

RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Location of transient ectodermal progenitor potential in mouse development

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ABSTRACT

Ectoderm is one of the three classic germ layers in the early mouse embryo, with the capacity to develop into both the central nervous system and epidermis. Because it is a transient phase of development with few molecular markers, the early ectoderm is the least understood germ layer in mouse embryonic development. In this work, we studied the differentiation potential of isolated ectoderm tissue in response to BMP signaling at various developmental stages (E6.5, E7.0 and E7.5), and identified a transient region in the anterior-proximal side of the embryo at E7.0 that possesses the ability to become neural or epidermal ectoderm in response to the absence or presence of BMP4, respectively. Furthermore, we demonstrated that inhibition of Nodal signaling could direct the pluripotent E6.5 epiblast cells towards ectoderm lineages during differentiation in explants *in vitro*. Our work not only improves our understanding of ectodermal layer development in early embryos, but also provides a framework for regenerative differentiation towards ectodermal tissues.

KEY WORDS: Ectoderm, BMP4, Epidermis, Nodal

INTRODUCTION

During early vertebrate development, initially pluripotent cells become progressively restricted in their developmental choices. Central to this transition is the process of gastrulation, during which the epiblast develops into the three primary germ layers (Tam and Loebel, 2007). In mouse embryos, the epiblast at embryonic day (E) 5.5 is pluripotent, and epiblast stem cells (EpiSCs) can be derived from this stage of development (Brons et al., 2007; Tesar et al., 2007). At E6.5, gastrulation is initiated with the formation of the primitive streak on the posterior side of the embryo. Epiblast cells that ingress through the primitive streak form the mesoderm and the endoderm. The cells that do not pass through the primitive streak and remain on the anterior side of the epiblast form the ectoderm (Lu et al., 2001; Tam and Loebel, 2007).

In *Xenopus*, the development of ectoderm proceeds through an ectodermal progenitor stage, which then differentiates to form the two major ectodermal lineages: surface ectoderm and neur ectoderm (Hemmati-Brivanlou and Melton, 1997a; Hemmati-Brivanlou and Melton, 1997b; Wilson and Hemmati-Brivanlou, 1995). BMP4, a member of the transforming growth factor β (TGF β) ligand superfamily, induces epidermal differentiation from the ectoderm. By contrast, suppression of bone morphogenetic protein (BMP) signaling, accomplished by BMP antagonists, leads to the specification of the neural ectoderm (Chang and Hemmati-Brivanlou, 1998; Wilson and Hemmati-Brivanlou, 1995).

In the mouse embryo, the pathways patterning the ectoderm have been less extensively studied. In early investigations, the fate map of the ectoderm was established using cell labeling and orthotopic and heterotopic grafts (Beddington, 1982; Beddington, 1981; Tam, 1989). At E6.5, the epiblast cells at the distal tip are fated to form neur ectoderm, whereas the cells at the adjacent region anterior to the distal cap contribute to surface ectoderm. Other epiblast cells at E6.5 are multipotent and do not appear to be restricted to a single lineage outcome (Lawson et al., 1991; Quinlan et al., 1995). At E7.5, the proximal part of the ectodermal layer, which is close to the extra-embryonic ectoderm (ExE), is mostly restricted to becoming surface ectoderm. The remaining regions of the anterior ectodermal layer can be mapped into progenitor regions for forebrain, midbrain, hindbrain and spinal cord (Tam, 1989; Tam and Quinlan, 1996). Based on these fate-mapping studies, it remains unclear whether a transient ectodermal progenitor potential region exists in mouse embryo. Recently, Cajal and colleagues have discovered a small number of cells in mouse embryo that could contribute to both surface ectoderm and neural ectoderm during normal embryonic development. These cells were positioned between the proximal and distal regions of the anterior ectoderm layer at late gastrulation stage (Cajal et al., 2012). This would suggest that the majority of the ectoderm cells are biased to neural or epidermal fate except for this small subset of cells positioned in the narrow intermediate zone. However, it is important to note that in the intact embryo, cell fate regionalization does not necessarily indicate lineage commitment. Although local signals may lead to early separation of the surface ectoderm and neur ectoderm cell fate in the intact embryo, these cells may retain a broader potential when explanted *in vitro* in response to new signals or the removal of repressive signals. For example, Osorno et al. revealed that presomitogenesis-stage embryo (E7.5–E8.0) tissue can also be cultured as pluripotent EpiSCs when explanted in activin/fibroblast growth factor (FGF) conditions (Osorno et al., 2012).

In this study, we isolated anterior ectodermal tissue from E6.5, E7.0 and E7.5 mouse embryos, and studied their differentiation potential by culturing these tissue fragments in chemically defined medium with or without BMP4. We found that, at E6.5, the anterior part of the mouse embryo still retained pluripotency, giving rise to

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all three germ layer derivatives. By E7.0, the anterior part of the ectodermal layer was no longer pluripotent but had the potential to become either epidermis or neur ectoderm, depending on the presence or absence of BMP4, respectively. However, this is a very transient phase of development because by E7.5, the ectoderm was no longer responsive to BMP4 and was restricted to either neural or epidermal fate, depending on its position in the embryo.

In mouse embryos, Nodal signaling is important for primitive streak formation and mesoderm/endoderm development (Brennan et al., 2001; Conlon et al., 1994; Dunn et al., 2004; Zhou et al., 1993). The anterior visceral endoderm (AVE) produces the Nodal antagonists *Lefty1* and *cerberus 1* (*Cer1*) to block mesoderm formation in the anterior region and promote neural development (Perea-Gomez et al., 2001; Perea-Gomez et al., 2002; Thomas and Beddington, 1996). Mouse embryos deficient for the activity of Nodal fail to form mesoderm and definitive endoderm. When explanted and cultured *in vitro*, *Nodal*^{-/-} epiblast cells readily differentiate into neurons (Camus et al., 2006). Studies in mouse EpiSCs and human embryonic stem cells (ESCs) also revealed that inhibition of Nodal signaling by SB431542 (Inman et al., 2002; Laping et al., 2002) attenuates mesoderm/endoderm differentiation and promotes neural induction (Chng et al., 2010; Patani et al., 2009; Vallier et al., 2009). A recent study further revealed that cells developing from EpiSCs in the absence of extrinsic signals (including Nodal signaling) express levels of core transcription factors similar to the anterior neural plate of ~E7.5 embryos (Iwafuchi-Doi et al., 2012). We show here that Nodal inhibition is not merely an enhancer of neural fate. The addition of Nodal inhibitor promotes ectodermal progenitor potential from the pluripotent anterior regions of E6.5 embryos. We propose that attenuation of Nodal signaling during mouse gastrulation promotes the initial formation of ectoderm and that subsequent exposure to different levels of BMP signaling determines the regional commitment to neural or epidermal fate.

RESULTS

Dynamic patterns of gene expression during ectoderm formation

During ectoderm formation, the loss of pluripotency and acquisition of specificity is a progressive, gradual process. In order to gain a detailed overview of the process, we examined gene expression patterns in mouse embryos from E6.0 to E7.5 using whole-mount *in situ* hybridization (Fig. 1).

The primitive streak marker *T* is expressed in the posterior side of the epiblast from E6.5 and gradually extends along the posterior edge to the distal point of the egg cylinder by E7.5 (Fig. 1A). The pluripotent marker *Pou5f1* (*Oct4*) is highly expressed in the whole epiblast until E7.5, from which stage it becomes weak in the anterior side (Fig. 1B). The expression of the epiblast marker *Fgf5* is reduced by E7.0 concurrent with the extension of the primitive streak (Fig. 1C). Although *Sox2* and *Oct6* (*Pou3f1*) are considered as neural markers after E7.5, they are initially expressed throughout the epiblast, but become restricted to the anterior side by E6.5 (Fig. 1D,E).

Other neur ectoderm markers, *Six3* and *Hesx1* (Oliver et al., 1995; Thomas and Beddington, 1996), are not seen until E7.5. *Six3* is only detectable at E7.5 in the anterior part of the ectodermal layer (Fig. 1F). *Hesx1* is expressed in the AVE at E7.0, and later found in the anterior part of the ectodermal layer at E7.5 (Fig. 1G). Epidermal markers, the cytokeratins *Krt8* (*K8*) and *Krt18* (*K18*), were previously reported to be expressed in the single-layer ectodermal cells from E8.5 (Aberdam et al., 2007b). However, our data show

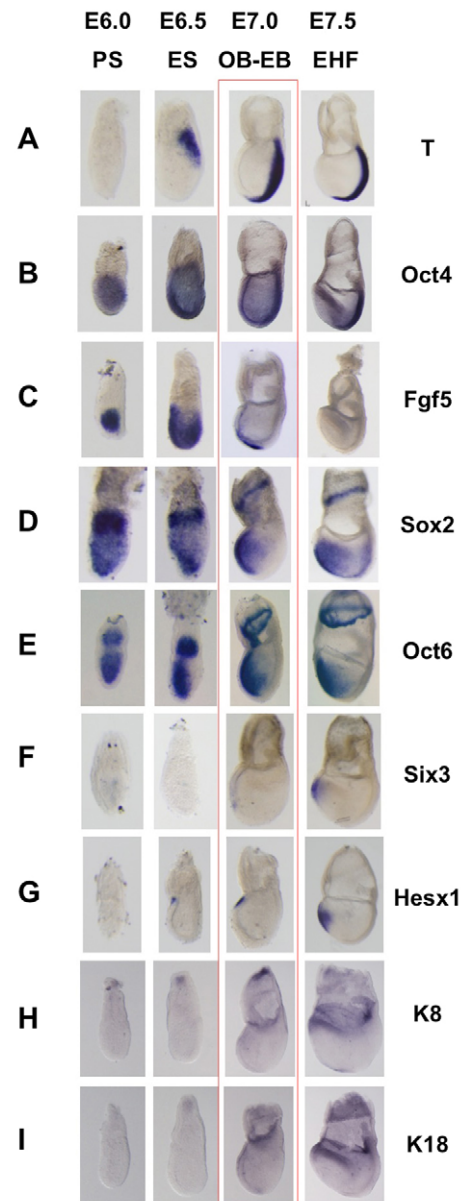


Fig. 1. Dynamic patterns of marker gene expression during ectoderm formation in mouse embryos. (A) *T* is expressed in the posterior side from E6.5. (B) *Oct4* is reduced in the anterior side by E7.5. (C) *Fgf5* expression is reduced in the epiblast by E7.0. (D,E) *Sox2* and *Oct6* are initially expressed in the epiblast and ExE, before being restricted to the anterior side from E6.5. (F) *Six3* is expressed in the anterior region at E7.5. (G) *Hesx1* is expressed in the AVE at E7.0, and then later also in the anterior ectoderm at E7.5. (H,I) *K8* and *K18* are expressed in the anterior region at E7.5. All are lateral views, with the anterior to the left. The red rectangle is used to highlight the E7.0 results.

that *K8* and *K18* expression is detectable in the anterior side as early as E7.5 (Fig. 1H,I). Notably, the *K8*- and *K18*-positive region is more proximal than the *Six3*- and *Hesx1*-positive region.

Our results show that the early epiblast marker *Fgf5* is downregulated by E7.0, but the neural markers *Six3* and *Hesx1* and the epidermal markers *K8* and *K18* are not yet expressed at this stage. This raises the possibility that an intermediate stage is present at E7.0, when cells can develop into either neural or epidermis depending on the cells' environment.

Explant cultures demonstrate progressive specification of ectoderm lineages from E6.5 to E7.5

To test the differentiation potential of the ectoderm at different developmental stages, we collected anterior ectoderm tissues at three time points: E6.5, E7.0 and E7.5. Reverse transcription quantitative PCR (RT-qPCR) analyses were performed on the explants to assess the purity of the dissected ectodermal tissue. Minimal contamination from non-ectodermal tissues was observed (supplementary material Fig. S1). Given the differences in gene expression profiles of the anterior (*Sox2*⁺; *Oct6*⁺) and posterior (*T*⁺) halves, we bisected the ectoderm layer into anterior and posterior portions, and cultured them as explants in order to compare variations in their developmental potential.

The epiblast tissue at E6.5 is not committed to ectoderm

The epiblast tissue was dissected away from the overlying visceral endoderm and ExE at E6.5, and then cut into anterior and posterior portions (Fig. 2A). Gene expression profiling showed that the epiblast tissue did not express the trophectoderm marker *Cdx2* (supplementary material Fig. S1Aa), but expressed the epiblast marker *Fgf5* in both anterior and posterior portions (supplementary material Fig. S1Ab). High expression of *T* in the posterior portion (supplementary material Fig. S1Ac) and enrichment for *Oct6* and *Sox2* in the anterior portion (supplementary material Fig. S1Ad,Ae) was also observed, complementing our *in situ* hybridization results.

During the 5-day culture, both anterior and posterior explants showed formation of stratified cells in BMP4-supplemented medium

that was not observed in controls (Fig. 2B). Time course experiments showed that *Oct4* was downregulated rapidly in both anterior and posterior explants, regardless of BMP4 supplementation (supplementary material Fig. S2A). The primitive streak marker *T* was transiently upregulated in anterior explants in both control and BMP4 conditions on day 1, and remained high in posterior explants. From day 3, *T* expression was sharply downregulated in all explants to barely detectable levels by day 5 (supplementary material Fig. S2B). The neural marker *Sox1* was upregulated only in anterior and posterior explants cultured without BMP4 (supplementary material Fig. S2C). By contrast, the epidermal marker *K18* and mesoderm marker *Flk1* (*Kdr*) were only upregulated in explants cultured with BMP4 (supplementary material Fig. S2D,E).

We assessed additional marker expression in anterior and posterior explants at day 5 of culture. In the absence of BMP4, the explants expressed the neural markers *Sox1* and *Pax6* (Fig. 2Ca,Cb). Intriguingly, their expression levels were higher in the anterior explants compared with the posterior, suggesting that there is already some restriction in potential towards a neural fate in the anterior versus posterior explants. However, in the presence of BMP4, both anterior and posterior explants expressed the epidermal markers *K18* and *Krt14* (*K14*) (Kirfel et al., 2003; Moll et al., 1982) (Fig. 2Cc,Cd), and the mesoderm/endoderm markers *Flk1* and *Gata6* (Fig. 2Ce-Cf), suggesting that the epiblast retains the capacity to respond to BMP4 and induce surface ectoderm and mesoderm/endoderm gene expression.

Explant culture of proximal and distal epiblast explants (supplementary material Fig. S2F) also showed equivalent induction

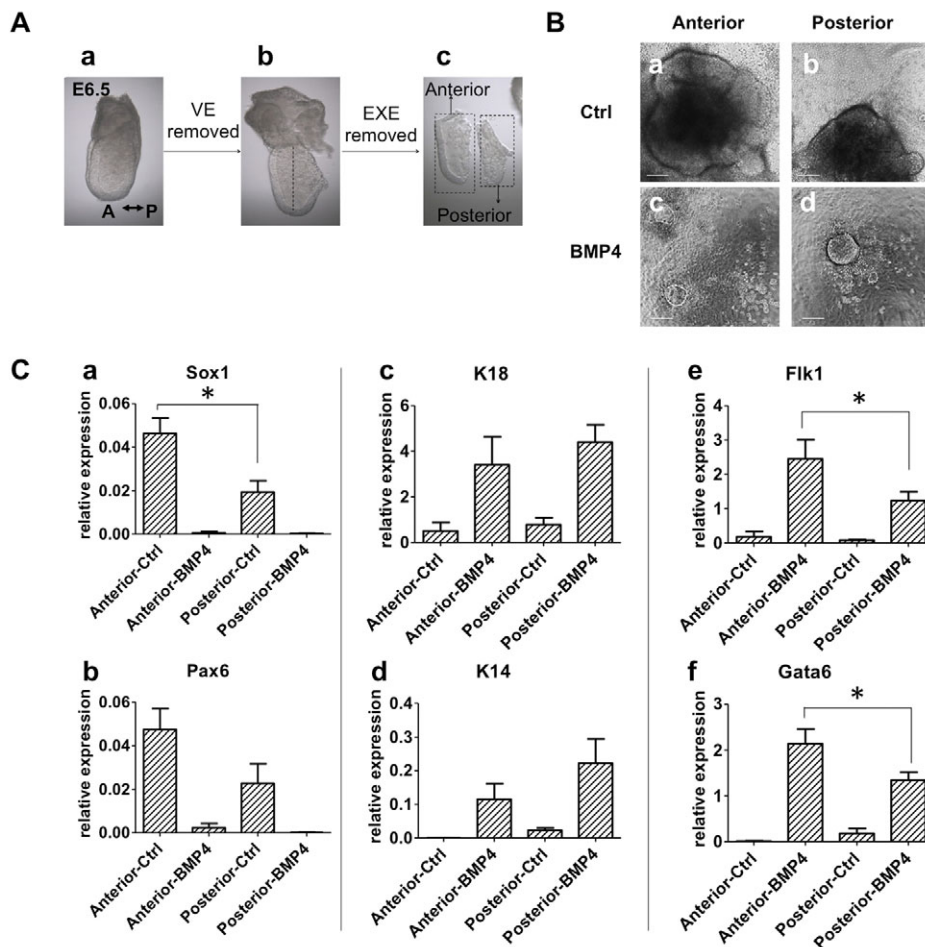


Fig. 2. The anterior part of the embryonic ectoderm at E6.5 is pluripotent.

(A) Dissection process of E6.5 mouse embryos. (B) Morphology of explants cultured in control (Ctrl; Ba,Bb) and BMP4-supplemented (Bc,Bd) medium. Scale bar: 100 μ m. (C) RT-qPCR analysis of marker gene expression of anterior and posterior explants cultured in control or BMP4-supplemented medium. *n*=4 biological replicates. **P*<0.05. Error bars represent s.d.

of mesoderm/endoderm markers in the presence of BMP4 (supplementary material Fig. S2G).

The data described above indicate that the epiblast at E6.5 has the ability to give rise to neural, epidermal and mesoderm/endoderm cells, and implies that the ectodermal germ layer restriction has not yet occurred in the epiblast at E6.5.

The anterior part of the E7.0 ectodermal layer has ectoderm but not mesoderm/endoderm potential in the presence of BMP4

In the E7.0 mouse embryo, embryonic tissue was first dissected into anterior and posterior portions (Fig. 3Aa,Ab). Mesoderm, endoderm and ExE were subsequently removed to generate anterior and posterior ectodermal explants (Fig. 3Ac,Ad). As expected, the ectoderm explants do not express cerberus 1 (*Cer1*), which is selectively expressed in the anterior visceral endoderm, or *Hex* (*Hhex*), which is found in the anterior definitive endoderm (Thomas et al., 1998) (supplementary material Fig. S1Ba,Bb). Consistent with

a previous study (Yamaguchi et al., 1993), the mesoderm marker *Flk1*, which is enriched in the anterior mesoderm at E7.0, was not detected in either anterior or posterior ectodermal fragments (supplementary material Fig. S1Bc). The primitive streak marker *T* was highly expressed in the posterior fragments but not in the anterior (supplementary material Fig. S1Bd). *Oct6* and *Sox2* were still enriched in the anterior fragments (supplementary material Fig. S1Be,Bf).

After 5 days of culture, we found that the anterior explants showed a homogeneous paving-stone-like morphology in BMP4-supplemented medium (Fig. 3B). Elevated expression of the neural markers *Sox2*, *Sox1*, *Pax6* and *Six3* were observed in the anterior explants cultured without BMP4 (Fig. 3Ca). By contrast, in the presence of BMP4, the expression of these neural markers was not found. Rather, epidermal markers such as *K8*, *K18*, *Krt5* (*K5*), *K14*, *Krt15* (*K15*) and *ΔNp63* (Aberdam et al., 2007a; Kirfel et al., 2003; Moll et al., 1982; Troy et al., 2011) (Fig. 3Cb) were

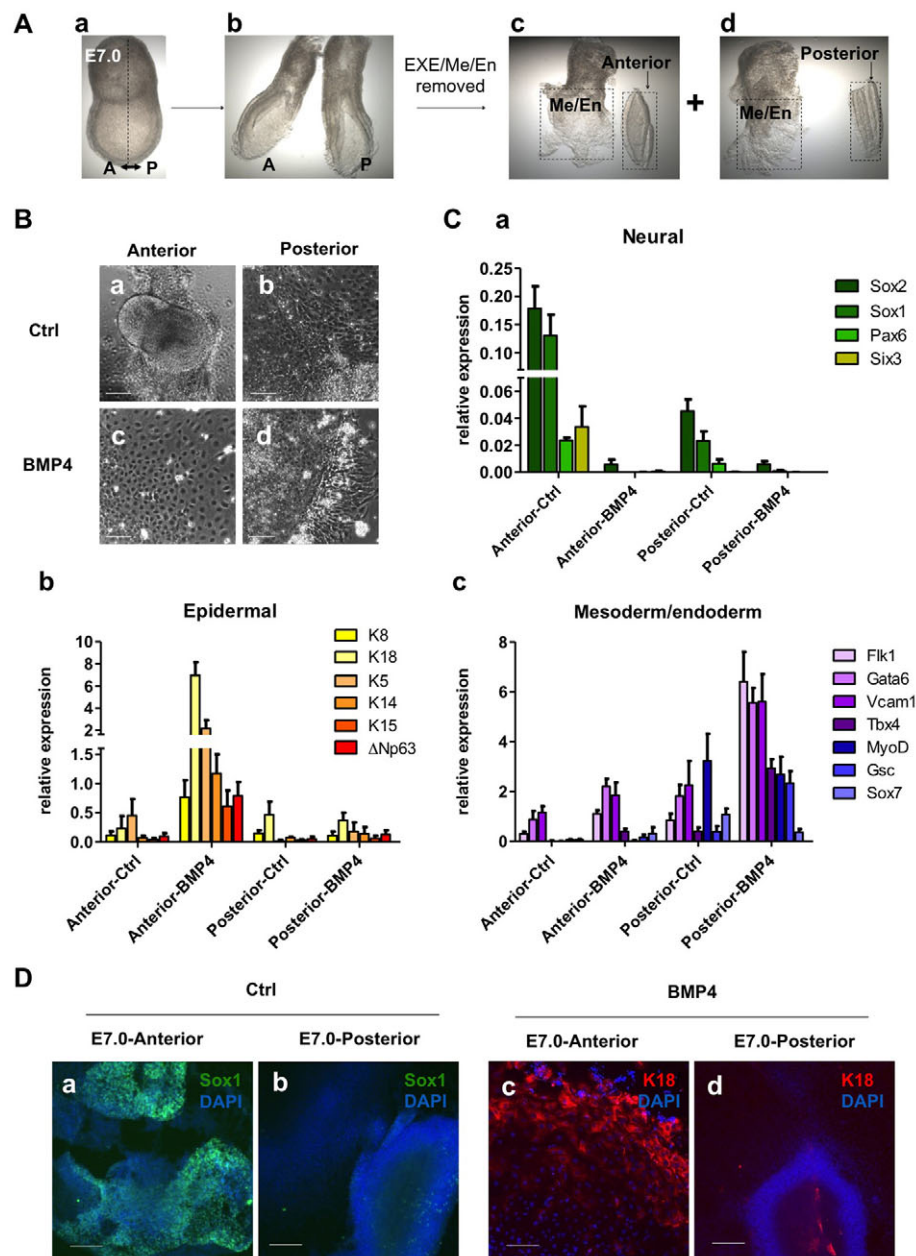


Fig. 3. The anterior ectoderm in E7.0 mouse embryo has the potential to give rise to neural and epidermal cells. (A) Dissection process of E7.0 mouse embryos. (B) Morphology of explants cultured in control (Ctrl; Ba,Bb) and BMP4-supplemented (Bc,Bd) medium. (C) RT-qPCR analysis of marker gene expression of anterior and posterior explants cultured in control or BMP4-supplemented medium. *n*=6 biological replicates. Error bars represent s.d. (D) Immunostaining for Sox1 and K18 in anterior and posterior explants cultured without (Ctrl) or with BMP4. Scale bars: 100 μm.

observed. Very low levels of mesoderm/endoderm markers, such as *Flk1*, *Gata6*, *Vcam1*, *Tbx4*, *MyoD* (*Myod1*), *Gsc* and *Sox7*, were expressed (Fig. 3Cc). These results suggest that the anterior ectoderm tissue of E7.0 mouse embryo has the potential to become either neural or epidermal lineage, and is restricted to an ectodermal fate.

The posterior explants expressed lower levels of neural markers in control medium (Fig. 3Ca), and failed to express epidermal markers in BMP4-supplemented medium (Fig. 3Cb). However, high levels of mesoderm/endoderm markers were found in response to BMP4 (Fig. 3Cc) suggesting that the posterior portion of the E7.0 ectodermal layer preferentially responds to BMP4 by forming mesoderm/endoderm. Immunostaining confirmed the expression of K18 (epidermal) and Sox1 (neural) markers in most cells in the anterior explants (with or without BMP4, respectively). Only a few cells in the posterior explants expressed these markers (Fig. 3D).

In order to expose transient changes that might be obscured by the 5-day culture regimen, we analyzed a more detailed time course of

changes in the expression of some important genes. The expression of *T* and the early mesoderm/endoderm marker *Mixl1* (Hart et al., 2002) were never activated in anterior explants during the 5-day culture period regardless of BMP4 supplementation. The higher expression level in the posterior explants was rapidly downregulated after the first day (supplementary material Fig. S3A,B). In the absence of BMP4, the neuronal markers *Sox2* and *Sox1* were upregulated in anterior explants. An initial transient increase in these markers was observed in posterior explants but the levels were lower than their anterior counterparts (supplementary material Fig. S3C,D). With supplementation of BMP4, *K18* was obviously activated from day 3 in anterior explants (supplementary material Fig. S3E), whereas *Flk1*, *Gata6* and the endoderm marker *Gsc* (Yasunaga et al., 2005) were highly activated only in posterior explants (supplementary material Fig. S3F-H).

In summary, our results suggest that the anterior part of the ectodermal layer becomes restricted to ectodermal progenitor potential by E7.0 with the ability to further differentiate into either neural or epidermal tissue in response to BMP4.

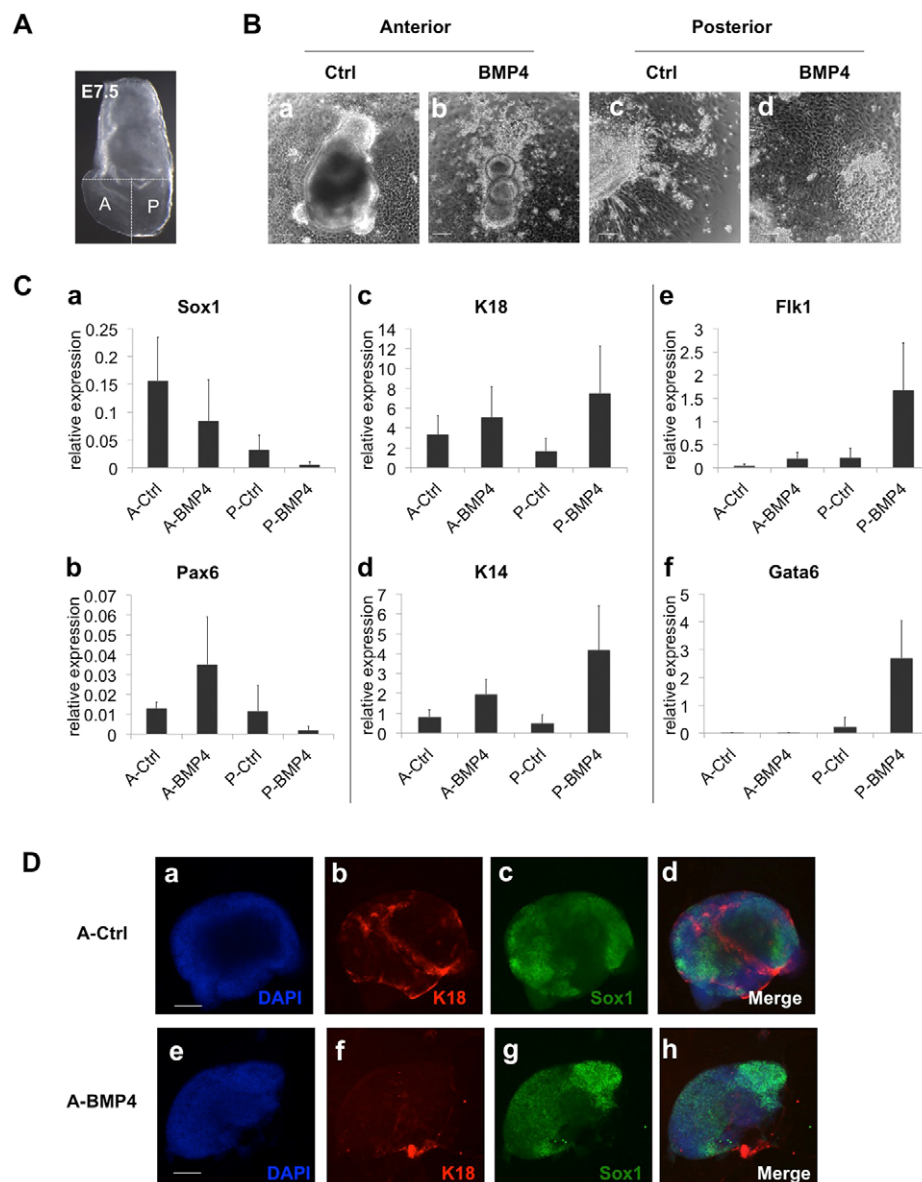


Fig. 4. BMP4 cannot repress neural induction or promote epidermal fate in the anterior ectoderm at E7.5. (A) Morphology of an E7.5 mouse embryo. Dotted line indicates position of dissection. (B) Morphology of explants cultured in control (Ctrl; Ba,Bc) and BMP4-supplemented (Bb,Bd) medium. (C) RT-qPCR analysis of marker gene expression of anterior explants (A-Ctrl, A-BMP4) and posterior explants (P-Ctrl, P-BMP4) cultured in control or BMP4-supplemented medium. $n=4$ biological replicates. Error bars represent s.d. (D) Immunostaining of Sox1 and K18 in anterior explants cultured in control (Da-Dd) and BMP4-supplemented (De-Dh) medium. Scale bars: 100 μ m.

Surface ectoderm and neural ectoderm are committed by E7.5

It is widely accepted that the neural plate has already formed by E7.5, which is supported by *in situ* hybridization of *Hesx1* and *Six3* in the anterior ectoderm at this developmental time point (Fig. 1E,F). We also observed expression of the epidermal markers *K8* and *K18* in the more proximal region of the ectoderm (Fig. 1G,H), suggesting that the epidermal region might also be specified. To test our model, we separated the ectodermal layer of E7.5 embryos into anterior and posterior portions, and tested their differentiation potential in culture (Fig. 4A).

In contrast to E6.5 and E7.0 explants, we found that the addition of BMP4 in the media could not prevent the formation of neur ectoderm outgrowths in the anterior explants of E7.5 embryos (Fig. 4Bb). RT-qPCR analysis also showed that BMP4 treatment did not cause a statistically significant difference in the expression of *Sox1*, *Pax6*, *K18* and *K14* in the anterior explants (Fig. 4Ca–Cd). The mesoderm/endoderm markers *Flk1* and *Gata6* were upregulated in the posterior, but not in the anterior, explants (Fig. 4Ce,Cf).

Immunostaining revealed the presence of two cell populations in the control anterior explants: Sox1-positive cells in neur ectoderm aggregates (Fig. 4Dc), and K18-positive cells surrounding them (Fig. 4Db). Addition of BMP4 did not noticeably change the ratio of K18- or Sox1-positive cells in the anterior explants (Fig. 4Df,Dg). Previous reports have suggested that, rather than inhibiting neuronal formation at this stage, BMP4 directs the differentiation and specification of neural progenitors (Hendrickx et al., 2009; Moon et al., 2009; Shan et al., 2011). This is consistent with our results, as BMP4 did not eliminate neural marker expression but only altered their expression level. In summary, our work supports the notion that neural ectoderm is

committed by E7.5 and also suggests that this is the time of the emergence of cells restricted to surface ectoderm.

BMP activity and refinement of the ectodermal progenitor region in E7.0 mouse embryo

BMP4 is expressed in the ExE from E6.5 to E7.5, and can presumably act at a distance in the associated epiblast to influence cell fate (Fig. 5Aa–Ac). The expression of the BMP4 direct targets *Id1* and *Id2* in the proximal region of the embryonic tissue from E7.0 (Fig. 5Ad–Ai) could define the boundaries of BMP4 activity. At E7.5, the proximal region of BMP signaling activity, as denoted by *Id1* and *Id2* expression, is coincident with the *K18*-positive region (Fig. 5Aj–Al), consistent with a role for BMP signaling in epidermis induction.

The differential downstream activity of BMP4 along the proximal-distal axis of the epiblast raises the possibility that the differentiation potential of anterior E7.0 ectodermal cells might be influenced by their proximity to the ExE. In order to test our hypothesis, we further dissected the anterior ectoderm into proximal (anterior/proximal, A/P) and distal (anterior/distal, A/D) portions (Fig. 5Ba). As expected, *Id1* and *Id2* are expressed at much higher levels in the A/P region compared with the A/D (Fig. 5Bb). After 5 days of culture, we found that both A/P and A/D explants expressed *Sox1*, *Pax6* and *Six3* in the absence of BMP4 (Fig. 5Ca). However, only A/P explants expressed the epidermal markers *K18*, *K14* and *K15* at high levels when cultured in the presence of BMP4 (Fig. 5Cb). Consistent with whole E7.0 anterior explants (Fig. 3Cc), both A/P and A/D expressed mesoderm/endoderm markers at lower levels compared with the posterior region (Fig. 5Cc). These results suggest that the A/P fragment possesses the potential to become both neurons and epidermis.

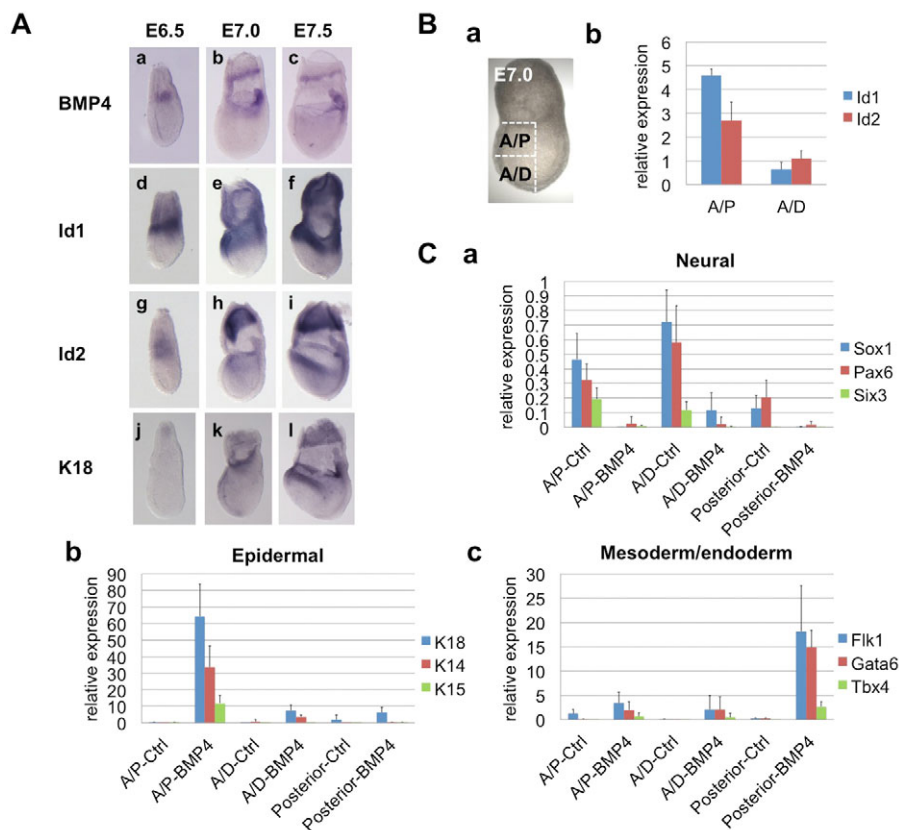


Fig. 5. The anterior/proximal (A/P) region of ectoderm at E7.0 has ectodermal progenitor potential. (A) Whole-mount *in situ* hybridization for *Bmp4*, *Id1*, *Id2* and *K18* in mouse embryos at E6.5, E7.0 and E7.5. (B) Separation of anterior ectoderm in E7.0 mouse embryo into proximal (anterior/proximal, A/P) and distal portions (anterior/distal, A/D) as indicated (Ba). *Id1* and *Id2* show different expression levels in A/P and A/D (Bb). (C) RT-qPCR analysis of neural marker genes *Sox1*, *Pax6* and *Six3* (Ca), epidermal marker genes *K18*, *K14* and *K15* (Cb), mesoderm/endoderm marker genes *Flk1*, *Gata6* and *Tbx4* (Cc) in A/P, A/D and posterior explants cultured in control or BMP4-supplemented medium. *n*=4 biological replicates. Error bars represent s.d.

To investigate the possibility of additional regional biases, we subdivided the A/P fragment into four smaller pieces (fragments 1, 2, 3 and 4) (Fig. 6Aa,Ba). Owing to the difficulty of culturing small ectoderm fragments in the chemically defined medium, we co-cultured these fragments with the much larger anterior ectoderm explants. Using mouse embryos ubiquitously expressing enhanced green fluorescent protein (EGFP) (Hadjantonakis et al., 1998), we isolated fragments 1, 2, 3 and 4 of the A/P region at E7.0 (Fig. 6Aa,Ab), aggregated each fragment with an anterior fragment from a wild-type E7.0 embryo (GFP negative), and co-cultured them in N2B27 with or without BMP4 for 5 days (Fig. 6Ac,Ad). We were able to obtain expansion of the GFP-positive cells within the aggregated populations.

We sorted out the GFP-positive cells derived from the small ectoderm fragments using fluorescence-activated cell sorting (FACS) and analyzed gene expression of various lineage markers. Three of the four fragments expressed similar levels of epidermal markers and neural markers when cultured with or without BMP4, respectively, with the exception of fragment 2, which expressed lower levels of epidermal markers (Fig. 6Bb,Bc). Consistent with the whole A/P explants (Fig. 5Cc), all four fragments expressed mesoderm/endoderm markers at much lower levels compared with the posterior explants (supplementary material Fig. S4). We conclude that, despite some heterogeneity in the differentiation efficiency of ectodermal derivatives, the entire A/P fragment can give rise to neural and surface ectoderm. By comparison, the small fragment of the A/D region co-cultured with large anterior ectoderm explant expressed very weak epidermal markers and relatively higher levels of mesoderm/endoderm markers when cultured with BMP4 (supplementary material Fig. S5). This result confirmed the difference between A/D and A/P regions. Moreover, it indicated that the large anterior fragment did not alter the differentiation potential of small fragments in an obvious manner.

Taken together, our data indicates that a proximal-distal BMP signaling gradient in the anterior ectoderm can influence its differentiation potential. The anterior/proximal region, which exhibits higher BMP activity, can give rise to neural and epidermal cells, whereas the anterior/distal region, which has lower BMP activity, preferentially generates neural tissues.

Derivation of the transient ectodermal progenitor stage by brief inhibition of Nodal signaling in E6.5 anterior epiblast

Given that Nodal antagonists are secreted from the AVE at E6.5–E7.0 to suppress Nodal activity at the anterior side (Perea-Gomez et al., 2001; Perea-Gomez et al., 2002; Thomas and Beddington, 1996), we hypothesized that Nodal inhibition probably directs the differentiation of epiblast cells towards ectodermal progenitor populations, which can then differentiate into neural or epidermal fate in the absence or presence of BMP4, respectively.

To verify this hypothesis, we tested the differentiation potential of E6.5 epiblast tissue pre-treated for 6 hours with the nodal inhibitor SB431542, prior to culturing the cells in our chemically defined media with or without BMP4. Consistent with previous reports (Camus et al., 2006), the epiblast tissue treated with Nodal inhibitor, regardless of anterior or posterior identity, expressed high levels of the neural markers *Sox1*, *Sox2*, *Pax6*, *Map2* and *Six3* after 5 days of differentiation in BMP4-negative medium, except that *Six3* was not expressed in the posterior portion (Fig. 7Aa,Ba). When cultured with BMP4, the anterior explants differentiated predominantly into epidermal-like cells, expressing very low levels of mesoderm/endoderm markers, and high levels of the epidermal markers *K8*, *K18*, *K5*, *K14*, *K15* and *ΔNp63* (Fig. 7Ab). This suggests that the E6.5 anterior tissue can be restricted to ectoderm by brief inhibition of Nodal signaling (Fig. 7C).

Surprisingly, Nodal inhibition had minimal effects on epidermal and mesoderm/endoderm marker expression in the posterior

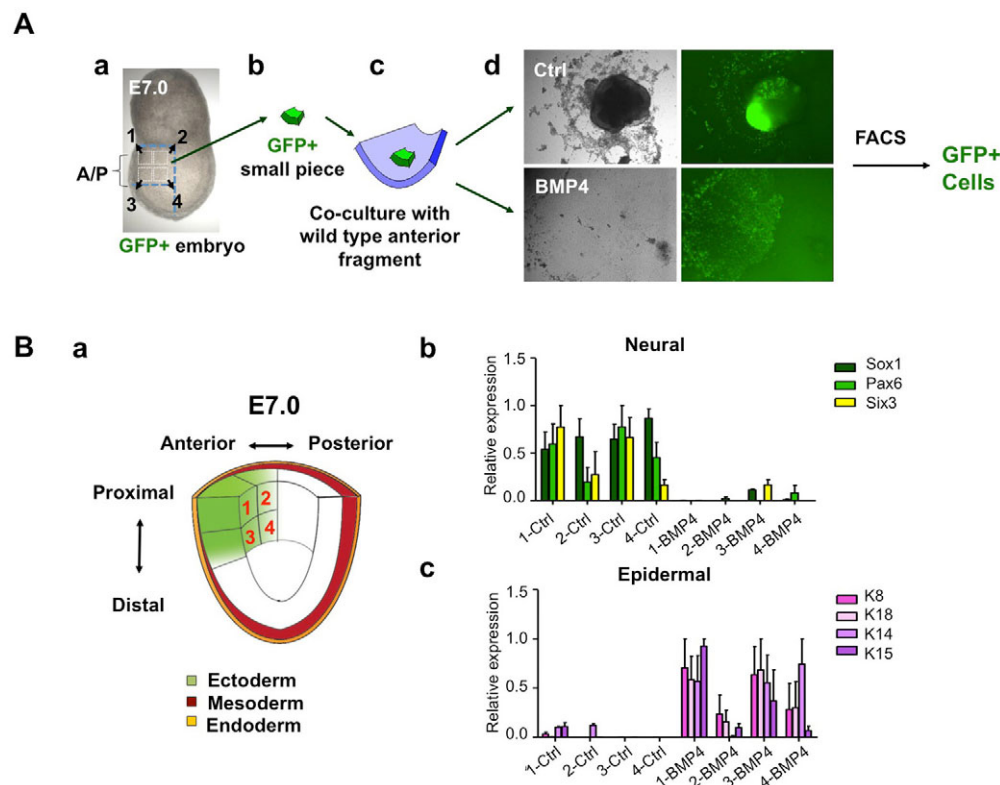


Fig. 6. Regionalization of ectodermal progenitor potential in E7.0 ectodermal layer in mouse.

(A) Schematic of the co-culture method used to obtain derivatives from EGFP-positive A/P fragments 1, 2, 3 and 4. (B) Schematic illustrating locations of fragments 1, 2, 3 and 4 in A/P region (Ba). RT-qPCR analysis of neural (Bb) and epidermal (Bc) marker gene expression in derivatives of fragments 1, 2, 3 and 4 cultured in control (Ctrl) medium or BMP4-supplemented medium. $n=4$ biological replicates. Error bars represent s.d.

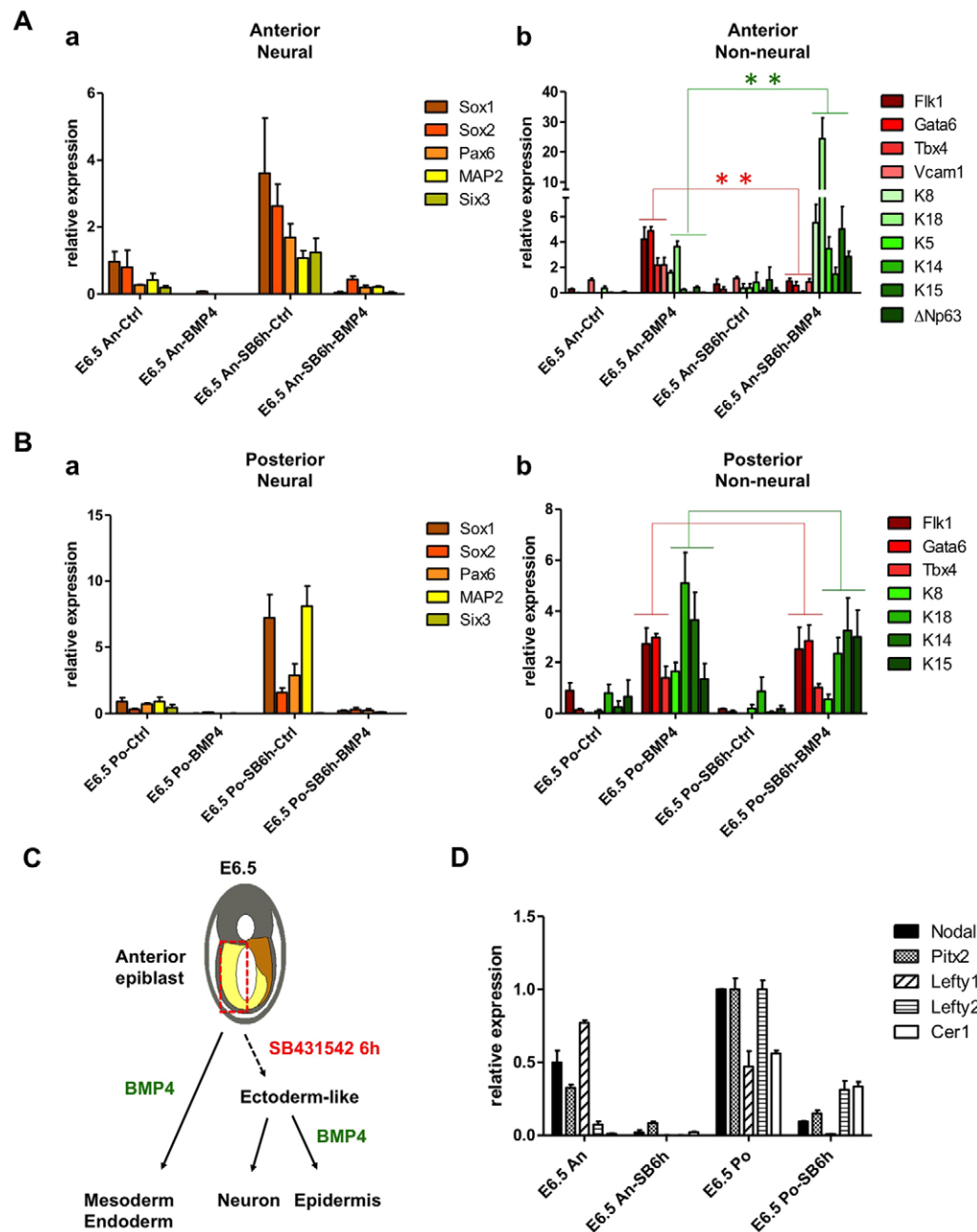


Fig. 7. Derivation of ectodermal progenitor-like cells by short-term inhibition of Nodal signaling.

(A) RT-qPCR analysis of marker gene expression in four groups of E6.5 mouse anterior explants that have differentiated for 5 days: the first group is cultured in N2B27 (E6.5 An-Ctrl), the second is cultured with BMP4 (E6.5 An-BMP4), the third and the fourth groups are pre-treated with SB431542 (10 μ M) for 6 hours, and then differentiated separately in control medium (E6.5 An-SB6h-Ctrl) or medium supplemented with 10 ng/ml BMP4 (E6.5 An-SB6h-BMP4). Each group of data has four biological replicates. ** $P < 0.01$. (B) Marker gene expression in E6.5 posterior explants cultured as described in A. $n = 4$ biological replicates. (C) Model of differentiation process in E6.5 anterior explants. Red rectangle indicates anterior epiblast. (D) RT-qPCR analysis of Nodal downstream targets in E6.5 anterior epiblast, posterior epiblast and explants cultured with SB431542 for 6 hours. Three embryos were collected for each group. Error bars represent s.d.

explants (Fig. 7Bb). In order to elucidate the molecular mechanisms underlying our observations, we analyzed several downstream target genes of Nodal signaling, *Nodal*, *Pitx2*, *Lefty1*, *Lefty2* and *Cer1* (Dickmeis et al., 2001; Katoh and Katoh, 2006; Whitman, 2001), in anterior and posterior explants. We found that 6 hours treatment with SB431542 reduced the expression of Nodal target genes efficiently in both anterior and posterior explants, but some residual expression of Nodal targets was observed in the posterior explants (Fig. 7D). This residual Nodal activity in the SB431542-treated posterior explants might be sufficient to allow differentiation into mesoderm/endoderm cells in the presence of BMP4.

In summary, our results suggest that attenuation of Nodal signaling during mouse gastrulation promotes initial formation of the ectodermal progenitor populations in the anterior region of the mouse embryo. Subsequent exposure to different levels of BMP signaling, based on their proximity to the ExE, could then determine the regional commitment to neural or epidermal fate.

DISCUSSION

The ectoderm, as defined by its potential to form neural and epidermal tissues, is essential for the formation of the central nervous system and the epidermis in the mouse embryo. However, unlike the mesoderm or endoderm, the exact domain of ectoderm potential is not clearly defined, and the mechanisms underlying its specification remain poorly understood. In this study, we aimed to address these questions through explant culture. We identified E7.0 as a key developmental stage when cells with potential to form neural and epidermal progenitors exist transiently in the anterior/proximal domain of the ectodermal layer. We further showed that brief attenuation of Nodal signaling promotes ectodermal formation and that subsequent exposure to BMP can determine epidermal versus neural fate in ectodermal explant cultures.

Heterotopic transplantation experiments have demonstrated that epiblast cells in E6.5 mouse embryo are capable of changing their developmental fate dependent on their final location after

transplantation (Parameswaran and Tam, 1995; Tam and Zhou, 1996). Transplantation performed in E7.0–E7.5 embryos showed that the anterior ectoderm can give rise to neur ectoderm when injected into the distal tip, but contributes preferentially to surface ectoderm when transplanted into the posterior/proximal region. This implied the possibility of an ectodermal progenitor population that can subsequently differentiate into the neural and surface ectoderm depending on its position in the embryo (Beddington, 1982). Up to now there have only been a few published studies that addressed the existence of a putative ectodermal population in the mouse embryo, such as the recent study by Cajal and colleagues (Cajal et al., 2012). They identified a small group of ectodermal cells positioned between proximal and distal regions in the anterior ectoderm at E7.0 in which single cells could contribute to both surface ectoderm and neural ectoderm during normal embryonic development. This work demonstrated that neural and non-neural lineages are not yet clonally separated at late gastrulation stage, and suggested that classical surface ectoderm and forebrain markers do not strictly define lineages at this stage.

We have been able to extend Cajal and colleagues' findings with our explant culture approach and demonstrated that the area of ectoderm with potential to generate both neural and surface ectoderm extends beyond the narrow strip bordering the proximal and distal region of the anterior ectoderm that is normally fated to do so. Our results suggest that the anterior/proximal region of the ectodermal layer in E7.0 [no allantoic bud (OB) and early allantoic bud (EB) stages] mouse embryo contains a transient cell population that can efficiently differentiate into epidermis or neural tissue depending on the local environmental cues (such as the presence or absence of BMP4). The restricted location of the cells in the intact embryo that show this mixed fate probably reflects the graded response in the epiblast to extra-embryonic secretion of BMP4. In explant cultures, we can provide elevated levels of BMP signaling to all cells and detect a broader responsive population with this dual potential.

In mouse embryos, Nodal signaling is important for primitive streak formation and mesoderm/endoderm development (Brennan et al., 2001; Conlon et al., 1994; Dunn et al., 2004; Zhou et al., 1993). The AVE produces Nodal antagonists Lefty1 and cerberus 1 (Cer1) to block mesoderm formation in the anterior region and promote

neural development (Perea-Gomez et al., 2001; Perea-Gomez et al., 2002; Thomas and Beddington, 1996). Epiblast cells from *Nodal*^{−/−} embryos readily differentiate into neurons in explant culture (Camus et al., 2006). Studies in mouse EpiSCs and human ESCs also revealed that inhibition of Nodal signaling promotes neural induction (Chng et al., 2010; Iwafuchi-Doi et al., 2012; Patani et al., 2009; Vallier et al., 2009).

Our data from E6.5 explant cultures suggest that transient Nodal inhibition can induce the formation of ectodermal progenitors with potential to form both neur ectoderm and epidermis. Explant cultures of both anterior and posterior E6.5 ectoderm were able to upregulate mesoderm/endoderm markers in the presence of BMP4 (Fig. 2Ce,Cf). However, transient chemical inhibition of Nodal signaling in the anterior E6.5 explants reduced induction of mesoderm/endoderm markers, converting them into cells that are reminiscent of the anterior ectoderm at E7.0 (Fig. 7A).

The time window during which the anterior ectoderm cells can respond to the BMP4 signal is small. In the E7.0 mouse embryo, BMP4 signaling is active in the proximal region, as shown by the upregulation of the target genes *Id1* and *Id2* (Fig. 5A). As a result of the persistent activity of BMP signaling, perhaps in conjunction with other pathways, the ectoderm in the proximal region becomes committed to an epidermal fate at E7.5. By contrast, the absence of BMP4 and the presence of antagonists secreted by the node (McMahon et al., 1998; Tam and Behringer, 1997) direct the distal ectoderm to commit and differentiate into the neural lineage. This is reflected by the reduced capacity of the anterior/distal region of the E7.0 ectodermal layer to give rise to epidermal cells (Fig. 5Cb).

In summary, our explant culture experiments demonstrate the progression from pluripotency to transient ectodermal progenitors to neural and surface ectoderm lineage restriction in the anterior ectoderm germ layer from E6.5 to E7.5. This leads to the model (Fig. 8) that the anterior region of the early streak embryo, although initially pluripotent, gradually loses its mesoderm/endoderm potential in response to Nodal antagonists secreted from the adjacent AVE. This anterior population of cells with the capacity to form either surface ectoderm or neural cells is subsequently directed by a BMP signaling gradient along the proximal-distal axis to become committed to surface ectoderm or neur ectoderm.

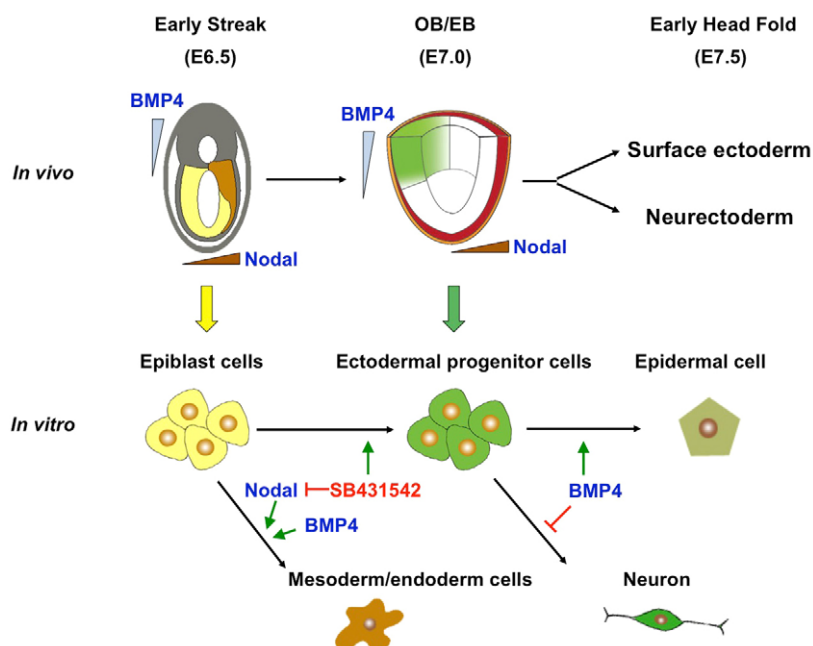


Fig. 8. Model for ectoderm formation during mouse embryonic development. The model shows the differentiation potential of the anterior ectoderm germ layer at different developmental stages. At early streak stage (E6.5), the anterior region of the epiblast is pluripotent. At OB/EB stages (E7.0), because of the low activity of Nodal signaling in the anterior side, the anterior/proximal ectoderm gradually loses its mesoderm/endoderm potential and becomes restricted to ectoderm with capacity to form either surface ectoderm (in the presence of BMP4) or neural cells (in the absence of BMP4). At early head fold stage (E7.5), the anterior ectoderm is subsequently directed by a BMP signaling gradient along the proximal-distal axis to become committed to surface ectoderm or neur ectoderm.

It remains to be determined whether it is possible to isolate and maintain in culture a transient ectoderm population with the capacity to form both neur ectoderm and surface ectoderm at the single-cell level. Our explant studies cannot exclude the possibility that individual cells within the potentially heterogeneous population are restricted to either neural or surface ectoderm. Additional studies will be required to demonstrate whether all cells of this transient ectoderm population possess bipotentiality to form neural and surface ectoderm *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mouse embryo dissection

Mouse embryos were obtained from ICR outbred mice. The stages of the embryos were determined by dissection date and morphology (Downs and Davies, 1993; Lawson and Pedersen, 1987). We refer to early streak (ES) embryos as E6.5 mouse embryos, no allantoic bud (OB) and early allantoic bud (EB) embryos as E7.0 mouse embryos, and early head fold (EHF) embryos as E7.5 mouse embryos. For E6.5 mouse embryo dissection, embryos were incubated for 15 minutes at 4°C in cell dissociation buffer (CDB; Gibco) and then transferred to Ca²⁺- and Mg²⁺-free flushing and handling medium (Chemicon). Visceral endoderm was peeled away using the tip of 30 1/2-gauge needle, and the extra-embryonic ectoderm was cut off from the epiblast tissue. The epiblast tissue was then bisected into anterior and posterior parts. For E7.0 and E7.5 mouse embryo dissection, the whole embryo was first cut into anterior and posterior parts in CDB. Then, the mesodermal and endodermal layers were peeled away from the ectodermal layer using the tip of the needle.

Mouse embryo explant culture

After dissection, the isolated ectodermal layers were cultured in N2B27 medium in 96-well plates for up to 5 days. The N2B27 medium was composed of 50% DMEM/F12 (Gibco) and 50% Neurobasal (Gibco), supplemented with 1/200 (by volume) of N2 (2.5 mg/ml insulin, 10 mg/ml transferrin, 5 mg/ml bovine serum albumin, 2 µg/ml progesterone, 1.6 mg/ml putrescine, 3 nM selenite) and 1/100 (by volume) of B27 (Gibco) with 500 µM Glutamax (Gibco) and 100 µM β-mercaptoethanol (Gibco). The 96-well plates were pre-coated with fetal bovine serum and then washed twice in PBS before use. For Nodal inhibition assays from E6.5 explants, the anterior and posterior epiblast tissues were cultured with 10 µM SB431542 (Sigma) for 6 hours. For assessment of epidermal versus neur ectodermal potential, the explants were cultured in N2B27 with or without BMP4 (10 ng/ml, R&D Systems, 314-BP-010) for 5 days.

Immunofluorescence analysis

Immunocytochemistry was performed as described previously (Gao et al., 2001). The following primary antibodies were used: mouse monoclonal anti-cytokeratin 18 (Krt18; also known as K18) (1:150; Abcam, ab668) and goat polyclonal anti-SOX1 (1:400, R&D systems, AF3369). Primary antibodies were detected with fluorescein isothiocyanate (FITC)- and Cy3-conjugated secondary antibodies (1:500; Jackson ImmunoResearch).

EGFP mouse and FACS

EGFP mouse embryos were ordered from Toronto Centre for Phenogenomics (TCP). Single-cell suspensions of co-cultured explants were obtained by dissociating with 0.05% trypsin at 37°C for 10 minutes. Flow cytometry was performed at the Sickkids - UHN Flow Cytometry Facility using a Becton Dickinson LSR II and Dako Cytomation MoFlo.

RNA preparation and quantitative PCR analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription was performed with 0.1 µg of total RNA using SuperScript III reverse transcriptase (Invitrogen). Quantitative PCR was performed in accordance with the manufacturer's instructions, using Opticon Monitor (Eppendorf). Each sample was analyzed in triplicate, with the Ct values averaged and then normalized to *Gapdh* control. In each experiment, there were at least three

biological replicates. Each experiment was performed at least three times. Values shown on the graphs represent the mean value ± s.d. Student's *t*-tests were used to compare the effects of all treatments. Statistically significant differences are shown as follows: **P*<0.05, ***P*<0.01. The primers are listed in supplementary material Table S1.

In situ hybridization

Whole-mount embryo *in situ* hybridization was performed as previously described (Yamanaka et al., 2007). The RNA probes used were: *Krt8* (from M. Llanos Casanova, Division of Epithelial Biomedicine, CIEMAT, Madrid, Spain), *Bmp4* (from Brigid Hogan, Department of Cell Biology, Duke University Medical Center, Durham, USA), *Id1* and *Id2* (Jen et al., 1996), *Pou5f1* (Kitamura et al., 2003), *T*, *Fgf5*, *Sox2*, *Oct6*, *Hesx1*, *Six3* and *K18* (*Krt18*) (PCR-amplified from cDNA). The primers are listed in supplementary material Table S2.

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.L. designed and performed the experiments, analyzed data and wrote the manuscript. C.L., S.B., Q.Z. and L.S. did *in situ* hybridization. F.L. contributed to experimental design. N.J. and J.R. supervised the project.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.092866/-/DC1>

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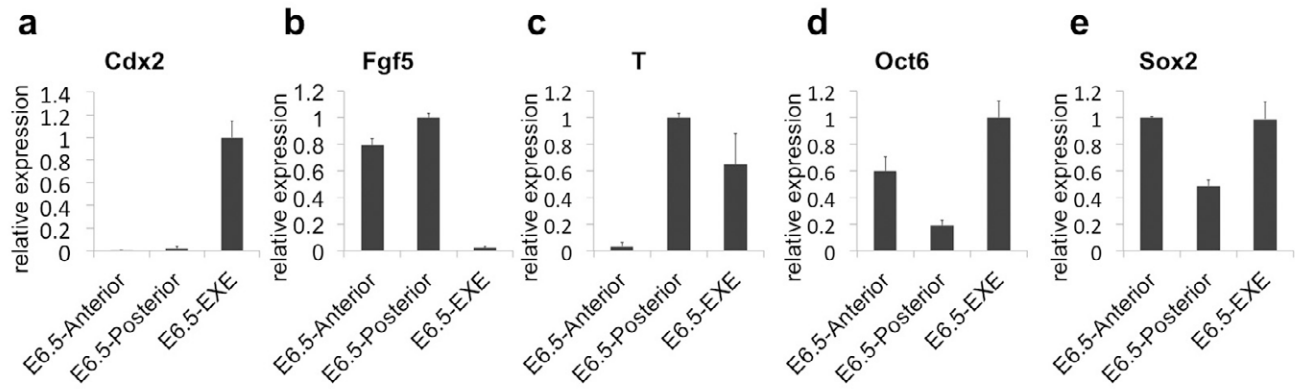
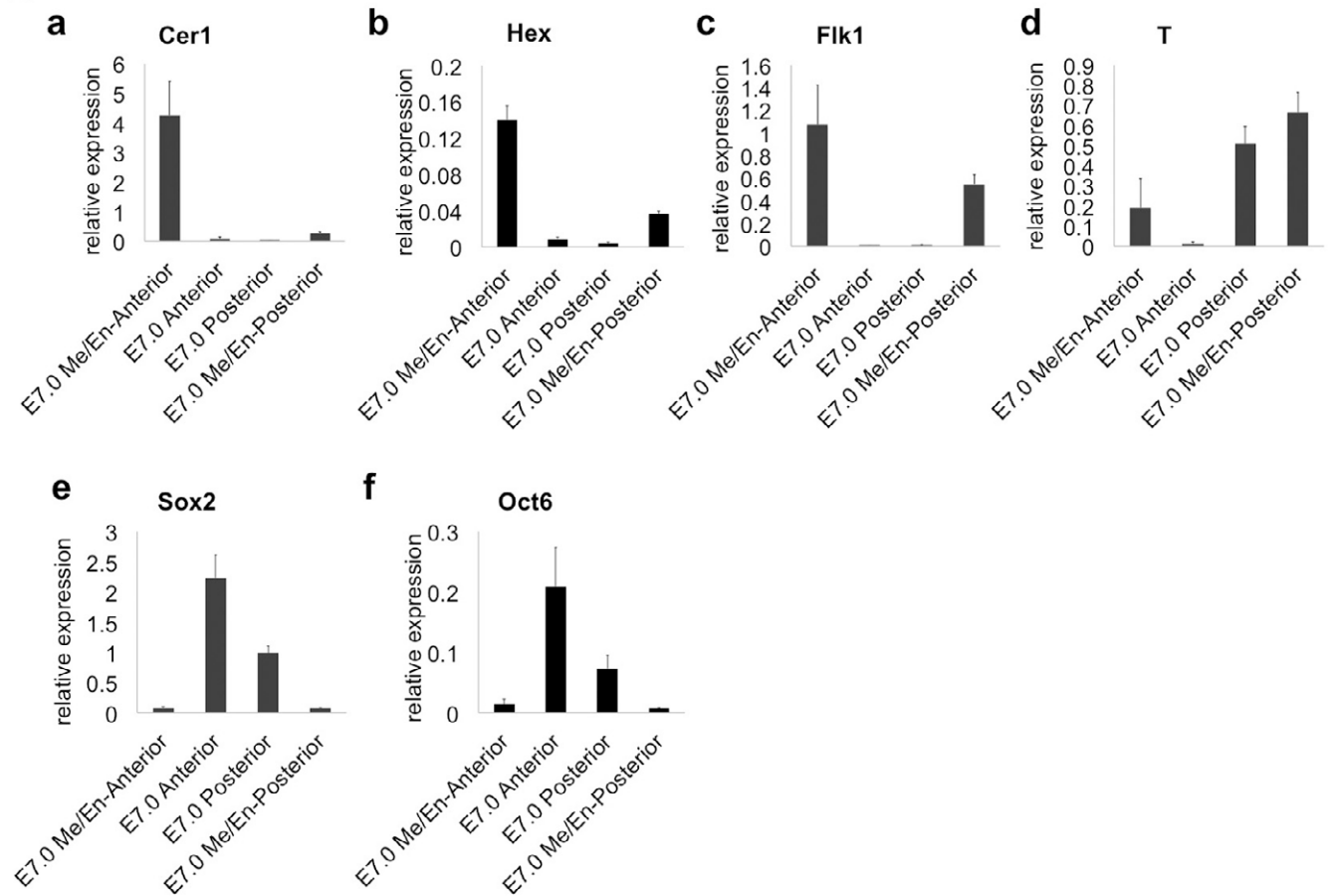
A**B**

Fig. S1. Detection of various lineage markers expression in harvested tissues to assess potential contamination from non-ectodermal tissues. (A) RT-qPCR analysis of marker gene expression in tissues separated from E6.5 mouse embryo, including the anterior portion of the embryonic ectoderm (E6.5-Anterior), the posterior portion of the embryonic ectoderm (E6.5-Posterior) and the ExE (E6.5-EXE). (B) RT-qPCR analysis of marker gene expression in tissues obtained from the E7.0 mouse embryo, including the anterior part of the mesoderm and endoderm (Me/En-Anterior), the anterior portion of the ectodermal layer (Anterior), the posterior portion of the ectodermal layer (Posterior) and the posterior part of the mesoderm and endoderm (Me/En-Posterior).

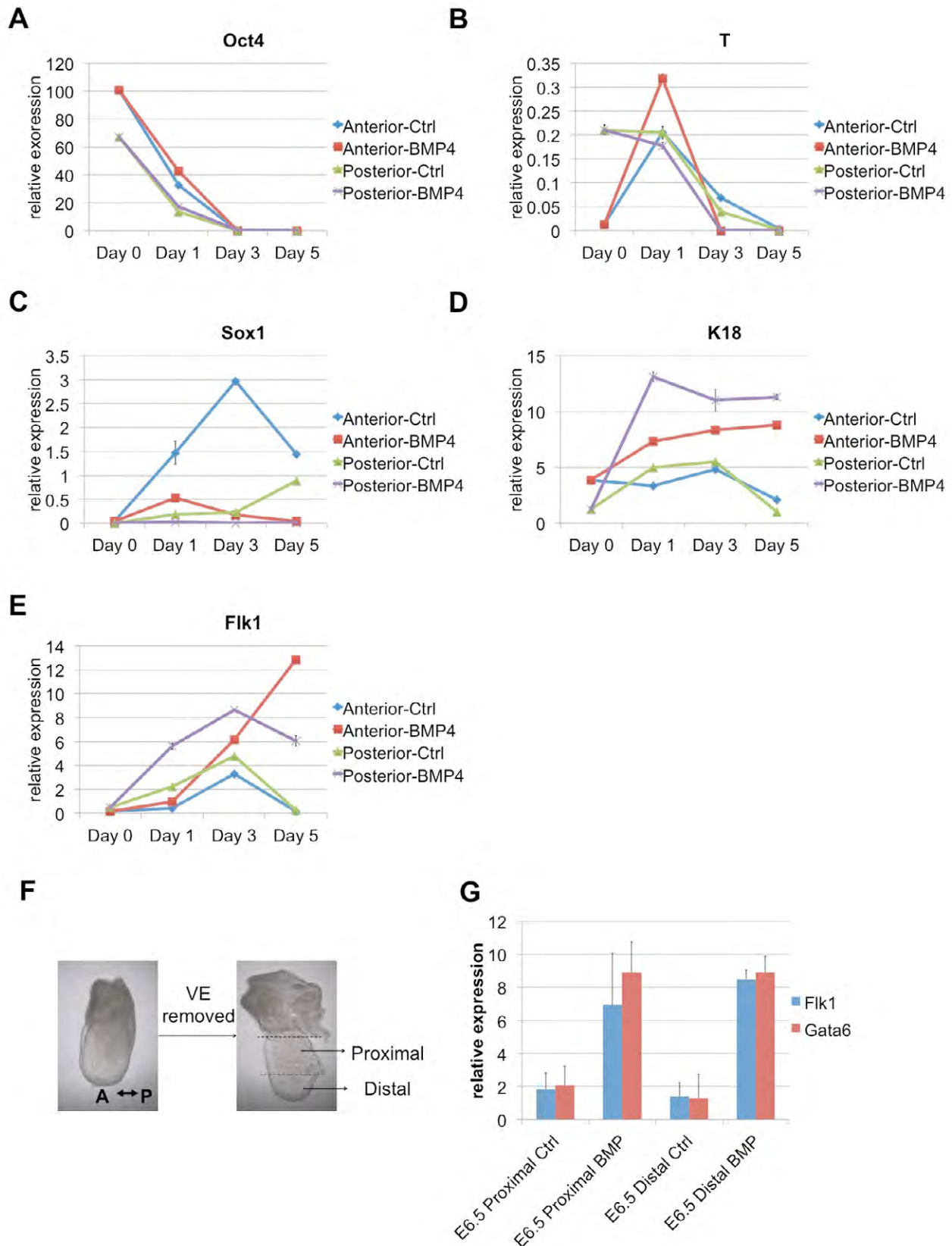


Fig. S2. E6.5 explants culture in control and BMP4-supplemented medium. (A-E) Marker genes expression (*Oct3/4*, *T*, *Sox1*, *K18*, *Flk1*) is analyzed during the 5 days culture of E6.5 anterior and posterior explants in the medium without or with BMP4. Each sample has epiblast tissue from three embryos. (F) A schematic showing how the epiblast is cut into proximal and distal portions. (G) RT-qPCR analysis of *Flk1* and *Gata6* in proximal and distal explants after 5 days culture with or without BMP4. There are three replicates for each group of data.

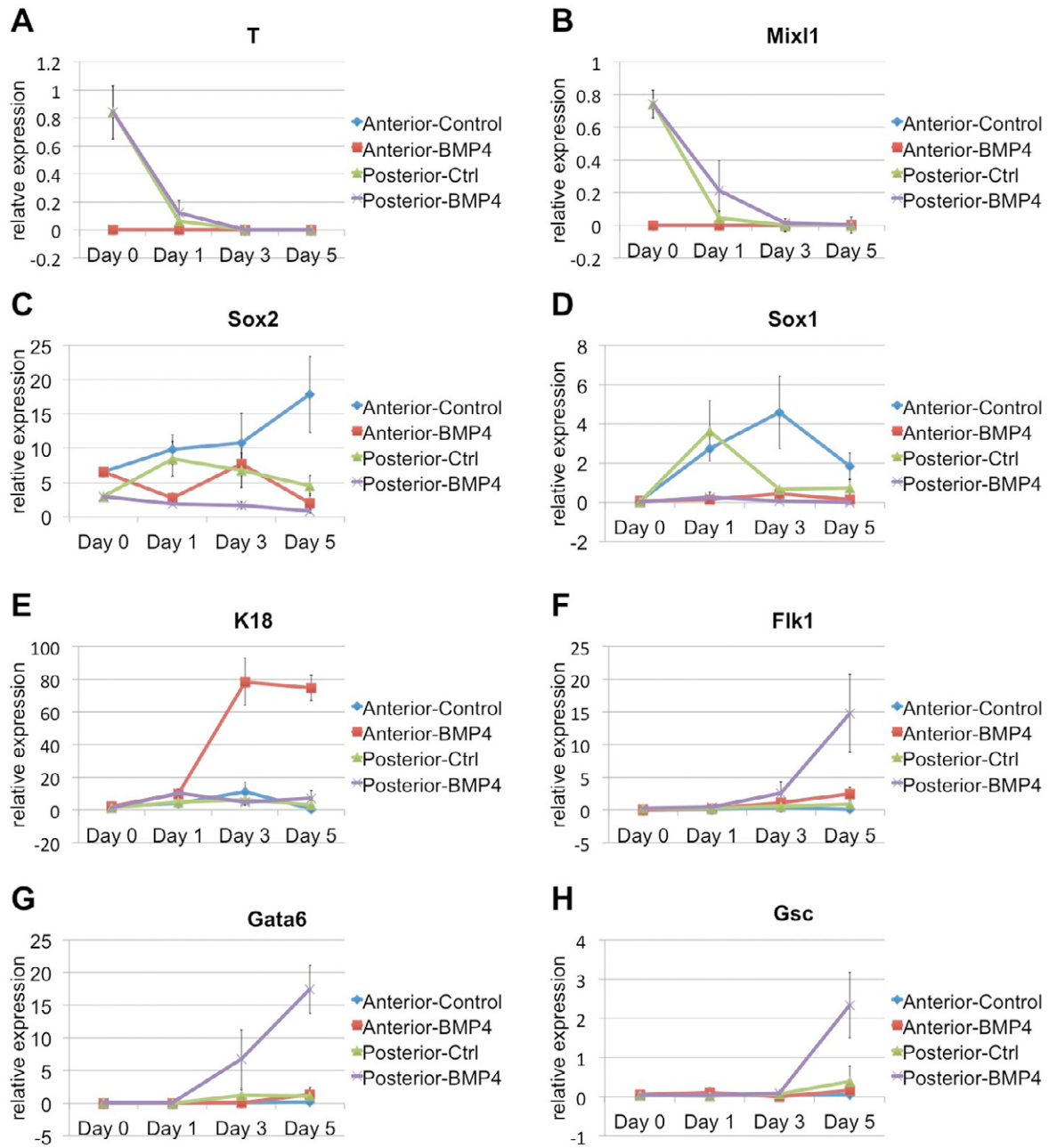


Fig. S3. The lineage progression of E7.0 anterior and posterior explants cultured without or with BMP4 for 5 days. (A-H) The expression of *T*, *Mixl1*, *Sox2*, *Sox1*, *K18*, *Flk1*, *Gata6* and *Gsc* is analyzed through RT-qPCR. There are four replicates for each group of data.

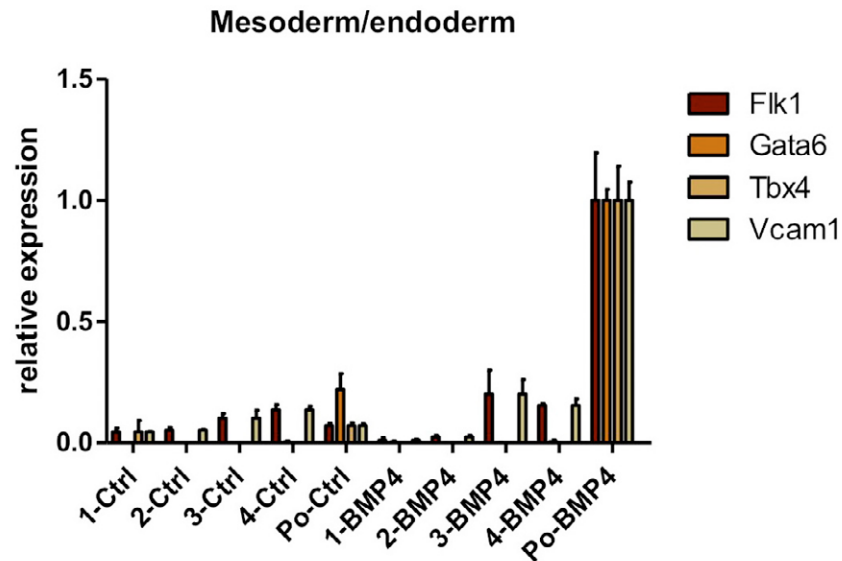


Fig. S4. Mesoderm/endoderm marker genes expression in the derivatives of E7.0 A/P fragments 1, 2, 3, 4. RT-qPCR analysis of mesoderm/endoderm marker genes expression in derivatives of fragments 1, 2, 3, 4 and posterior explants that have been cultured in control or BMP4-added medium for 5 days.

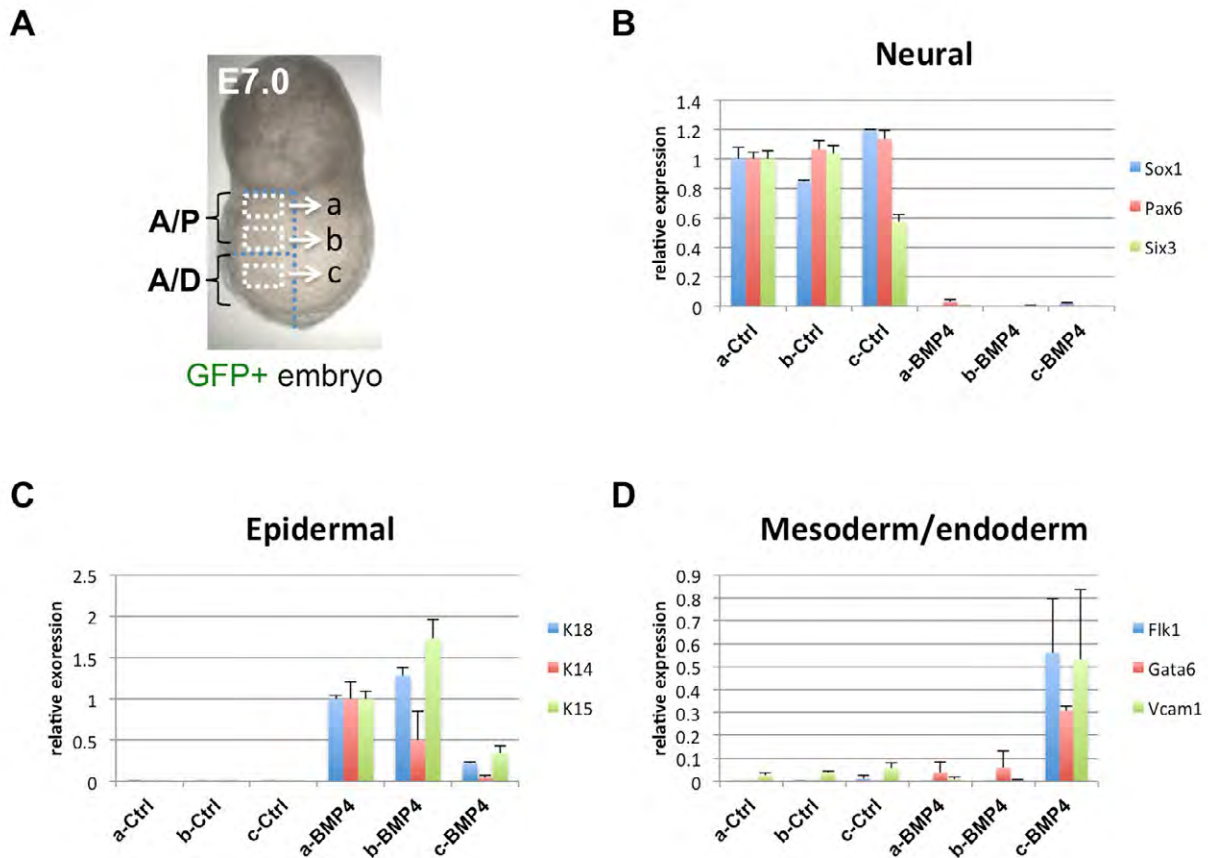


Fig. S5. Regionalization of ectodermal progenitor potential in E7.0 ectodermal layer. (A) Schematic illustrating locations of fragments a, b and c in anterior ectoderm region. (B-D) RT-qPCR analysis of neural (*Sox1*, *Pax6*, *Six3*), epidermal (*K18*, *K14*, *K15*) and mesoderm/endoderm (*Flk1*, *Gata6*, *Vcam1*) marker genes expression in derivatives of fragments a, b and c co-cultured with large anterior ectoderm explants for five days.

Table S1. Primer sequences (5'→3') for RT-qPCR

Genes	5' primer	3' primer
Hhex/Hex	CGGACGGTGAACGACTACAC	CGTTGGAGAACCTCACTTGAC
Pou5f1/Oct4	AGTTGGCGTGGAGACTTTGC	CAGGGCTTTCATGTCCTGG
Pou3f1/Oct6	TCGAGGTGGGTGTCAAAGG	GGCGCATAAACGTCGTCCA
Nanog	TTGCTTACAAGGGTCTGCTACT	ACTGGTAGAAGAATCAGGGCT
Nodal	CCTGGAGCGCATTTGGATG	ACTTTTCTGCTCGACTGGACA
Fgf5	GCTGTGTCTCAGGGGATTGT	CACTCTCGGCCTGTCTTTTC
Sox2	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
Sox1	GCACACAGCGTTTTCTCGG	ACATCCGACTCCTCTTCCC
Krt18/K18	CAGCCAGCGTCTATGCAGG	CTTCTCGGTCTGGATTCCAC
Krt19/K19	GGGGGTTCAGTACGCATTGG	GAGGACGAGGTCACGAAGC
Brachyury/T	CTCGGATTACATCGTGAGAG	AAGGCTTTAGCAAATGGGTTGTA
Kdr/Flk1	GGGTCGATTTCAAACCTCAATGT	AGAGTAAAGCCTATCTCGCTGT
Sox17	CGAGCCAAAGCGGAGTCTC	TGCCAAGGTCAACGCCTTC
Gata6	TTGCTCCGGTAACAGCAGTG	GTGGTCGCTTGTGTAGAAGGA
Gata4	CCCTACCCAGCCTACATGG	ACATATCGAGATTGGGGTGTCT
Eomes	CCTGGTGGTGTTTTGTTGTG	TTTAATAGCACCGGGCACTC
Cdx2	GCTACGGCGAACTTGGACA	GTGATGGTGCGCGTGGTAT
Krt8/K8	TCCATCAGGGTGA CTCAGAAA	CCAGCTTCAAGGGGCTCAA
Krt5/K5	TCCAGTGTGTCCTTCCGAAGT	TGCCTCCGCCAGAACTGTA
Krt14/K14	AAGGTCATGGATGTGCACGAT	CAGCATGTAGCAGCTTTAGTTCTTG
Krt15/K15	AGCTATTGCAGAGAAAAACCGT	GGTCCGTCTCAGGTCTGTG
ΔNp63	TGTACCTGGAAAACAATGCCCA	GACGAGGAGCCGTTCTGAATCT
Tbx4	TCCCCAGCTACAAGGTAAAAGT	ACCATCCATTTGTTGTACAGAA

Vcam1	AGTTGGGGATTCTGGTTGTTCT	CCCCTCATTCTTACCACCC
Mtap2/MAP2	GCCAGCCTCGGAACAAACA	GCTCAGCGAATGAGGAAGGA
Cer1	CTCTGGGGAAGGCAGACCTAT	CCACAAACAGATCCGGCTT
Krt17/K17	ACCATCCGCCAGTTTACCTC	CTACCCAGGCCACTAGCTGA
Six3	CCGGAAGAGTTGTCCATGTTC	CGACTCGTGTTTGTGATGGC
Cdh1/E-cadherin	CAGGTCTCCTCATGGCTTTGC	CTCCGAAAAGAAGGCTGTCC
Pax6	GCAGATGCAAAAGTCCAGGTG	CAGGTTGCGAAGAACTCTGTTT
Id1	GGTCCGAGGCAGAGTATTACA	CCTGAAAAGTAAGGAAGGGGGA
Id2	ATGAAAGCCTTCAGTCCGGTG	AGCAGACTCATCGGGTCGT
Hand1	GGCAGCTACGCACATCATCA	CCTGGCATCGGGACCATAG
Mesp2	CGGCGTTCTCTCACCGATG	CACCCCACTACTCATGGCTG
Mixl1	ACGCAGTGCTTTCCAAACC	CCCGCAAGTGGATGTCTGG
Gsc	CAGATGCTGCCCTACATGAAC	TCTGGGTACTTCGTCTCCTGG
Sox7	ATGCTGGGAAAGTCATGGAAG	CGTGTTCTGGTCACGAGAGA
MyoD	CCACTCCGGGACATAGACTTG	AAAAGCGCAGGTCTGGTGAG

Table S2. Primer sequences (5'→3') for *in situ* probes

T: Fwd: ATCAAGCTTCTGGGAGCTCAGTTCTTTTCGAGGC
T: Rev: ATCGGATCCGTGGACGAATTCCAGGATTTCAAAG
Hesx1: Fwd: CCGAATTCGGGAAGGTGCTCAGCTC
Hesx1: Rev: GCTCTAGAACTGTGATTCTCTACGGGAC
Sox2: Fwd: GGAATTCAAAGGAAAAAAATCTCCG
Sox2: Rev: GCTCTAGAACGTTTGCCTTAAACAA
Oct6: Fwd: TTAAACAAAACCAAACACCCG
Oct6: Rev: CGATTTAAATTAAGGGCGCG

Krt18: Fwd: CTCCAGACAAGATGAGCTTCACA
Krt18: Rev: CTCCATCTGTGCCTTGTATCG
Six3: Fwd: GCGCACTACCAGGAGGCCGAGAA
Six3: Rev: GTGTGTATCTGTCTGTGTATCCTGATT
Fgf5: Fwd: CCGGAAGAATGAGCCTGTCCTT
Fgf5: Rev: GAGCATCATCCAAAGCGAAACTTC