B). This dynamic is reminiscent of the progressive restriction of Nodal expression to the posterior epiblast and the PS in gastrula stage embryos (Collignon et al., 1996).

Nodal expression in the post-implantation epiblast depends firstly on its own signaling pathway and secondly on a signaling cascade where BMP4 activates \( \text{Wnt3} \) and \( \text{Wnt3} \) increases Nodal expression (Ben-Haim et al., 2006; Norris et al., 2002). We found that inhibiting ACTIVIN/NODAL signaling with SB431542 or WNT secretion with IWP2 similarly impaired the induction of Nodal expression in B-stimulated colonies, confirming that this expression is, like in the embryo, dependent on both signaling pathways (Figure 3D). Together with our previous observation that \( \text{Wnt3} \) expression is most induced in B-stimulated colonies (Figure 2B), these results are consistent with m2Dgas adequately replicating the roles played by BMP4 and \( \text{Wnt3} \) upstream of Nodal expression in the post-implantation embryo.

The effect of Nodal on its own expression is mediated by the asymmetric enhancer (ASE) (Figure 3A [Norris et al., 2002; Yamamoto et al., 2001]), whereas the effect of \( \text{Wnt3} \) is mediated by the proximal epiblast enhancer (PEE) (Ben-Haim et al., 2006). In nascent epiblast, Nodal expression is initially under the control of the highly bound element (HBE) enhancer, but ASE becomes the predominant Nodal enhancer during epiblast maturation.

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**Figure 3. Key aspects of Nodal regulation are recapitulated during m2Dgas patterning**

(A) Schematics of the Nodal alleles used in this study. Exons are in blue, enhancers in green. White-filled enhancers are inactive at gastrulation stages. Genotypes of the cell lines used below: WT, Nodal\(^{+/+}\); Nodal-YFP, Nodal\(^{+/yfp}\); \( \Delta \text{ASE-Nodal} \), Nodal\(^{\Delta \text{ASE}/\Delta \text{ASE}} \), \( \Delta \text{ASE-Nodal-YFP} \), Nodal\(^{\Delta \text{ASE}/\Delta \text{ASE-yfp}} \), and Nodal knockout (KO), Nodal\(^{23\Delta V/23\Delta V}\).

(B) Nodal reporter expression in BMP4-stimulated Nodal-YFP EpiLC colonies. (Top) Timeline of BMP4 stimulation. (Lower left) Expression of the fluorescent reporter at \( t = 23 \) and 44 h. (Lower right) Averaged \( (n = 4) \) radial profiles of the fluorescent intensity at regular time intervals. For readability, profiles of day 1 and 2 are presented on separate graphs and SDs are only shown for \( t = 5 \) and 44 h.

(C) Same as (B) for WNT3A stimulation.

(D) Effect of an ACTIVIN/NODAL signaling inhibitor (SB) and a WNT secretion inhibitor (IWP2) on Nodal reporter expression in BMP4-stimulated Nodal-YFP EpiLC colonies. Averaged \( (n = 4) \) fluorescence intensity profiles.

(E) RT-qPCR quantification of Lefty2 expression in WT, \( \Delta \text{ASE-Nodal} \) and Nodal KO ESCs. Error bars represent the SEM of three independent experiments.

(F) Nodal reporter expression dynamics in Nodal-YFP and \( \Delta \text{ASE-Nodal-YFP} \) differentiating EpiLC colonies after WNT3A or BMP4 stimulation. Averaged profiles of \( n = 4 \) colonies for each condition. Experiments of (B–D and F) were repeated and gave similar results at least twice. Scale bar, 100 \( \mu \)m.
(Papanayotou et al., 2014). This regulatory shift is recapitulated during the conversion of ESCs into EpiLCs.

To investigate how Nodal expression levels affect cell-fate specification, we generated homozygous ASE deletions in both WT and Nodal+/YFP ESC lines (Figure 3A). We also generated an ESC line homozygous for a loss-of-function mutation of Nodal, noted Nodal23, by deleting on both alleles a sequence that encodes most of the mature ligand (Figure 3A). Nodal+/ASE/ΔASE ESCs expressed the ACTIVIN/Nodal signaling target Lefty2 at a level comparable with that of WT ESCs (Figure 3E). In contrast, Lefty2 expression was barely detectable in Nodal23/Δ23 cells, demonstrating their inability to produce a functional ligand. Nodal+/ΔASE and Nodal23/Δ23 EpiLCs were obtained in N2B27 + knockout serum replacement (KSR) without AF. They appeared similar to wild-type (WT) EpiLCs (Figures S4A and S4B).

Nodal+/ΔASE/ΔYFP cells showed a 75% reduction in YFP expression after B or W stimulation, relative to Nodal+/YFP cells (Figure 3F). Nodal+/ΔASE/ΔASE-YFP cells nevertheless showed a small increase in YFP expression after stimulation, presumably mediated by the WNT signaling-dependent PEE, because WNT-stimulated cells responded faster than BMP-stimulated ones. The expression of Nodal was similarly affected in Nodal+/ΔASE cells, whereas no post-stimulation bumps were detected in Nodal23/Δ23 cells (Figures S5A and S5B), suggesting that ASE is not the only Nodal enhancer mediating the influence of NODAL signaling at this stage.

The 2Dgas thus correctly recapitulate in vitro the regulation of Nodal expression as it has been characterized in the embryo, and Nodal+/ΔASE and Nodal23/Δ23 EpiLCs are suitable models to investigate in vitro the contribution of Nodal to patterning.

**Nodal is required to form posterior mesoderm in BMP4-stimulated colonies**

We compared the differentiation of WT, Nodal+/ΔASE/ΔASE, and Nodal23/Δ23 EpiLCs in B-stimulated colonies. As before, we performed three independent experiments and analyzed the first with 29 markers; 18 of these were then selected to complete the analysis of all three experiments. The resulting dataset was then used to draw and compare colony differentiation trajectories for each cell line (Figures 4A–4C and S5A).

The analysis of the first experiment grouped the markers into four clusters (Figures 4A and 4B). In the first were early epiblast markers, which tended to be more highly expressed before stimulation. In the second were markers of the maturing epiblast, which normally peaked at t = 0 h and decreased after stimulation. In the third were PS and posterior PS derivatives markers, which were most expressed 48 and 72 h after stimulation in WT colonies. In the fourth cluster were markers associated with a variety of cell identities, from mature epiblast and non-neural ectoderm to PS and cardiac mesoderm. Nodal23/Δ23 colonies, like the unstimulated WT colonies above, failed to activate Bra expression and showed no evidence of forming a PS and mesoderm derivatives, adopting instead what we identified in cluster 4 as a signature of non-neural ectoderm. This observation supports a strict requirement for Nodal in the formation of posterior mesoderm and is consistent with studies that found no sign of mesoderm in Nodal−/− embryos (Ben-Haim et al., 2006; Brennan et al., 2001).

The mean developmental trajectories obtained by PCA (Figure 4C) largely confirmed this analysis. They also showed that Nodal23/Δ23 and Nodal+/ΔASE/ΔASE colonies reached closer endpoints than expected given the dissimilarity of the corresponding embryonic phenotypes (Brennan et al., 2001; Conlon et al., 1994; Norris et al., 2002). This resulted from the fact that Nodal+/ΔASE/ΔASE colonies at the end of the culture had lower expression of PS markers and higher expression of ectodermal markers than WT colonies. Although they expressed Bra and other PS and posterior mesoderm markers, most peaked at 48 h and declined afterward (Figures 4C and S5A–S5C). Expression of cluster 4 genes Tp63, Pax3, Tflap2a, and Id1 was also closer to that observed in Nodal+/ΔASE/Δ23 colonies than in WT colonies. Immunostaining confirmed the differentiation of Nodal+/ΔASE/ΔASE colonies toward an ectodermal identity as they expressed TFLAP2A homogeneously, as Nodal23/Δ23 colonies did. Most Nodal+/ΔASE/ΔASE colonies also expressed BRA, GATA6, and CDX2 in irregular patches of varying size and number, but the proportion of cells expressing these markers was drastically reduced at the end of the culture (Figures 4D–4F and S5C).

These results demonstrate a strict requirement for Nodal to form the PS and posterior PS derivatives. They also reveal that the patterning of m2Dgas is more sensitive to Nodal expression levels than that of the epiblast, because no gastrulation defects were reported in Nodal+/ΔASE/ΔASE embryos (Norris et al., 2002).

**Low Nodal expression is sufficient to specify anterior PS derivatives**

We then compared the differentiation of colonies from the same three cell lines after W stimulation. The gene expression dynamics of the markers used in a first experiment identified three clusters (Figures 5A and 5B): the first contained markers of early and maturing epiblast; the second, markers for the anterior ectoderm and the neur ectoderm; and the third, markers of the PS and its derivatives. As before, W stimulation promoted the emergence of epiblast and PS derivatives of anterior character in WT colonies. In Nodal23/Δ23 colonies, anterior ectodermal and neural markers were more strongly induced, while expression
Figure 4. Nodal is required to form posterior mesoderm in m2Dgas
(A) (Top) Timeline of BMP4 stimulation. (Bottom) Gene expression matrix obtained via RT-qPCR of pooled colonies at similar time points for the WT, ΔASE-Nodal, and Nodal K0 cell lines. Expression dynamics comparison led to the grouping of markers into four distinct clusters.
(B) Projection of the RT-qPCR data shown in (A) in the space defined by the first two principal components of the dataset shows the developmental trajectories of WT and mutant colonies.
(C) Average developmental trajectories obtained from three independent experiments using a selection of 18 markers. The overlap of the 95% confidence ellipses at two endpoints indicates some similarity.
(D) Maximum intensity projections (MIPs) of representative WT and Nodal mutant colonies immunostained 48 h after the start of BMP4 stimulation.
(E and F) MIPs of representative WT and Nodal mutant colonies immunostained 72 h after the start of BMP4 stimulation and corresponding average (n = 4) radial profiles. Experiments of (D–F) were repeated and gave similar results at least twice. Scale bar 100 µm. See Figure S4 for additional examples of the patterns obtained for ΔASE-Nodal colonies.
Figure 5. Low Nodal expression does not prevent the emergence of anterior PS identities
(A) (Top) Timeline of WNT3A stimulation. (Bottom) Gene expression matrix obtained via RT-qPCR of pooled colonies at similar time points for the WT, ΔASE-Nodal, and Nodal KO cell lines. Expression dynamics comparison led to the grouping of markers into three distinct clusters.
(B) Projection of the RT-qPCR data shown in (A) in the space defined by the first two principal components of the dataset shows the developmental trajectories of WT and mutant colonies.
(C) Average developmental trajectories obtained from three independent experiments using a selection of 18 markers. The overlap of the 95% confidence ellipses at two endpoints indicates some similarity.
(D) Maximum intensity projections (MIPs) of representative WT and Nodal mutant colonies immunostained 48 h after the start of WNT3A stimulation.
(E and F) MIPs of representative WT and Nodal mutant colonies immunostained 72 h after the start of WNT3A stimulation and corresponding average (n = 4) radial profiles. Experiments of (D–F) were repeated and gave similar results at least twice. Scale bar 100 μm. See Figure S4 for additional examples of the patterns obtained for ΔASE-Nodal colonies.
of most PS and PS derivative markers remained at very low levels. A notable exception was Cdx2, which was transiently activated shortly after stimulation. *Nodal*^ASE/ASE^ colonies again showed a stronger phenotype than expected, activating the expression of ectodermal and neural markers to levels similar to those of *Nodal*^223/223^ colonies. Although they expressed PS and anterior PS derivatives markers (such as Bra, Noto, Cer1, and Foxa2), they did not reach the levels seen in WT colonies.

The 18 markers were selected to analyze three independent experiments (Figure S5A). PCA of the resulting dataset allowed us to plot mean developmental trajectories (Figure SC). While that of the *Nodal*^ASE/ASE^ colonies was initially close to that of the WT colonies, its endpoint was closer to that of *Nodal*^223/223^ colonies (Figure SC). The similarity between *Nodal*^ASE/ASE^ colonies and WT colonies was confirmed by immunostaining, showing that SOX1 and SOX2 were expressed from the center to the edge in both types of colonies (Figures 5D–5F). However, W-stimulated *Nodal*^ASE/ASE^ colonies displayed similar patches of BRA or FOXA2 expression as B-stimulated *Nodal*^ASE/ASE^ colonies, with the same variability in size and expression levels (Figures 4D, 5D, and S5C), except that this time FOXA2 was clearly co-expressed with BRA, as befits anterior PS derivatives (Figure 5D). This, and the detection of small clusters of DE cells co-expressing FOXA2 and SOX17 in some colonies after 72 h culture (Figure 5E), suggest that the drastic decrease in *Nodal* expression resulted in a reduction of the number of anterior mesendoderm cells but not in their replacement by cells of a more posterior PS identity.

These results show that the formation of anterior PS derivatives can be obtained in a context where *Nodal* is expressed at low level. Their maintenance, as well as the robustness and reproducibility of colony patterning, are, however, critically dependent on the ASE enhancer.

**DISCUSSION**

We exposed micropatterned EpiLC colonies to different morphogens to model mouse gastrulation in vitro. Although the protocol we used was slightly different from that described in another report (Morgani et al., 2018), we obtained similar differentiation patterns, attesting to the robustness of the approach. The fact that *Nodal*^−/−^ EpiLC colonies failed to express markers of the PS and its derivatives when stimulated with BMP4 or WNT3A underlines a strict requirement for *Nodal* for both PS formation and the specification of mesendodermal identities. This is in agreement with embryological studies that found no evidence of expression of PS or nascent mesoderm markers in *Nodal*^−/−^ embryos (Blen-Haim et al., 2006; Brennan et al., 2001) and finally demonstrates that neither BMP4 nor WNT3A can compensate for NODAL absence. The fact that *Nodal*^−/−^ colonies form ectodermal and neural cell identities is also consistent with their premature emergence in *Nodal*^−/−^ embryos (Camus et al., 2006). These results position Nodal as the determining factor in a binary choice between ectodermal and mesendodermal identities. They are consistent with Nodal acting upstream of the TBX factors Eomes and BRA, which have recently been shown to govern the same binary choice via their impact on chromatin state (Tosic et al., 2019).

Our results, in line with previous reports (Faial et al., 2015; Morgani et al., 2018), strongly suggest that BMP4, in addition to promoting posterior PS cell fates, actively suppresses anterior ones. This is consistent with anterior PS identities emerging from a region of the embryo that is initially beyond the reach of diffusing BMP4 molecules and later sees local activation of the expression of BMP antagonists (Bachiller et al., 2000; Zhang et al., 2019). There are several examples of BMP signaling antagonizing ACTIVIN/NODAL/Nodal signaling during embryogenesis (Furtado et al., 2008; Pereira et al., 2012; Yamamoto et al., 2009). These situations involve a component common to both pathways being limiting and competition for it favoring BMP signaling. The situation is, however, different in micropatterned colonies, and presumably in the PS, because the WNT and ACTIVIN/NODAL signaling pathways were still active in BMP4-stimulated colonies, possibly even more so given the increase in the expression of their respective feedback antagonists Axin2 and Lefty2. Although this should dampen WNT and NODAL signaling, it also suggests the possibility of a selective impact of BMP signaling on the expression of some of their targets. Furthermore, BMP4-stimulated *Nodal*^ASE/ASE^ EpiLC colonies, where *Nodal* expression is drastically reduced, failed to maintain the posterior PS derivatives they formed initially, indicating that maintaining a higher level of *Nodal* expression is required to do so, in addition to its prior requirement for PS formation. These observations suggest that, although BMP4 prevents Nodal and WNT3a from activating the anterior PS developmental program, it does not block their signaling pathways but rather recruits them to assist in promoting posterior PS development.

The fact that *Nodal*^ASE/ASE^ EpiLC colonies were unable to form radial differentiation patterns is evidence that the ability of cells to respond to and reflect local Nodal exposure is essential for patterning. The random patches of mesoderm that formed on some colonies may, however, suggest that the remaining active *Nodal* enhancers, HBE and PEE, sometimes manage to initiate a small autoregulatory response. This is consistent with reports that ACTIVIN/NODAL signaling is part of the regulatory input of these enhancers (Funa et al., 2015; Papanayotou et al.,...
and with the small bump of Nodal expression detected in these colonies 24 h after stimulation. With its activation taking place after stimulation, a limited PEE-driven auto-inductive response could explain the formation of unstable patches of BRA-positive cells. The fact that a similar phenotype was not seen in Nodal<sup>AASE/ΔASE</sup> embryos strongly suggests that Nodal depletion was compensated by ligands of extra-embryonic or maternal origin in these embryos (Norris et al., 2002). Two other transforming growth factor β (TGF-β) family members present at these stages are GDF1 and GDF3, but neither of them appears capable of compensating for Nodal absence (Andersson et al., 2007; Granier et al., 2011; Levine et al., 2009).

Furthermore, we found that both types of Nodal mutant colonies developed their drastic phenotypes despite maintaining WT levels of Gdf3 expression. A better candidate may be the ACTIVIN produced by extra-embryonic and decidual cells (Albano et al., 1994; Pijuan-Sala et al., 2019).

The PS derivatives that formed on Nodal<sup>AASE/ΔASE</sup> colonies within 48 h had identities that matched the stimulation, posterior for BMP4 and anterior for WNT3A, despite Nodal expression remaining well below its normal levels. However, their maintenance was found to depend in both conditions on sustained higher Nodal expression as their cell numbers had dwindled by the end of the culture. This does not seem to fit models where differences in the level or duration of Nodal signaling are what leads to the specification of distinct PS cell identities. These observations suggest instead a two-step process where emerging cell identities initially reflect the combination of signals their progenitors were exposed to, whereas their expansion depends on Nodal signaling reaching certain levels. Interestingly, the list of transcription factors partnering with SMAD2/3 and known to play a role in PS patterning has expanded in recent years. It now includes, in addition to SMAD4 and FOXH1 (Chu et al., 2004; Hoodless et al., 2001; Yamamoto et al., 2001), transcription factors, such as OCT4, NANOG, Eomes, and BRA, some of which are also SMAD1 partners (Faial et al., 2015; Mulas et al., 2017; Mullen et al., 2011; Suzuki et al., 2006). This mix of effectors, and the potential for both competition and cooperation it implies, appears permissive to PS cell fate specification involving some combinatorial process. Further investigations will be necessary to determine whether this is actually the case.

We noticed that below a certain cell density, mouse colonies do not get patterned whereas human ones do. The patterning of h2Dgas was shown to be critically dependent on BMP signaling being restricted to the edge of colonies. This results from TGF-β receptors remaining accessible to apically applied ligands at the edge of colonies, but not at their center, and from the expression of the BMP antagonist NOGGIN (Etoc et al., 2016). However, Noggin<sup>−/−</sup> mouse embryos display no gastrulation phenotype (McMahon et al., 1998). This is consistent with the comparatively late onset of Noggin expression during mouse development and in our m2Dgas and the fact that, unlike in hESCs, it is not directly induced by BMP4 in EpiSCs (Etoc et al., 2016).

The early absence of this antagonist could be what makes m2Dgas patterning more reliant on cell density and epithelialization to restrict BMP signaling to the edge of colonies. It could also explain why Nodal expression is first induced in the entire colony before being restricted at the edge of m2DGas, while it moves inward from the colony edge in h2DGas (Chhabra et al., 2019). Differences between mouse and human colonies in how their patterning unfolds may thus reflect actual differences in how gastrulation proceeds in the two species.

To summarize, the patterning of m2Dgas is largely consistent with the embryological data. The discrepancies we identified highlighted gaps in our understanding of the mechanisms underlying the patterning of the PS. The m2Dgas model system is, therefore, complementary to embryological studies and will address unresolved issues of gastrulation in vitro.

**EXPERIMENTAL PROCEDURES**

**Cell culture and cell lines**

All ESC lines used are HM1 (Selfridge et al., 1992), except the Nodal<sup>YFP</sup> and ΔASE-Nodal-YFP lines, which are CK35 (Papanayotou et al., 2014). ESCs were cultured on 0.15% gelatin-coated plates in N2B27 medium supplemented with MAPK/ERK pathway and glycogen synthase kinase 3 (GSK3) inhibitors and leukemia inhibitory factor (2i+LIF) (Silva et al., 2008). The Nodal<sup>−/−</sup> and Nodal<sup>AASE/ΔASE</sup> lines were generated via CRISPR/Cas9 editing. A 1196 bp deletion spanning exons two and three prevents Nodal production in the Nodal<sup>−/−</sup> line. The Nodal<sup>AASE/ΔASE</sup> line contains a 600 bp ASE deletion similar to that previously described (Norris et al., 2002).

**EpiLCs differentiation on micropatterned adhesive substrates**

ESCs were seeded on fibronectin-coated (15 μg/mL for 30 min) plates and cultured in N2B27 + 1% KSR, optionally supplemented with 20 ng/mL ACTIVIN and 12 ng/mL FGF2. After 24 h, cells were trypsinized and seeded at a density of 8,000 cells/mm<sup>2</sup> on micropatterned substrates produced by microcontact printing of fibronectin on polydimethylsiloxane (PDMS)-coated glass coverslips. After 1 h, unattached cells were removed. Colonies were cultured for an additional 24 h before stimulation began (BMP4: 50 ng/mL, WNT3A: 200 ng/mL, ACTIVIN: 20 ng/mL, and FGF2: 12 ng/mL). The medium was renewed every 24 h. See supplemental information and Simon et al., 2022 for detailed protocols.

**Quantitative analysis of gene expression**

Standard RT-qPCR was used to quantify gene expression. A custom R script was used to compute the following steps. Markers expressed at...
low level—with a difference of at least 10 between their cycle quantification (Cq) value and that of glyceraldehyde-3 phosphate dehydrogenase (GAPDH)—were removed from the analysis in all samples considered. For each marker in each sample, a relative gene expression value was computed with respect to the mean expression of the gene in the experiment (pooling all samples) and normalized by the expression of GAPDH in the sample. This value was log2-transformed and then centered and reduced with respect to the expression value of the gene in all samples considered in order to compute and display the expression matrix and the PCA of this matrix.

See supplemental information for a complete list of reagents, primers, and antibodies used.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.05.009.

AUTHOR CONTRIBUTIONS
Conceptualization, B.S., J.-L.P., and J.C.; methodology, B.S., J.-L.P., G.S., and M.V.; investigation, J.-L.P., G.S., M.V., and B.S.; formal analysis, J.-L.P., G.S., J.C., and B.S.; writing, J.C. and B.S.; and supervision, J.C. and B.S.

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CONFLICTS OF INTEREST
The authors declare no conflict of interest.

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Supplemental Information

Dissecting signaling hierarchies in the patterning of the mouse primitive streak using micropatterned EpiLC colonies

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Figure S1 (related to Fig. 1): Characterization and reproducibility of EpiLC colonies production and differentiation

(A) Representative immunostained EpiLC colonies 48h after the start of conversion. EpiLC characterization 48h after replacement of 2i+LIF by in ACTIVIN+FGF (AF) homogenous stain for OCT4, NANOG OCT6 OTX2 UTF-1 SOX2 and ECAD. Representative image of n=7 pictures.

B – qRT-PCR characterization of the expression of markers characteristic of naïve and formative pluripotency, for mESC maintained in N2B27+2i+LIF medium and for micropatterned EpiLC colonies 48h after initiation of their conversion in either N2B27+ACTIVIN+FGF+KSR (AF) or N2B27+KSR. In both case, the EpiLC conversion is characterized by down-regulation of core pluripotency markers Nanog and Sox2 but not Pou5f1, upregulation of epiblast markers Fgf5 and Otx2, while primitive endoderm markers such as Gata4 remains low, as previously reported in (Hayashi et al., 2011) error bars: standard error of the mean of n=3 separate experiments. Significance of difference between the 2 EpiLC induction protocols was assayed using the student t-test and the threshold for significance set at p=0.05.

(C) BRACHYURY immunostaining of multiple EpiLC colonies cultured with ACTIVIN and FGF2 and stimulated with BMP4 for 48h. For low colony density (each colony is separated from its closest neighbor by two colony diameters) and homogenous seeding, the observed pattern is highly reproducible inside a well.

(D) Gene expression matrix of multiple replicates obtained by qPCR 0/7/24/48/72h after BMP stimulation. Duplicates 1&2 belong to the same experiment. The replicate belongs to and independent experiment. Genes were clustered according to the similarity of their expression patterns in all replicates and 3 groups were defined based on the clustering. The experimental timeline is displayed above the gene expression matrix.

(E,) Temporal trajectory of the replicates along the first 2 principal components of the data set.

Scale bar 100µm.
Plouhinec et al. Figure S2
Figure S2 (related to Figure 2): Evolution of developmental trajectories depending on stimulation – full replicate.

(A,B) replicate experiment of figure 2. (A) Gene expression matrix obtained by qRT-PCR 0/6/29/52/94h after various stimulations: B: BMP4, A: ACTIVIN, F: FGF2, W: WNT3a. BAF and BAFo are two replicates of the same protocol but with cells with not the same passage number (BAF: 4 passages in N2B27+2i+LIF, BAFo: 27 passages in N2B27+2i+LIF) all other conditions are done with early passage cells.

(C) Individual expression profiles of the 12 genes subset used to plot the averaged developmental trajectories presented in figure 2C. Error bars: standard error of the mean of n=3 separate experiments. The stimulation received by the cells is presented using the following code (medium used for EpiLC induction – medium used during the first 48h of EpiLC colonies differentiation) e.g. in AF-BAF experiment, EpiLC are induced for 48h in N2B27+ACTIVIN+FGF then differentiated for 48h with N2B27+ACTIVIN+FGF+BMP4.
Figure S3 (related to Fig 3). Representative time series of Nodal-yfp in various conditions

A - Example of the evolution of spatiotemporal Nodal-yfp signal in various conditions, specified at the top of each column. Bottom. BRA stain at the end of the experiment. Shows that BRA expression doesn’t necessarily localize with Nodal-yfp at a given time point.

B - Nodal-yfp vs p-Smad2 co-stains at t=48h. Again, p-SMAD2 signal not necessarily highest where Nodal-yfp is the highest, see BMP. Bottom, average radial profiles for n=4 colonies.
Figure S4 (related to Figure 3): characterization of EpiLC derived from wt and mutant ESC lines.

(A) EpiLC characterization 48h after replacement of 2i+LIF by in ACTIVIN+FGF homogenous stain for OCT4, NANOG, OCT6, OTX2, UTF-1, SOX2 and ECAD. Representative image of n=7 pictures

(B) Quantification of immunofluorescence levels of the markers presented in (A), for the 3 genotypes in mESC vs. mEpiLC states. Error bars represent the standard deviation of the average single cell fluorescence of n=7 colonies. Significance of difference between pairs of conditions designed by brackets was assayed using the student t-test and the threshold for significance set at p=0.05.
Figure S5 (related to figure 4 and 5) Individual expression profiles of the 18 genes used to plot PCA presented in figure 4C and 5C

(A) Individual expression profiles of the 18 genes used to plot PCA presented in figure 4C (BMP4 stimulation) error bars: standard error of the mean of n=3 separate experiments. The experimental timeline is presented at the top of the panel

(B) Individual expression profiles of the 18 genes used to plot PCA presented in figure 5C (WNT3a stimulation). Error bars: standard error of the mean of n=3 separate experiments. The experimental timeline is presented at the top of the panel. As a reference, the trajectory of a WT unstimulated colonies has been added (in orange-brown) showing that mutant colonies follow a neural differentiation path much like unstimulated WT, with the notable exception of Otx2. This is consistent with the caudalizing effect of WNT/βcat stimulation on neural progenitors (see (Gouti et al., 2014) for instance)

(C) Immunostaining against BRACHYURY of BMP stimulated Nodax^{ASE/ASE} (ASE KO) colonies. BRACHYURY expression is spatially heterogeneous with one or a few patches of positive cells per colony. Below each image is indicated the frequency at which each phenotype has been observed in WNT and BMP4 stimulated Nodax^{ASE/ASE} colonies

Scale bars : 500µm
Table S1 (related to figures 2,4&5): average single cell expression of markers used in this study in the mouse gastrula (E6.5 to E8.5), arranged by tissue types

These data were extracted from the publicly available dataset from (Pijuan-Sala et al., 2019)

Cell types form the public data sets have been aggregated the following way:

"Caudal epiblast+" = "Caudal epiblast", "Caudal Mesoderm"

"Nascent mesoderm+" = "Nascent mesoderm", "Mixed mesoderm"

"Paraxial mesoderm+" = "Paraxial mesoderm", "Somitic mesoderm"

"Pharyngeal mesoderm+" = "Pharyngeal mesoderm", "Cardiomyocytes"

"ExE mesoderm+" = "ExE mesoderm", "Allantois", "Mesenchyme"

"Haematoendothelial progenitors+" = "Haematoendothelial progenitors", "Endothelium", "Blood progenitors 1/2", "Erythroid1/2/3"

"Neurectoderm+" = "Caudal neurectoderm","Rostral neurectoderm", "Forebrain/Midbrain/Hindbrain", "Spinal cord","Neural crest"

The color represents the relative expression of the gene (in log) normalized by the maximal expression (in log) of the same gene across all cell types. The minimal value used for the maximal expression is log(2) = 0.69.

Relative expression between 0 and 0.2: White
Relative expression between 0.20 and 0.40: Light Orange
Relative expression between 0.40 and 0.60: Orange
Relative expression between 0.60 and 0.80: Light Red
Relative expression between 0.80 and 1: Red

For each cell type, genes are ordered in decreasing order based on their relative expression.
Supplementary movie S1 (related to Figure 3): spatiotemporal dynamics of Nodal-YFP

Supplementary movie S1 shows the spatiotemporal dynamics of Nodal-YFP signal in EpiLCs colonies stimulated at t=0 with 50ng/ml BMP4. 6 colonies from 2 separate experiments are shown, highlighting the reproducibility of the spatiotemporal dynamics of Nodal-YFP expression (these colonies were picked randomly at the start of the experiment): Nodal-YFP is first upregulated in the entire colony, peaking around t=24h after BMP stimulation. During the second day of stimulation, the signal decreases faster at the center of the colony. At t=48h Nodal-YFP persist only in a ring at the periphery that will move inward of the colony before completely vanishing during the 3rd day of stimulation.

The overall spatio-temporal dynamics of Nodal-YFP expression is quite reproducible among colonies of a single experiment and between independent replicates. One can note however that the downregulation of Nodal-YFP expression at the center of colonies starts earlier in experiment 2 than in experiment 1, presumably because the cell density is higher at the time of stimulation in this experiment.
Extended Materials and Methods

Cell culture

We used mESCs line HM1 (Selfridge et al., 1992), kindly provided by Vanessa Ribes (Institut Jacques Monod) for wt cells and Nodal-/- and NodalΔASE/ΔASE mutants. Nodal-YFP line was established in a CK35 background (Papanayotou et al., 2014). mESCs were cultured on 0.15% gelatin coated tissue culture grade plates in N2B27 medium (DMEM/F12 and Neurobasal media (1:1, Life Technologies) supplemented with 1× B27 1×N2 (Life Technologies), 2 mM L-glutamine (Life Technologies), 0.1 mM β-mercaptoethanol, penicillin and streptomycin (Life Technologies) LIF [1000U/ml cell guidance systems GFM200], PD0325901 [1µM, cell guidance systems, SM26 ] and CHIR99021 [3µM, cell guidance systems, SM13]. They were passaged every 2-3 days by dissociating them in 0.05% Trypsin [gibco, 25300-054 ] for 5 min, neutralizing trypsin with 15% serum supplemented DMEM, concentrating cells by spinning, and transferring between ten and fifty thousand cells to a new plate.

Generation of Nodal-/- and NodalΔASE/ΔASE cell lines

mESC mutant lines and were established using a CRISPR plasmid containing guide RNA and a Cas9-2A-OFP (Life Technologies A2117). The choice of gRNA was made using the CRISPR MIT tool (http://crispr.mit.edu/ of The Laboratory of Professor Zhang (Hsu, Lander and Zhang, 2014) ). The targets selected in this study were chosen with a potential off-target score greater than 80/100 to minimize the risk of adverse effects. For the Nodal-/- line, we used 5’ GTGAGCAGAAAGTGTGGG and 3’ ACCGGGTTCCTTCAACGTGC as gRNA pair. The 1196bp deletion includes part of the exon2 until the beginning of the exon3, so a whole intron and a piece of exons 2 and 3 is missing, including the major part of the region coding for Nodal propeptide. For the NodalΔASE/ΔASE line, we used ACGATTTCTAAACTACAGAT and CGGCGGGCGGCTCAGAC as gRNAs to reproduce the ASE deletion described in the literature (Norris et al., 2002). The cloning of gRNAs into the plasmid was carried out following the recommendations of the manufacturer. For transfections, 500,000 cells were seeded in a 35mm diameter petri dish in 1.5ml of ES-serum medium (DMEM+10% FBS+ LIF), 1 hour before the addition of transfecting agent. A mixture of 3µl of lipofectamine2000 and 1µg of each transfected plasmid was prepared in parallel in 1ml of DMEM. The mixture was left to incubate 30 minutes before being dropped on the cells. The medium was replaced 24 hours after the transfection. 48 hours after transfection, Fluorescent cells were FACS-sorted to select cell that received the plasmid. These are then seeded at low-density (1000 to 5000 cells per petri dish 10cm in diameter) in ES-serum medium. When the colonies are large enough, they were picked manually and further expanded in 96 wells plates. After passing, a fraction of each well was recovered in order to perform the genotyping of the cells. DNA extraction was achieved using Sigma RedExtract-N-Amp for tissue (Sigma XNAT-1K) kit, following manufacturer’s instructions

Genotyping was performed by PCR reaction, using PrimeSTAR GXL buffer, 1.2µl dNTP Mixture TAKARA, 0.2µl of each primer at 10 µM, 1µl of PrimeSTAR GXL polymerase and 9.4µl of distilled sterile water (TAKARA RO50A). The following pairs of primers were used for genotyping Nodal-/- line : (fwd TGAGGTTGAGAGTGGGTG rev CTGCTGGATCGAAACTCAGG) and NodalΔASE/ΔASE line (fwd AATTGTTCCTCGTGGCATG rev: AGCATCCACTGATTTCCCA) Genotyping confirmed homozygous deletions.
Production of Micropatterned adhesive substrates

The micro-patterned substrates used in the present study were produced using standard micro-contact printing approach, presented in details in (Simon, et al., 2022). First, a mold with the desired pattern was produced by photo-lithography of SU8 resin on a silicon wafer. Uncured PDMS was then poured on these molds to obtain a negative replica of the SU8 master, after curing at 65°C for at least 1h. Cured PDMS was then peeled off the SU8 master and inked with a solution of fibronectin (20μg/ml in PBS, incubation 30 minutes). The inked stamps were then washed twice with PBS and once with water and allowed to dry for 5 minutes. During that time, PDMS-coated glass cover slips (obtained by spin coating PDMS on glass and curing) were activated in a UV-Ozone cleaner. The Fibronectin coated stamp and the activated coverslip were then put into contact, for a few seconds and separated. To prevent cell adhesion outside of the desired areas, stamped coverslips were then incubated in PBS + 1% Pluronics F127 for at least 30 minutes. After 3 washes with PBS, the micropatterned substrates were ready for cell seeding or could be stored at 4°C for a future experiment.

Epil.Cs differentiation on micropatterns

ESC cultures in N2B27+2i+LIF were harvested by trypsinization and seeded cells were first cultured for 24h on plates coated with fibronectin (15μg/ml for 30 min) in N2B27 supplemented with 1% KSR [Life Technologies, cat#10828010] and optionally with 20ng/ml ACTIVIN [Cell Guidance Systems] and 12ng/ml FGF2 [Cell Guidance Systems]. After 25h, cells were then dissociated using trypsin and seeded on micropatterned substrates at a density of 8000 cells per mm², this high seeding density ensured to achieve a dense and uniform surface coverage. After 1h, cells that did not attach were removed by gentle flushing and changing the culture medium and remaining cells were allowed to form colonies for an additional 24h in 250μl of the same base medium. The medium was renewed every 24h during the course of the experiment. Colonies were stimulated by adding different combinations of factors to the medium (50ng/ml BMP4 [R&D System], 200ng/ml WNT3A [R&D System], 20ng/ml ACTIVIN and 12ng/ml FGF2, [Cell Guidance Systems]). The stimulation was renewed after 24h, and colonies were cultured again in base medium. See also (Simon, et al., 2022) for a detailed step by step protocol of seeding on micropatterned substrates.

Immunostaining and microscopy

Cells were fixed with PBS + 4% paraformaldehyde at room temperature (RT) for 30 min, then washed twice with PBS for 10 min at RT, and blocked in PBS containing 0.1% Triton-X100 (PBST) and 3% bovine serum albumin for 1h at RT. They were then incubated overnight with the primary antibodies at 1:250 in the blocking solution (PBST+3% BSA). The cells were then washed 2 times 10 min in PBST and once for 40 min with the blocking solution, incubated for 2 hours with a cocktail of Alexa fluorophore-coupled donkey anti-rabbit, goat, and mouse secondary antibodies at 1:500 [Invitrogen] and DAPI, wash three times in PBS (quick wash followed by 10 min and 50 min washes), and mounted in Prolong Anti-fade mounting medium [Invitrogen]. Fluorescent images of the colonies were acquired with an Olympus IX81 inverted microscope equipped with a
Yokogawa CSU-X1 spinning disk head. Radial profiles of fluorescence were extracted from maximum intensity projection using a custom matlab script.

**Quantitative analysis of gene expression**

Cells from one well, containing between 7 and 10 colonies, were first dissociated in 200µl of 1M guanidinium by pipeting multiple times until the dissociation of all aggregates and the resulting solution was frozen at -20°C until all samples were collected. Cell total RNA was then extracted using the RNASpin RNA extraction kit (Macherey-Nagel), and quantified using a Nanodrop. Between 25 and 250ng of total RNA were then reverse transcribed (RT) using the Superscript IV VILO reverse-transcriptase (Thermo Fisher Scientific) and random hexamers according to manufacturer's instructions. Gene expression was quantified by quantitative PCR on a LightCycler 480 Instrument II (Roche) using 1:250 of the reverse-transcribed RNA sample mixed with a specific primer pair (1uM final each, sequence in supplemental table) and 2X KAPA SYBR FAST qPCR Master Mix (Roche) according to manufacturer's conditions. For each experiment, duplicates samples were analyzed. For each gene, the quality of the amplification was tested by quantifying its expression in a serially-diluted pool of all RT (1:10 to 1:320) to quantify amplification efficiency and target mRNA concentration relative to the RT pool in each sample. A custom R script was used to compute the following steps. In order to remove genes with low expression from later analysis, we removed genes which had a difference of at least 10 between their cycle quantification (Cq) value and the one of GAPDH in all samples considered. Then we computed for each sample and gene a relative gene expression value with respect to the mean expression of the gene in the experiment (obtained by pooling all samples belonging to the same experiment) and normalized by the expression of the GAPDH gene in the sample. This value was log2-transformed, then centered and reduced with respect to the expression value of the gene in all samples considered in order to compute and display the expression matrix and the principal component analysis of this matrix.

Averaged developmental trajectories, provided in Fig 2, 4 and 5 are based on 3 independent replicates. To avoid reprocessing all the conditions for all the different markers assayed to generate the gene expression matrices in each figure, we used the PCA and clustering to define what would be the minimal set of markers that would allow to discriminate the different experimental conditions. For each cluster, we picked a few genes having the highest expression correlation with the first two principal components. This defined a list of 12 genes for Fig. 2 and 18 for Fig. 4 and 5. Average temporal profiles for these individual genes in the different conditions are now presented in Fig. S2 and Fig. S5. Having averaged trajectories from 3 replicates allowed to compute the 95% confidence area for each condition. Overlapping confidence ellipses indicate that the conditions are not statistically distinguishable with the combination of markers used.
Table of PCR primer pairs used in this study
| Gene       | Forward Sequence | Reverse Sequence |
|------------|-----------------|-----------------|
| MmAxin2   | CTAGACTACGCCATACGAGA | GCTGGCGACAGACGACATA |
| MmBmp2    | AAAGCGTCAGGCAAACACAA | AGTCCACATACAAAGGGTGTCCTTT |
| MmBmp4    | CTCAAGGGAGTGGAAGTTGG | ATGCTGGAAGTGACCTTT |
| MmT       | TGCTGAGTCCCTCATGATAACTG | ATGACTCAGGAGCAGCATGCT |
| MmCdx2    | TGTTACAGGACATCCAGCGG | CCAAATTTAACCTGCTCTC |
| MmCer1    | GTCTGACCATACAAAGGGTGTCTCTT | CACAACAGATCGCAGCTT |
| MmCripto  | GTCTTCTTCAGTTTGTGCTCCTTC | GTTACAGAGTTCGCTCATA |
| MmDkk1    | CCATCTGCGCAATCTCTTTC | CATTCCCTCTTCCAAATAC |
| MmDnmt3b  | CTCGCAAGGTGTGGGCTTTTG | GTTCTCCGAGTTTTCGATCCT |
| MmEn1     | ACACAACCTGCGATCTCATT | GAGACGCTGAGATGTTGAC |
| MmFgf5    | TGTACTGCAAGATGGGCAATC | ACAATCCCCTGAGACACAG |
| MmFoxa2   | GAGACGCAACTACCAACAG | CGTAGGCGCTGAGGTCAT |
| MmFzrb    | AGCCCTGAAGTCTGTGTTG | CCCCTCTGAGTCTGACAT |
| MmFst     | TACCTGTTGACCTGTAATCGGA | TGATACACTCTCTGACAGCT |
| MmGata4   | TAAACCCGCAGATTTGCCTTAC | GTGCTGAGTCTGACAGT |
| MmGbx2    | GCAAGGGAAAGAGCGTCAA | GCAATTTTGTACATGAGC |
| MmGdf4    | CCACCCCCAAGGTCCTCATT | GTGCTGAGTCTGACAGT |
| MmGsc     | CATGACGCTGACCATACAGACG | CGTAGGCGCTGAGGTCAT |
| MmHand1   | TGGCTACAGTACATCGCC | TGCTGAGCAACTCCTTTC |
| MmHhex    | CTACAGCAGCCTCCGTTAC | CAGAGTGCGCTGACAGT |
| MmId1     | GAATCTGAAATCGGCGACAC | GAGTCGTCCTGTCCTTAC |
| MmLefty1  | CCAACCCGCACTGCCGAGGGA | CAGCGAACGCAACACAGT |
| MmLefty2  | CAGCGACAGATTTGCAGAGGAT | AGTTCGAGTGAGGACCTC |
| MmMesp1   | GCCTGCCTGACCTCCGACG | GTGACATCATCCCTTGC |
| MmNanog   | TTGCTTACAAGGGTCTTCCTACT | ACTCGTGAAGAATCAAGGGCT |
| MmNkx2-5  | AAAGACCCCTGCGGCCGATAA | ATCCCGCTCGCTTGCTTAC |
| MmNodal   | CTGTGAGGGCGAGTGTTCTCTA | CAGTGGCTTGCTCTCACAG |
| MmNog     | CGAGGGCATGGGTTGTAAG | TGAGGGTGACTGAGGTA |
| MmNoto    | CTAGCAGGCACTACAGGTGAGG | GATCTGGGATCAGACTG |
| MmOtx2    | CATGATGCTCTTATCAAAGCAACC | GTCGAGCTGCTCCTT |
| MmPdf3    | TGAGGCGTGAGTCTTCCAGG | ATCTGCTGAGGCTCTACAG |
| MmPax3    | AACAAGCAGGTCGACAGG | CTCGAGATGAGGCTCTAG |
| MmPou3f1  | GGGCGATATAAAGCTGCTC | TGAGGCTGAGGCTCTACAG |
| MmPou5f1  | ATGGGCGTGGATGCTTGT | CAGGGCGCTGACAGG |
| MmPrdm1   | AGCATGACCTGACATTGACACC | CTCAACACTCTCATGTAAGAGG |
| MmSfrp2   | ATCTCGAGAGAAGAGAAGAAGAGACC | TGACAGATACAGGGAGGTTGATG |
| MmSix1    | CGAAAAATTTGCGCTGCCG | TTTTCGGTGTTCTTTCCTT |
| MmSix3    | CCAGAGAAGAAGCAGGAACT | GATCTGGAGCCCTTCTTCT |
| MmSox1b   | CAAGATGGGCGAGAGAAAC | TCGGAGGATCAGCTTACCT |
| MmSox2    | GTCCGCCACAGGTCAAGAC | GCTGCGACTAGAGGAC |
| MmTal1    | CACTAGGGCAAGTGGCTTTG | GTTGAGGAGGACACTGAAATCT |
| MmTbx4    | TCAAGCGCGCTCAGTCCGCCG | GGAAGAGAGGACCTGAG |
| MmTbx6    | ACCGGCTACCTGAAGTTGAATA | AGATGGGAGAAGGGAAG |
| MmTfap2a  | TCAACCGCAACATCCGCTCC | TGAAGTGGGCTCAAGG|
| MmUtf1    | ATTCAGTCTTCCCAGGACG | GGAGAGATTCCGAGTATG |
| MmWnt1    | TCCATCGAGTCTGACCTG | ACACGGTCTGCTGCTT |
| MmWnt3    | CACAACACGAGAGGAGGAGAAA | CGCAAAACCTACCTCCCTT |

Table of antibodies used in this study

| antigen      | species | supplier       | cat#     | dilution |
|--------------|---------|----------------|----------|----------|
| BRACHYURY/TBX2 | Goat    | R&D Systems    | AF2085   | 1:250    |
| CDX2         | Mouse   | Abcam          | ab157524 | 1:250    |
| E-CAD        | Rabbit  | Cell Signaling | 3195S    | 1:250    |
| FOXA2        | Rabbit  | Cell Signaling | 81865    | 1:250    |
| GATA6        | Goat    | R&D Systems    | AF1700   | 1:250    |
| HAND2        | Rabbit  | Novus Bio      | NBP2-58062 | 1:500  |
| KLF4         | Goat    | R&D Systems    | AF3158   | 1:250    |
| NANOG        | Goat    | R&D Systems    | AF2729   | 1:250    |
| NANOG        | Rabbit  | Abcam          | ab80892  | 1:250    |
| OCT3/4       | Mouse   | BD Biosciences | 611203   | 1:250    |
| OCT6         | Rabbit  | Abcam          | ab221964 | 1:250    |
| OTX2         | Goat    | R&D Systems    | AF1979   | 1:250    |
| pSMAD2       | Rabbit  | Cell Signaling | 18338    | 1:250    |
| SOX1         | Goat    | R&D Systems    | AF3369   | 1:250    |
| SOX17        | Goat    | R&D Systems    | AF1924   | 1:250    |
| SOX2         | Rabbit  | Abcam          | ab97959  | 1:250    |
| SOX2         | Rat     | Thermofischer  | 14-9811-82 | 1:500  |
| TFAP2A       | Mouse   | SCBT           | SC-12726 | 1:250    |
| UTF1         | Mouse   | Thermofischer  | 14-9849-82 | 1:250  |

secondary: donkey anti:

| target species | fluorophore | supplier       | ref         | dilution |
|----------------|-------------|----------------|-------------|----------|
| Mouse          | Alexa488    | Abcam          | ab150109    | 1:500    |
| Rat            | DyLight488  | Thermofischer  | SA5-10026   | 1:500    |
| Rabbit         | Alexa555    | Abcam          | ab150062    | 1:500    |
| Goat           | Alexa555    | Abcam          | ab150134    | 1:500    |
| Mouse          | Alexa647    | Abcam          | ab150111    | 1:500    |
| Goat           | Alexa647    | Abcam          | ab150135    | 1:500    |
| Rabbit         | Alexa647    | Abcam          | ab150063    | 1:500    |

MmGapdh: TTCAACAGCAACTCCCACTCTTC

CCCTGTTGCTGTAGCCGTATTC

```python
MmGapdh TTCAACAGCAACTCCCACTCTTC CCCTGTTGCTGTAGCCGTATTC
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