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ORIGINAL ARTICLE

# AP2γ regulates neural and epidermal development downstream of the BMP pathway at early stages of ectodermal patterning

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**Bone morphogenetic protein (BMP) inhibits neural specification and induces epidermal differentiation during ectodermal patterning. However, the mechanism of this process is not well understood. Here we show that AP2γ, a transcription factor activator protein (AP)-2 family member, is upregulated by BMP4 during neural differentiation of pluripotent stem cells. Knockdown of AP2γ facilitates mouse embryonic stem cell (ESC) neural fate determination and impairs epidermal differentiation, whereas AP2γ overexpression inhibits neural conversion and promotes epidermal commitment. In the early chick embryo, AP2γ is expressed in the entire epiblast before HH stage 3 and gradually shifts to the putative epidermal ectoderm during HH stage 4. In the future neural plate AP2γ inhibits excessive neural expansion and it also promotes epidermal development in the surface ectoderm. Moreover, AP2γ knockdown in ESCs and chick embryos partially rescued the neural inhibition and epidermal induction effects of BMP4. Mechanistic studies showed that BMP4 directly regulates AP2γ expression through Smad1 binding to the *AP2γ* promoter. Taken together, we propose that during the early stages of ectodermal patterning in the chick embryo, AP2γ acts downstream of the BMP pathway to restrict precocious neural expansion in the prospective neural plate and initiates epidermal differentiation in the future epidermal ectoderm.**

***Keywords*:** AP2γ; BMP; neural and epidermal development; ectodermal patterning; ESC; chick embryo
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## Introduction

During gastrulation, an early phase in vertebrate embryonic development, cell movements result in a massive reorganization of the embryo from the single-layered blastula to the gastrula with three germ layers: the outer ectoderm, the inner endoderm and the interstitial mesoderm. The ectodermal precursor cells are patterned under the action of inductive signals from the neighboring tissues to produce neural or epidermal cells [1, 2]. It has been shown in the chick embryo that different ectodermal regions have differential competency to respond to inductive signals [3-6]. Studies in *Xenopus*, chick and zebrafish demonstrate that bone morphogenetic protein (BMP) signaling plays important roles in neural/epidermal fate determination [7-9]. The "default model" proposes that BMP inhibits the "default" neural tendency of the ectodermal cells and induces them to adopt an epidermal fate, while BMP antagonists (noggin, chordin, and follistatin) from the organizer inhibit BMP signaling to protect the default neural fate [7, 10, 11]. The study in mouse embryo indicates that BMP signaling is required for inhibiting premature neural differentiation [12], and the involvement of Fgf and Wnt signaling pathways in neural induction has been established by the evidence from *Xenopus* and chick embryo [1, 2, 13-16].

BMP signal is transduced from the extracellular environment to the nucleus via Smad1/5/8 phosphorylation to regulate the expression of many target genes. Among them, Id1 sustains mouse embryonic stem cell (ESC)

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self-renewal [17], and Dlx5 and Tlx2 act downstream of BMP to regulate mesoderm development [18]. Only *Xenopus* Msx1 [19] and zebrafish ΔNp63 [20] are negative regulators of neural differentiation. To the best of our knowledge, there are no reports on BMP downstream targets that are involved in neural and epidermal differentiation of mammalian cells, and the functional effectors downstream of BMP signaling in ectodermal patterning remain unclear.

AP2γ (also known as *Tcfap2c*) belongs to the AP2 transcription factor family [21], which plays important roles in proliferation, differentiation and embryonic development with diverse expression patterns [22-24]. Among the AP2 factors, AP2α, AP2β and AP2γ are reported to be the key regulators in neural crest development [25-28], which is generally thought to occur following the neural plate formation. Conditional disruption of Smad4 in neural crest cells results in AP2α downregulation from embryonic day 9.5 (E9.5) in mouse embryo [29]. AP2α also regulates neural border specification during the neuralization stage in *Xenopus* and lamprey [30, 31]. Mouse AP2γ is expressed in both extraembryonic and embryonic tissues [32, 33] and displays multiple functions in extraembryonic development, neural crest induction and terminal epidermal differentiation [26, 34, 35]. Moreover, disruption of AP2γ leads to mouse embryonic lethality at approximately E7.5, showing extraembryonic cell defects and abnormal embryonic gastrulation [36]. However, it is unclear whether AP2γ is involved in ectodermal patterning at earlier stages of embryonic development and what is the relationship between AP2γ and BMP signaling.

Here we show that *AP2γ* is upregulated by BMP4 during pluripotent stem cell differentiation and that AP2γ partially mediates the BMP4 functions of neural inhibition and epidermal promotion. *In vivo*, chick AP2γ (cAP2γ) is expressed in the epiblast before HH stage 3 and gradually shifts to the peripheral ectoderm during HH stage 4, and AP2γ acts downstream of BMP signaling to regulate neural and epidermal development in different regions of the epiblast in the early chick embryo.

## Results

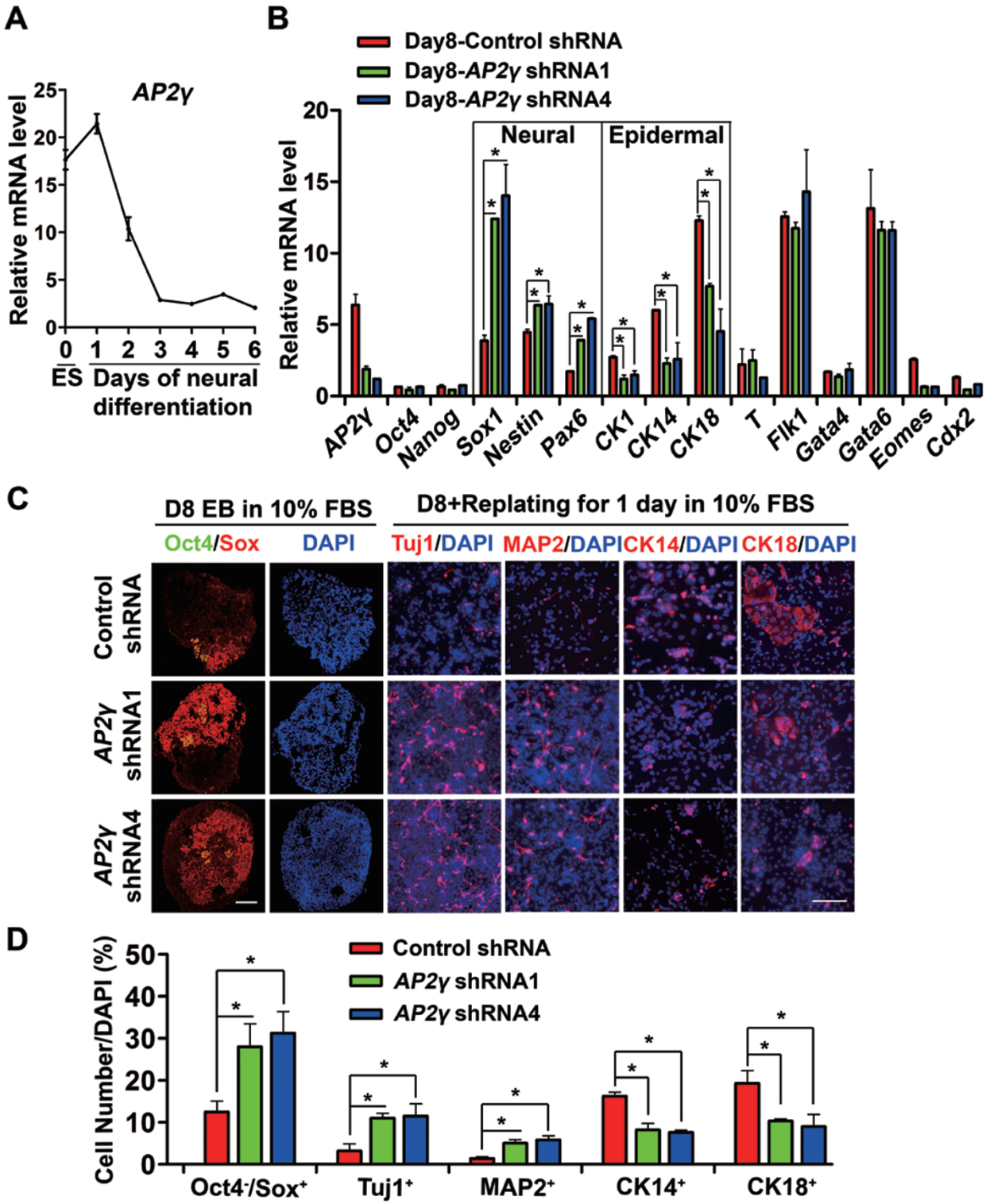
### *AP2γ knockdown facilitates neural differentiation and impairs epidermal commitment of ESCs*

Previously, we established methods of inducing a high percentage of neural progenitor cells (NPCs) from P19 embryonic carcinoma cells and ESCs, and showed that BMP4 inhibited the neural differentiation of pluripotent stem cells [37, 38]. To search for functional factors involved in neural inhibition by BMP4, differential gene expression microarray was performed with or without BMP4 treatment during P19 cell neural differentiation. Within the differentially expressed genes, AP2γ was found to be markedly upregulated by BMP4 at both mRNA and protein levels (Supplementary information, Figure S1). ESC neural differentiation in knockout serum replacement (KSR) medium was used as an *in vitro* model [38, 39] to study AP2γ function in early embryonic development. We found that *AP2γ* mRNA was expressed in undifferentiated mouse ESCs and that its level gradually decreased with progressing neural conversion (Figure 1A), suggesting that AP2γ might be involved in the neural differentiation of ESCs.

To test this hypothesis, shRNAs specifically targeted to *AP2γ* were introduced into ESCs using lentivirus, and two shRNAs (shRNA1 and shRNA4) could efficiently knock down AP2γ expression (Supplementary information, Figure S2A). The control and shRNA1/4-expressing ESCs showed comparable expression levels of pluripotency and differentiation markers (Supplementary information, Figure S2B) and were used for further studies.

Using an unbiased differentiation method, shRNA-expressing ESCs were differentiated as embryoid bodies (EBs) in DMEM containing 10% FBS for 8 days. qRT-PCR analysis showed that *AP2γ* knockdown upregulated the expression of NPC markers *Sox1*, *Pax*6 and *Nestin*

**Figure 1** *AP2γ* knockdown facilitates neural commitment and impairs epidermal fate determination during ESC differentiation. **(A)** qRT-PCR analysis of *AP2γ* mRNA level during neural differentiation of ESCs. EBs were cultured in KSR medium for 0-6 days and subjected to analysis. **(B)** ESCs were cultured in standard ES medium and transfected with control shRNA or *AP2γ* -specific shRNA lentivirus (shRNA1 & 4) coexpressing GFP. GFP-positive cells were then sorted by FACS and proliferated. These ESCs were cultured as EBs in DMEM containing 10% FBS. The expression of differentiation markers on day 8 was analyzed using qRT-PCR. Relative gene expression levels were normalized to the expression level of Gapdh. **(C**, **D)** Double immunostaining of Oct4 (green, artificial color) and Sox (red) proteins in day 8 EBs cultured under the conditions described in **B**. For EB staining in all of the following experiments, sections from thousands of EB aggregates were stained and statistical analyses were performed. Cells were replated for adherent culture in DMEM containing 10% FBS on Matrigel-coated culture dishes for 1 day and were analyzed by immunostaining for Tuj1 (red), MAP2 (red), CK14 (red) and CK18 (red). Nuclei were stained using DAPI (blue). The percentages of $Oct4^-/Sox^+$ NPCs, $Tuj1^+$, $MAP2^+$, $CK14^+$ and $CK18^+$ cells are shown in **D**. Scale bar, 50 μm.




(Figure 1B). Immunostaining of day 8 EBs confirmed that control shRNA-expressing ESCs produced approximately 15% Oct4$^-$/Sox$^+$ NPCs, whereas ESCs with *AP2γ* shRNAs displayed enhanced neural differentiation, generating 30% Oct4$^-$/Sox$^+$ NPCs (Figure 1C and 1D). Furthermore, the percentages of Tuj1$^+$ and MAP2$^+$ neurons were increased in *AP2γ* knockdown cells after EB replating (Figure 1C and 1D). EB differentiation was also performed in serum-free KSR medium, which normally generates approximately 80% NPCs at day 6.

*AP2γ* knockdown accelerated neural differentiation as measured by the generation of more Oct4$^-$/Sox$^+$ NPCs at day 4 and more Tuj1$^+$ neurons at day 6 (Supplementary information, Figure S2C-S2F). The examination of the expression of other germ layer markers showed that the expression of the epidermal markers *CK1*, *CK14* and *CK18* was downregulated in *AP2γ* shRNA-expressing cells (Figure 1B), which was also observed in KSR neu-

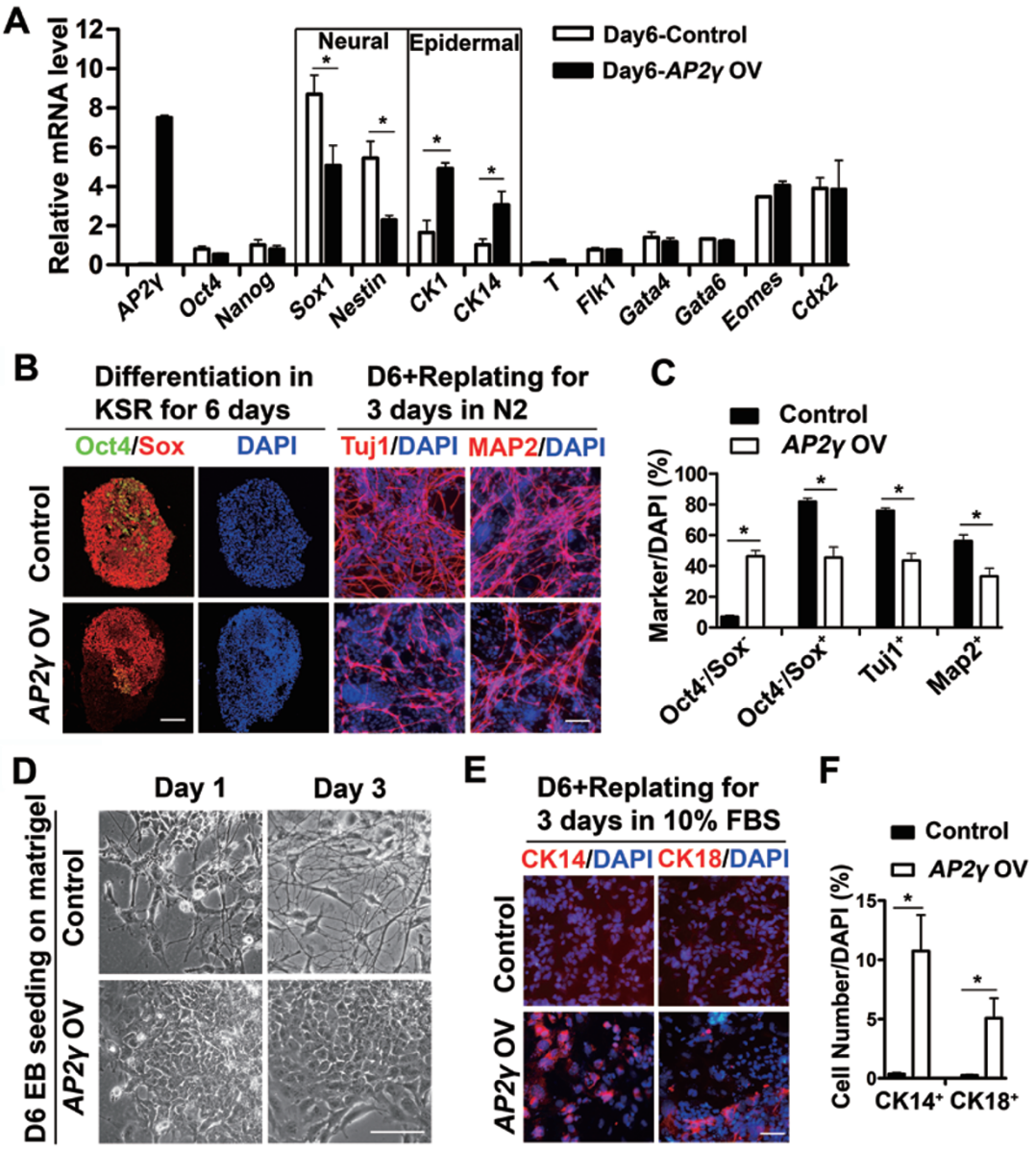



**Figure 2** *AP2γ* overexpression suppresses neural commitment and promotes epidermal marker expression during ESC neural differentiation. **(A)** The expression of neural and epidermal markers was analyzed by qRT-PCR in day 6 EBs derived from control or *AP2γ*-overexpressing (OV) ESCs in 8% KSR medium. **(B**, **C)** Control and *AP2γ*-overexpressing ESCs were cultured in KSR medium as EBs for 6 days and were replated in N2 medium for 3 days. Double immunostaining of Oct4 (green) and Sox (red) for day 6 EBs and immunostaining of Tuj1 (red) and MAP2 (red) for replated cells were performed. The percentages of Oct4$^-$/Sox$^+$, Tuj1$^+$ and MAP2$^+$ cells are shown in **C**. **(D)** Control and *AP2γ*-overexpressing ESCs were induced in KSR medium as EBs for 6 days, and these EBs were seeded in DMEM containing 10% FBS on Matrigel-coated dishes for 1 or 3 days. The different morphologies are shown in bright field. Scale bar, 100 μm. **(E**, **F)** Immunostaining of CK14 (red) and CK18 (red). Day 6 EBs from the conditions described in **B** were replated in DMEM containing 10% FBS on Matrigel-coated dishes for 3 days. The percentages of CK14$^+$ and CK18$^+$ cells are shown in **F**. D, day. Scale bar in **B** and **E**, 50 μm.

ral differentiation (Supplementary information, Figure S2G and S2H). Consistently, the percentages of $CK14^+$ and $CK18^+$ epidermal cells were reduced by *AP2γ* knockdown (Figure 1C and 1D). However, the expression of the pluripotency markers Oct4 and Nanog, the mesoderm markers *T* (also known as *Brachyury*) and *Flk1*, and the endoderm markers *Gata4* and *Gata6* was not affected by *AP2γ* shRNAs (Figure 1B). Together, these data suggest that AP2γ might be necessary for epidermal commitment and be a negative regulator of neural specification during ESC differentiation.

*AP2γ overexpression inhibits neural conversion and promotes epidermal differentiation of ESCs*

To examine whether AP2γ is sufficient for neural inhibition and epidermal induction, *AP2γ* was overexpressed in ESCs by lentivirus. Similarly to the *AP2γ*-knockdown ESCs, *AP2γ*-overexpressing ESCs showed comparable expression levels of pluripotent markers and proliferation rates compared with those of control cells (Supplementary information, Figure S3). After 6 days of neural differentiation in KSR medium, *AP2γ*-overexpressing cells showed decreased expression of the NPC markers *Sox1* and *Nestin* (Figure 2A). Immunostaining confirmed that 80% of control ESCs differentiated into $Oct4^-/Sox^+$ NPCs, whereas only approximately 40% NPCs were generated from *AP2γ*-overexpressing cells (Figure 2B and 2C). Subsequent neuronal differentiation was also inhibited by *AP2γ* overexpression as measured by decreases in the percentages of $Tuj1^+$ and $MAP2^+$ cells (Figure 2B and 2C). In contrast, the expression of epidermal markers (*CK1* and *CK14*) was increased by *AP2γ* overexpression (Figure 2A). Replated cells from *AP2γ*-overexpressing EBs showed epithelial-like morphology, whereas control cells grew many neurite-like processes (Figure 2D). It was further confirmed that some epithelial-like cells expressed *CK14* and *CK18* (Figure 2E and 2F). As in the *AP2γ*-knockdown cells, the expression of other germ layer markers was not affected by *AP2γ* overexpression (Figure 2A). Taken together, these results suggest that AP2γ inhibits neural conversion and promotes epidermal differentiation of ESCs.

*AP2γ partially mediates BMP4 functions during ESC differentiation*

Given that AP2γ phenocopies BMP's effects of promoting epidermal conversion and inhibiting neural commitment during ESC differentiation [40], and that *AP2γ* expression is upregulated by BMP4 (Supplementary information, Figure S2), we speculated that AP2γ mediates BMP functions in neural and epidermal differentiation.

Previously, we showed that BMP4 had different effects at different stages of ESC neural differentiation and that day 2-3 is the most sensitive window of BMP inhibition of neural commitment [38]. To determine whether *AP2γ* was most efficiently induced by BMP4 during the same time period, ESCs were cultured in KSR medium supplemented with BMP4 at different time points for 24 h and were analyzed by qRT-PCR at day 6 (Figure 3A, left panel). We found that the highest level of *AP2γ* mRNA was induced by BMP4 supplementation at day 2-3, during which BMP4 most efficiently induced the expression of *CK1* and *CK14* and inhibited the expression of *Sox1* and *Nestin* (Figure 3A, right panel). The BMP4-elicited *AP2γ* upregulation at day 2-3 was confirmed at the protein level by western blot (Figure 3B). Given that *AP2γ* responsiveness to BMP4 correlates with the neural inhibition and epidermal induction by BMP in ESC differentiation, we further propose that AP2γ might mediate BMP functions during the BMP4-sensitive window.

To test this hypothesis, we cultured *AP2γ* shRNA-expressing ESCs in KSR medium with or without BMP4 at day 2-3 and examined marker expression in day 6 EBs using immunostaining. We found that BMP4 reduced the percentage of $Oct4^-/Sox^+$ NPCs from 80% to approximately 5% in control shRNA-expressing cells, but this reduction was partially recovered to approximately 25% $Oct4^-/Sox^+$ NPCs in *AP2γ*-knockdown cells (Figure 3C and 3D). This neural inhibition recovery was confirmed by qRT-PCR analysis for *Sox1* and *Nestin* (Supplementary information, Figure S4A). BMP4 also induced approximately 20% of $CK14^+$ and $CK18^+$ cells from control ESCs, but it could induce only 10% of those cells from *AP2γ* shRNA-expressing cells (Figure 3C and 3D), suggesting that the epidermal promoting activity of BMP4 on the expression of the markers *CK1* and *CK18* (Supplementary information, Figure S4A) was also impaired by *AP2γ* knockdown. Moreover, the most efficient induction of the mesoderm marker *T* and the endoderm markers *Gata6* by BMP4 at day 2-3 was observed (Supplementary information, Figure S4B). However, we could not observe the expression change of mesendodermal markers *T* and *Gata6* when *AP2γ*-knockdown EBs were treated with BMP4 at day 2-3 (Supplementary information, Figure S4C), suggesting that AP2γ may not mediate BMP functions during mesendodermal specification.

Together, these data suggest that AP2γ partially mediates the BMP4 functions of neural inhibition and epidermal induction during the BMP4-sensitive window in ESC neural differentiation.

*AP2γ expression shifts from the epiblast to the putative epidermal ectoderm of the early chick embryo*

To explore the biological functions of AP2γ *in vivo*,

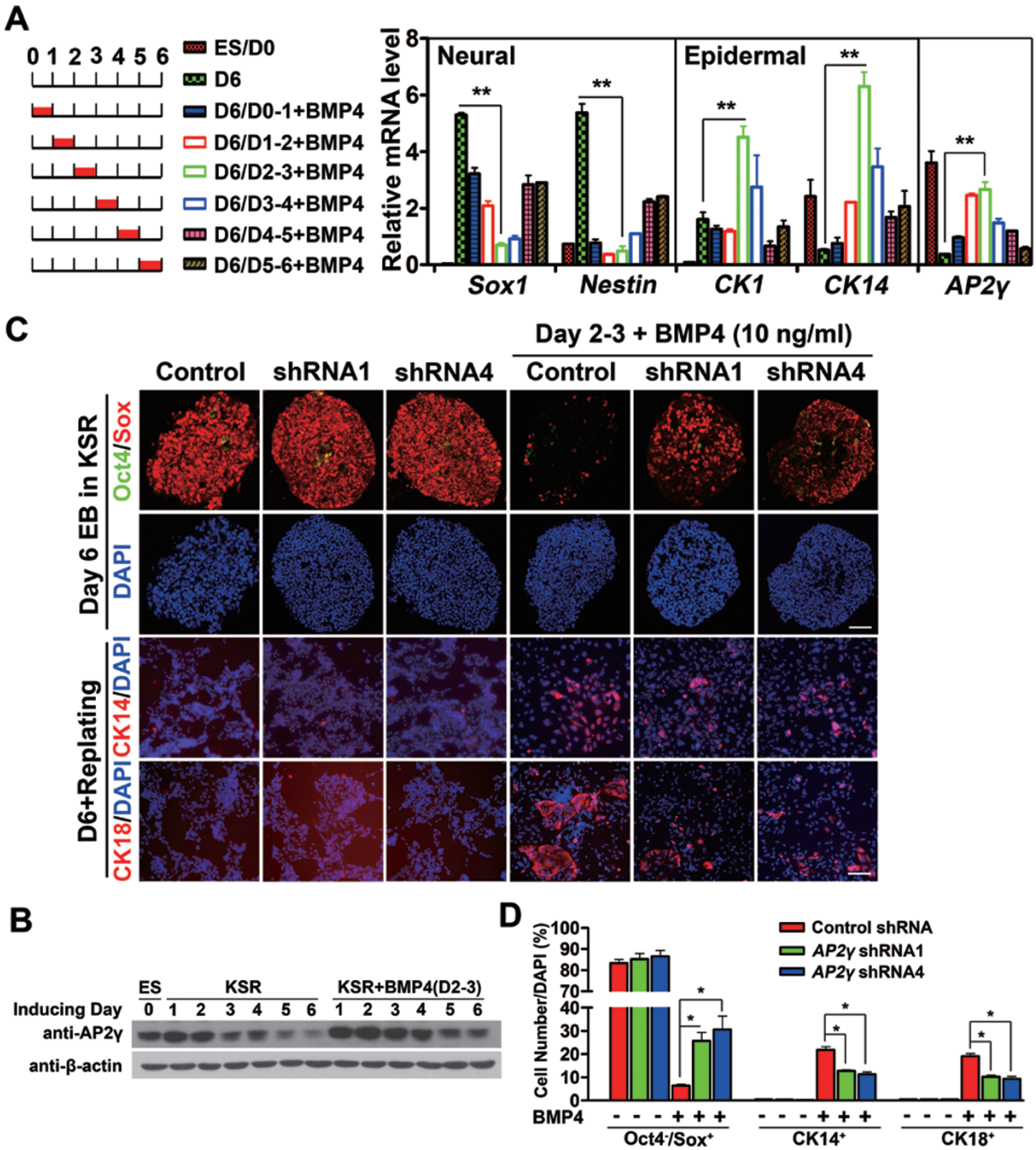



**Figure 3** AP2γ partially mediates BMP4 functions in ESC neural differentiation. **(A)** qRT-PCR analysis of ectoderm markers and *AP2γ* expression in EBs following culture in KSR medium for 6 days with or without BMP4 (10 ng/ml) treatment. BMP4 was added to the medium on the indicated days for 24 h. Undifferentiated ESCs without feeders served as a negative control and normally-induced day 6 EBs served as a positive control. **(B)** *AP2γ* expression in ESCs and EBs cultured in KSR medium with or without BMP4 (10 ng/ml) was analyzed by immunoblot from day 1-6. BMP4 was added to the medium at day 2-3 (D2-3) for 24 h. These cells were continuously cultured in KSR medium and harvested on the indicated days. **(C**, **D)** ESCs with control or *AP2γ* shRNAs were cultured in KSR medium as EBs for 6 days without or with BMP4 and were replated in DMEM containing 10% FBS on a Matrigel-coated dish for 2 days. Day 2 EBs were treated with BMP4 (10 ng/ml) for 24 h. Immunostaining of Oct4 (green)/Sox (red) for day 6 EBs and immunostaining of CK14 (red) and CK18 (red) for replated cells were performed, and the percentages of $Oct4^-/Sox^+$, $CK14^+$ and $CK18^+$ cells are shown in **D**. D, day. Scale bar, 50 μm.

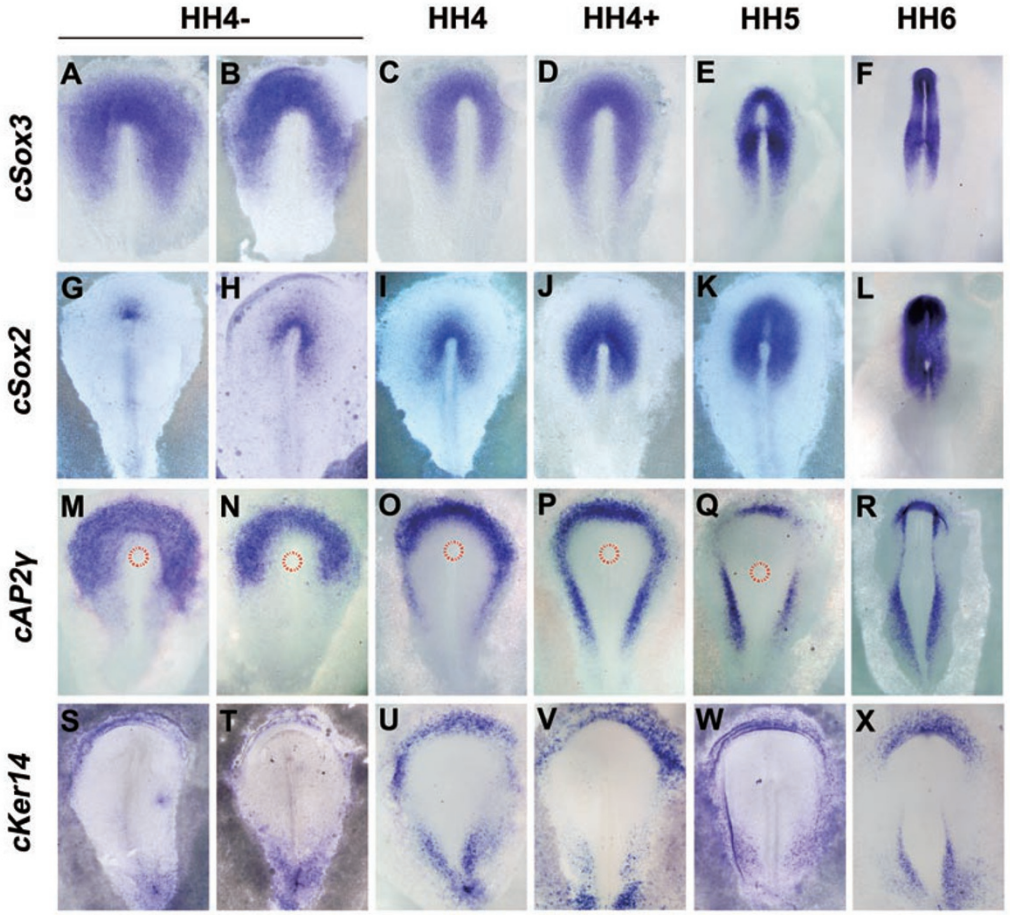



**Figure 4** *AP2γ* shifts from the epiblast to the peripheral ectoderm concurrent with neural plate expansion in the chick embryo. Expression of *cSox3* **(A-F)**, *cSox2* **(G-L)**, *cAP2γ* **(M-R)** and *cKer14* **(S-X)** is shown by ISH using digoxigenin-labeled riboprobes in HH stage 4-6 chick embryos. The broken red line indicates the area **(M-Q)** of Hensen's node.

the spatial-temporal relationship between *AP2γ* and neural/epidermal markers was examined by whole-mount *in situ* hybridization (ISH) in early chick embryos. Prior to the detection of the definitive neural plate marker *cSox2*, *cAP2γ* was expressed throughout the epiblast marked by the early epiblast marker *cSox3* [41] and *cAP2γ* expression was distributed in the interior of *cDlx5*-expressing region [42] before HH stage 3 (Supplementary information, Figure S5A and S5B).

During neural plate formation accompanying with *cSox2* expansion from the anterior tip of the primitive streak and *cSox3* shrinking to the neural plate (Figure 4A-4L), *cAP2γ* expression progressively shifted from the epiblast to the peripheral ectoderm at HH stage 4 (Figure 4M-4P). Double ISH showed that *cAP2γ* transcripts were complementary to *cSox2* expression and partially overlapped with that of *cSox3* at the early stage of neural plate formation (HH stage 4−, Supplementary information, Figure S5Ca,c). At HH stage 4+ *cAP2γ* staining was complementary to *cSox3* expression and was separated from *cSox2* transcripts with a visible distance (Supplementary information, Figure S5Cb,d). From the full primitive-streak to later stages (HH 5-6), *cAP2γ* transcripts were restricted to the future epidermal ectoderm (Figure 4Q and 4R). *Keratin14* that was previously identified as an epidermal marker [24] was first detected at HH stage 4− concurrently with the appearance of *cSox2* in the chick embryo. Chick *Keratin14* (*cKer14*) expression was restricted to the presumptive epidermis (Figure 4S-4X), similar to the expression of *cAP2γ* and the presumptive epidermal markers *Gata2/3* at later stages (Supplementary information, Figure S6). Therefore, *cKer14* was used as the other epidermal marker in the following studies.

Together, our results show that *cAP2γ* expression shifts from the epiblast to the putative epidermal ectoderm during neural plate formation at HH stage 4, suggesting that cAP2γ might play roles in neural plate

expansion. *cAP2γ* is also consistently expressed in the epidermal ectoderm, indicating that it might be involved in epidermal development.

*AP2γ inhibits neural expansion and promotes epidermal commitment in different regions of epiblast in the chick embryo*

To examine the *in vivo* function of AP2γ, chemically synthesized siRNAs targeted to *cAP2γ* were co-electroporated with the electroporation tracer GFP into HH stage 3 chick embryos, and gene expression was analyzed by ISH at later stages (Supplementary information, Figure S7A). We found that *AP2γ* siRNA could efficiently knockdown *cAP2γ* expression (Supplementary information, Figure S7B).

In medial epiblast, when *AP2γ* siRNA was electroporated along a continuous line extending outward from Hensen's node before the onset of *cSox2* expression (HH stage 3), we observed a prolonged extension of *cSox2* into the peripheral ectoderm (Figure 5A-5B′), which was confirmed by the histological section (Figure 5B″). However, *AP2γ* siRNA electroporation in a separated region of peripheral ectoderm could not induce *cSox2* expression (Supplementary information, Figure S7C). In contrast, *AP2γ* overexpression in the prospective neural plate suppressed *cSox2* expression (Figure 5G-5H′), suggesting that AP2γ can inhibit excessive neural expansion neighboring the *cSox2*-expressing region at early stages of neural plate formation.

In the future epidermal ectoderm *cKer14* and *cGata2* were suppressed by *AP2γ* siRNA (Figure 5C-5F′), and the inhibitory effect on *cKer14* was fully rescued by co-

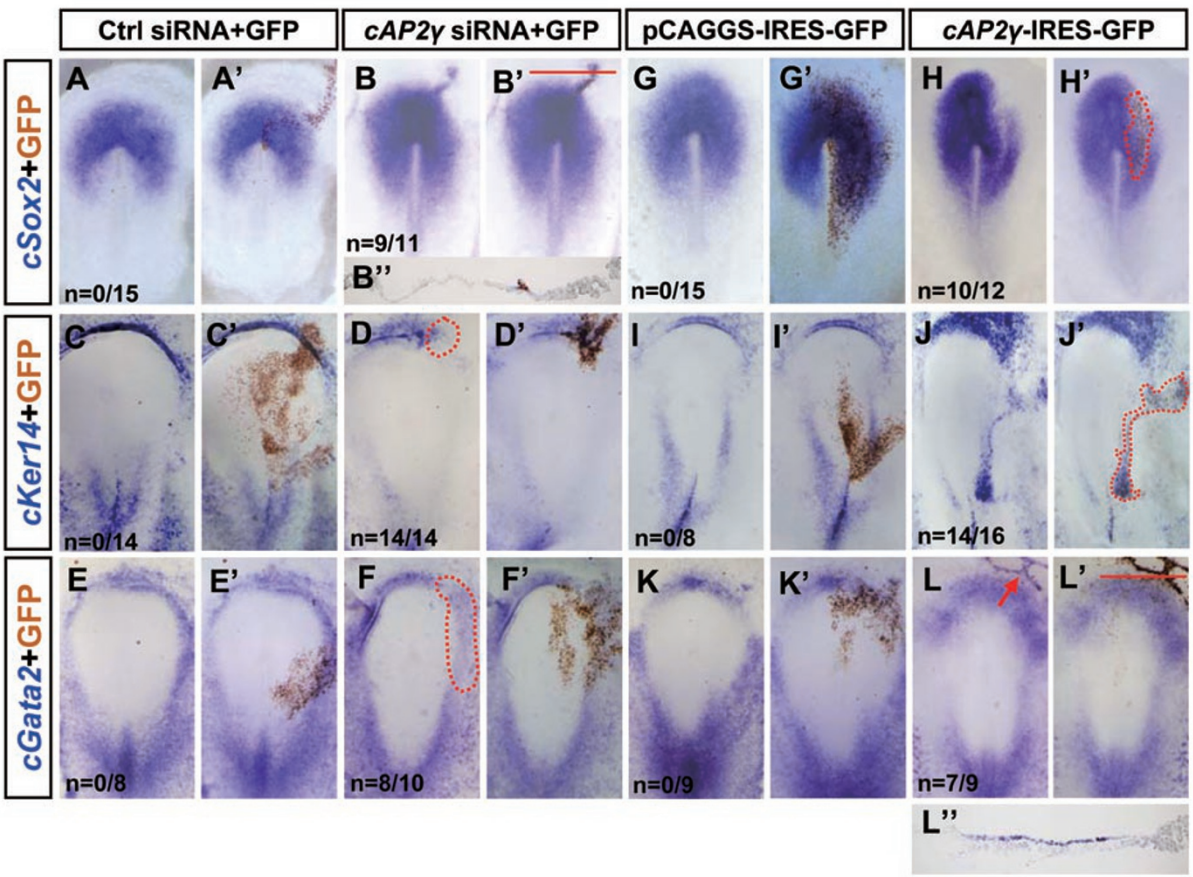



**Figure 5** AP2γ inhibits neural expansion and promotes epidermal commitment at different regions of epiblast. Whole-mount ISH (blue) was performed in chick embryos that had been previously electroporated in the epiblast layer at HH stage 3. The same embryo was stained for GFP expression (brown), which marks the electroporated field (some images are highlighted by broken lines). **(A-F′)** Control or *AP2γ* siRNA (50 ng/μl; mixed with GFP, 0.15 μg/μl) electroporated embryos were stained using *cSox2*, *cKer14* and *cGata2* probes. Transverse section (red line) of the embryo in **B′** is shown in **B″**. **(G-L′)** Embryos electroporated with GFP or cAP2γ in the epiblast and stained for *cSox2*, *cKer14* and *cGata2*. Transverse section (red line) of the embryo in **L′** is shown in **L″**.

electroporated mouse AP2γ (mAP2γ) (Supplementary information, Figure S7D). To determine whether AP2γ is sufficient for epidermal induction, we overexpressed *cAP2γ* in the peripheral epiblast and found that cAP2γ could efficiently induce the ectopic expression of *cKer14* and *cGata2* (Figure 5I-5L′,5L″). *cSox3* expression was not affected by *AP2γ* overexpression or knockdown (Supplementary information, Figure S7F), similarly to BMP4 misexpression's effects on the early expression of *Sox3* [13]. Collectively, AP2γ restricts excessive neural

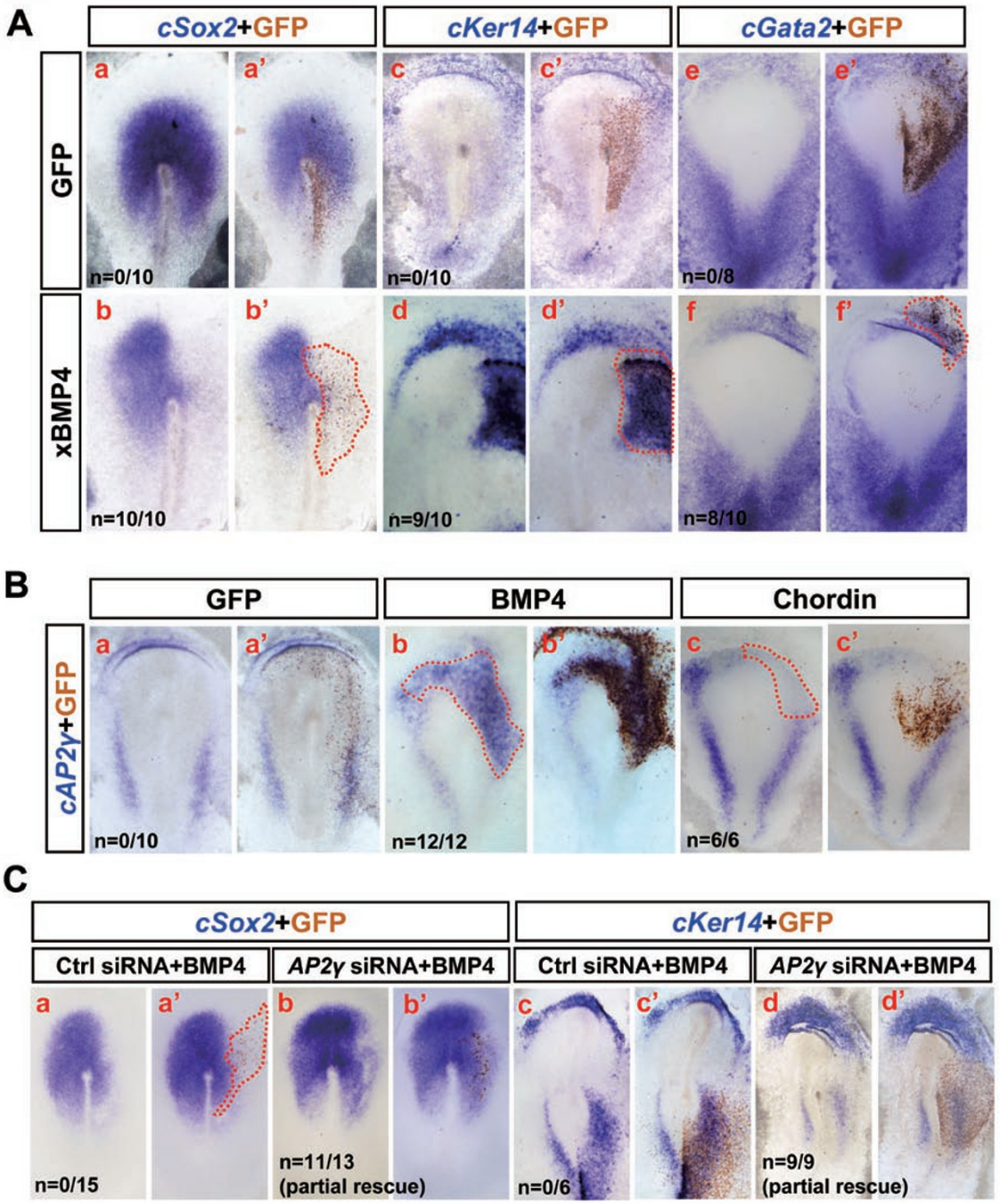



**Figure 6** AP2γ partially mediates BMP4 functions in ectodermal patterning. **(A)** Whole-mount ISH analysis of *cSox2*, *cKer14* and *cGata2* in control GFP- or xBMP4-electroporated chick embryos at HH stage 5. **(B)** Whole-mount ISH analysis of *cAP2γ* in control GFP-, xBMP4- or Chordin-electroporated chick embryos. **(C)** Control or *AP2γ* siRNA (50 ng/μl) and xBMP4 (0.3 μg/μl) were co-electroporated into the chick embryo epiblast. Embryos were subsequently stained for *cSox2* and *cKer14*.

plate expansion during young neural plate generation in the medial epiblast. Furthermore, AP2γ is both sufficient and necessary for epidermal induction in putative epidermal ectoderm.

*AP2γ partially mediates BMP functions in ectodermal patterning of the chick embryo*

Given that AP2γ can partially mediate the BMP4 functions of neural inhibition and epidermal conversion during ESC differentiation (Figure 3), we asked whether AP2γ has similar functions *in vivo*. We first electroporated *Xenopus* BMP4 (xBMP4) into HH stage 3 chick embryos and found that forced expression of xBMP4 completely blocked *cSox2* expression (Figure 6Aa-b′) and induced the ectopic expression of the epidermal markers (*cKer14* and *cGata2*) in non-neural ectoderm (Figure 6Ac-f′). *cAP2γ* expression could be induced by xBMP4 and inhibited by BMP inhibitor chordin (Figure 6B), suggesting that the dynamic expression pattern of *cAP2γ* (Figure 4) might be controlled by BMP signaling activity.

To explore whether AP2γ is necessary for BMP4 functions, we co-electroporated *AP2γ* siRNA and xBMP4 into the prospective neural plate of HH stage 3 embryos and found that xBMP4 with control siRNA strongly blocked *cSox2* expression (Figure 6Ca,a′), whereas *AP2γ* siRNA partially rescued xBMP4 inhibition of *cSox2* expression (Figure 6Cb,b′). Similarly, xBMP4-induced *cKer14* expression was weakened by *AP2γ* siRNA (Figure 6Cc-d′). qRT-PCR analysis confirmed that the BMP effects of neural inhibition and epidermal induction were partially impaired by AP2γ siRNA (Supplementary information, Figure S8). Together, these results suggest that AP2γ partially mediates BMP functions in ectodermal patterning of the chick embryo.

*AP2γ is a direct downstream target of BMP signaling*

To test whether *AP2γ* is directly regulated by BMP, we cultured P19 cells in N2B27 serum-free medium and added BMP4 for 0.5-3 h. We found that *AP2γ* transcription was elevated within 0.5 h of BMP4 treatment (Figure 7A), suggesting that *AP2γ* is an early BMP4 responsive gene. We also found that *AP2γ* expression was suppressed in a dose-dependent manner by the BMPR1a inhibitor dorsomorphin [43], similar to the known direct BMP targets *Msx2* and *Id1* [44, 45] (Supplementary information, Figure S9A). Moreover, *Msx2*, *Id1* and *AP2γ* were induced by BMP4 in the presence of the protein synthesis inhibitor cycloheximide (CHX) (Figure 7B and Supplementary information, Figure S9B), whereas the indirect target *Wnt3* [46] was not responsive to BMP4 with CHX pre-treatment (Supplementary information, Figure S9B).

To further show that *AP2γ* is a direct target of BMP4, we screened the upstream and downstream of the *mAP2γ* transcribed region and found four putative Smad-binding sites (P1-P4) (Figure 7C). We then cloned a 3.8-kb (WT), a 3.5-kb (ΔP) and a mutation fragment (Mut: site mutation at the possible Smad-binding site) of the upstream of the *mAP2γ* transcribed region (Figure 7C), and constructed reporter plasmids to perform luciferase assays in P19 cells. We found that BMP4 and constitutively active (CA)-BMPR1a increased WT fragment driven-luciferase activity in a dose-dependent manner, but the ΔP and mutation fragments showed no responsive activity (Figure 7D), suggesting that AP2γ expression can be induced by BMP/Smad signaling that is dependent on the responsive element within this −3.8- to −3.5-kb fragment. Furthermore, WT fragment electroporated into the chick epiblast drove GFP expression in the presumptive *AP2γ*-expressing area, whereas the ΔP fragment had no such activity (Figure 7E, WT: 6/9, ΔP: 0/10). Chromatin immunoprecipitation (ChIP) assays with Smad1 antibody showed that Smad1 was mainly enriched at the putative site P1 under BMP4 stimulation and that the neighboring P2 site showed weak binding activity (Figure 7F). Smad1 was also enriched in the *Id1* and *Msx2* promoter regions as a positive control (Supplementary information, Figure S9C). Together, these results indicate that *AP2γ* is a direct downstream target of BMP signaling and is upregulated by BMP4 through the recruitment of Smad1-containing complex to the binding site in the *AP2γ* promoter.

## Discussion

*AP2γ* is expressed in the inner cell mass (ICM) and trophectoderm of mouse embryo [32, 47], and previous studies have focused on its functions in extraembryonic development. However, the functions of AP2γ during development from ICM to neural/epidermal ectoderm are not fully understood. Using ESC differentiation as an *in vitro* model, we found that *AP2γ* knockdown facilitates neural conversion and impairs epidermal commitment, while *AP2γ* overexpression inhibits neural conversion and promotes epidermal differentiation (Figures 1 and 2). Our finding suggests that AP2γ might be involved in ectodermal patterning, but its exact *in vivo* function required investigation in an animal model.

Early chick embryo was chosen as the *in vivo* model. Detailed spatiotemporal expression analysis showed that *AP2γ* is expressed in the entire epiblast before HH stage 3 and gradually shifts outwards to the putative epidermal ectoderm at HH stage 4, accompanying with comple-

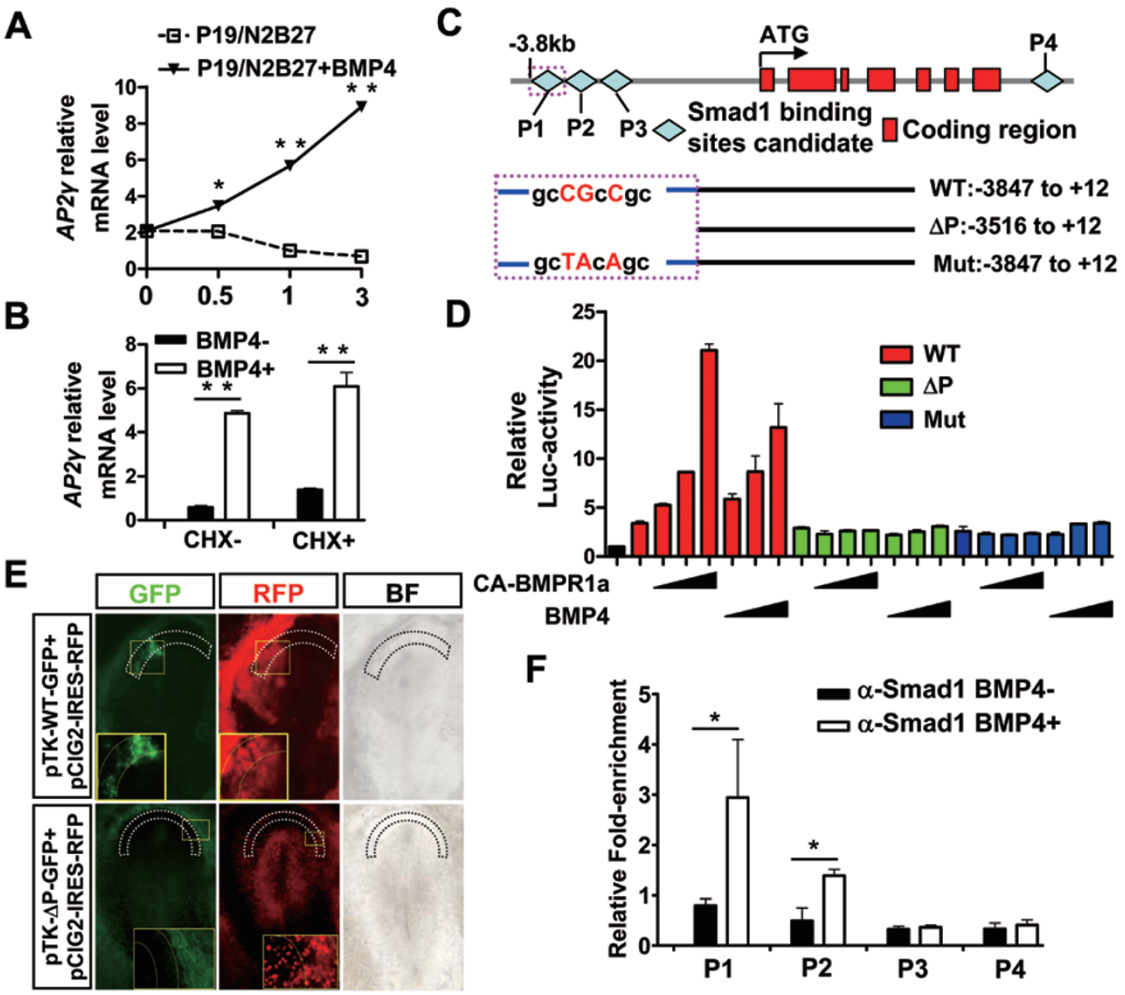



**Figure 7** *AP2γ* is directly regulated by BMP4. **(A)** *AP2γ* expression was examined using qRT-PCR in N2B27 serum-free medium-induced P19 cells with or without BMP4 (10 ng/ml) treatment for 0.5, 1 or 3 h. **(B)** qRT-PCR analysis of *AP2γ* expression with or without BMP4 (10 ng/ml) in the presence of CHX (10 ng/ml). P19 cells were cultured in standard medium and induced by N2B27 medium for 3.5 h. CHX was added to the medium 30 min prior to BMP4 treatment (for 3 h) to block new protein synthesis. **(C)** Diagram of predicted Smad1-binding site candidates (P1-P4) in the upstream and downstream of the AP2γ transcribed region. The 3.8-kb (WT: −3 847 to +12) fragment, 3.5-kb (ΔP: −3 516 to +12) fragment and mutation (Mut: sites mutation in the putative Smad binding site from −3 602 to −3 595) fragment were constructed into a pGL3-basic vector. **(D)** Luciferase (Luc) reporter assay was performed by co-transfection of pRL-TK (10 ng) with WT-Luc (50 ng), ΔP-Luc (50 ng) or Mut-Luc (50 ng) with increasing amounts of CA-BMPR1a (0.1, 0.2 or 0.4 μg) or with increasing concentrations of BMP4 (5, 10 or 20 ng/ml). P19 cells were starved in N2B27 serum-free medium for 12 h and transfected with the indicated plasmids. After 12 h of transfection the cells were treated by BMP4 for 24 h and the relative luciferase activity was analyzed using the dual luciferase reporter (DLR) system. **(E)** The WT fragment or ΔP fragment was constructed into the pTK-GFP vectors and co-electroporated with pCIG2-IRES-RFP into HH stage 3 embryos. The presumptive *AP2γ*-expressing region was indicated by broken lines, and the specified regions were enlarged. BF, bright field. **(F)** ChIP was performed using a Smad1 antibody versus normal IgG as a control. N2B27-induced P19 cells were treated with or without BMP4 for 3 h and then subjected to the ChIP assay. qRT-PCR was performed for regions within the possible Smad-binding sites P1-P4, and values were normalized to the input.

mentary *cSox2* expansion. Similarly, mouse AP2γ might have expression changes from ICM at E3.5 [47] to non-neural ectoderm at the late gastrulation stage [48]. The dynamic expression pattern of *cAP2γ* suggests that AP2γ might play multiple roles in ectodermal patterning in different regions of the epiblast.

During the early phase of neural plate generation (HH 4− and 4) the expression patterns of *cAP2γ* and *cSox2*

are complementary to each other, and *cAP2γ* acts as a gatekeeper to restrict excessive neural expansion. When *cAP2γ* was knocked down along a continuous trail in the nascent neural plate, *cSox2* escaped from the gate into the outer territory of the ectoderm (Figure 5), similar to *Gata2/3* morpholino-induced neural extension by inhibiting BMP signaling [49]. The continuity requirement is consistent with the notion that cell communication within the neural plate is necessary for the induction of neural markers [49] and that BMP inhibition was not sufficient for neural induction [13]. Moreover, the ectopic expression of *cAP2γ* restricts normal neural expansion (Figure 5). These results indicate that cAP2γ is sufficient and necessary for the inhibition of excessive neural plate expansion during early neuralization. This raises the question of how cAP2γ inhibits *cSox2* extension. There are two possibilities: (1) AP2γ functions as a transcriptional repressor through direct binding to the *cSox2* enhancer N2, which possesses a characterized AP2-binding site [22, 50, 51]; (2) AP2γ biases the epiblast to differentiate into non-neural tissues [52]. At HH stage 4+, the expression of *cAP2γ* and *cSox2* was separated and confined to their predisposed regions, which implies that neural expansion is a multi-step process [13] under the strict restriction of AP2γ in the nascent neural plate.

*cAP2γ* is also expressed in the surface ectoderm throughout HH stage 4 and might play important roles in epidermal development. Epidermal development undergoes epidermal progenitor commitment from the surface ectoderm. Previously, epidermal progenitors were thought to be determined around E8-E12 in mouse embryos [53], which is later than neural induction. *Keratin* (*K5* and *K14*) expression is first detected at E9.5 in the surface ectoderm and is recognized as the hallmark of the stratified epithelia [24]. Here, we found that *cKer14* begins to be expressed at the onset of neural induction and can be ectopically induced by xBMP4 in non-neural ectoderm of the chick embryo (Figures 4 and 6), implying that epidermal and neural commitment might occur simultaneously in different regions. AP2γ has been implicated in regulating keratin expression in terminal epidermal development [34, 54], and AP2-binding sites exist in the epidermis-specific enhancer of the *K14* promoter [55, 56]. However, it is unknown whether AP2γ is involved in the initial epidermal commitment. We show that *cKer14* expression generally overlapped with *cAP2γ* (Figure 4) and that AP2γ was sufficient and necessary for the expression of epidermal markers in the future epidermis (Figures 2 and 5). *cKer14* may be one of the earliest markers for primarily committed epidermal progenitors in chick ectodermal patterning and AP2γ might act upstream of *cKer14* to initiate epidermal differentiation in the surface ectoderm. *cGata2* was detected at early stages and was ectopically induced by xBMP4 and cAP2γ (Figures 4-6). It is possible that *Gata2* induction occurs in parallel with primary epidermal commitment. TAp63α is required for the stratification program of epidermal morphogenesis [57, 58], whereas its transcripts appear later at HH stage 6 in the chick embryo [59]. We propose that AP2γ triggers the initial step of epidermal development in the surface ectoderm. Thereafter when *p63* and *AP2γ* are expressed in the committed surface ectoderm after HH stage 6, AP2γ might act downstream of p63 in the stratification program [52] and combine with other epidermal factors such as p63, Msx1 and AP2α to regulate the subsequent epidermal maturation at later stages [19, 26, 34, 54, 60, 61].

During the *AP2γ* expression shift from the epiblast to the surface ectoderm, *AP2γ* transitionally occupies the gap between the peripheral epidermis and the neural plate named pre-placodal region. We observed that BMP4 and AP2γ could inhibit the expression of prepladocal markers *cEya2* and *cSix1* (data not shown), suggesting that AP2γ might be also involved in pre-placode development [62]. Furthermore, AP2γ has redundant activities with AP2α in neural crest induction and non-neural ectoderm derivative development [26]. Other factors such as *Pax7*, *Msx1*, *Zic1*, *Dlx5* and *AP2α* were shown as neural border specifiers [30, 63-68]. During early neuralization of the chick embryo, some factors are mainly expressed in the peripheral (*Pax7*, *Dlx5* and *AP2α*) or caudal (*Msx1*) ectoderm and the expressions of others (*Zic1*) partially overlaps with *cSox2* expression [68]. However, there are no reports of gene expression complementary to early *cSox2*-expressing territory. We show that during the expression shift of *AP2γ*, its expression consistently surrounds the expanding neural plate in the anterior and lateral region. At the onset of neuralization, *AP2γ*-expressing cells in the future neural plate have the competence to differentiate into neural cells and the excessive expansion of *cSox2* is restricted by AP2γ. Only when *AP2γ* is cleared from neighboring cells around the *cSox2*-expressing region can *cSox2* expand moderately. *AP2γ* might function as one of the earliest neural border genes, and its expressing territory might represent the "pre-border" state proposed by Streit and Stern [63]. The *AP2γ*-expressing epiblast at HH stage 3+/4− may differentiate into one of three directions: neural plate (the epiblast around Hensen's node gradually loses *AP2γ* expression and expresses *cSox2*), neural crest or pre-placode (AP2γ might collaborate with some neural border factors to specify the neural crest or pre-placodal ectoderm [26, 62]) or epidermis (AP2γ combines with epidermal factors to determine epidermal fate in the sur-

face ectoderm [34, 52, 69]).

BMPs are the most important morphogens involved in ectodermal patterning, neural border establishment and maintenance and neural crest specification [13, 42, 63, 70]. However, the intracellular effectors of BMP signaling during these processes are not well known. Here, we identified *AP2γ* as a novel BMP downstream target that is directly regulated by BMP4 through Smad1 binding to the *AP2γ* promoter (Figure 7). *BMPR1a*-knockout mice die at E8.0 while *BMPR1b*-null mice are viable [71, 72], implying that BMPR1a was the possible receptor mediating BMP functions during ectodermal patterning. In the early chick embryo, *cAP2γ* can be ectopically induced by BMP4 and suppressed by chordin (Figure 6), suggesting that clearance of *AP2γ* transcripts from the medial epiblast might be elicited by BMP antagonists secreted from the organizer with gradually enhanced expression [63, 73]. It is also possible that *AP2γ* is concurrently regulated by other signals, such as FGF and Wnt [74]. BMP is proved to inhibit prematuration of neural tissues in the mouse embryo [12]. AP2γ might be the executor of BMP signaling in the nucleus to guarantee the normal progress of neural development by suppressing excessive *cSox2* expansion in the young neural plate. Moreover, AP2γ partially mediates the BMP4 function of epidermal induction in the peripheral ectoderm. Further investigation is needed to determine whether AP2γ mediates BMP functions in pre-placodal development and neural crest specification, during which AP2α might play important roles downstream of BMP signaling [29, 30].

To summarize, we propose a model for AP2γ function in ectodermal patterning of the chick embryo (Supplementary information, Figure S10). During neural induction, BMPs are expressed mainly in the non-neural ectoderm, and the BMP inhibitors chordin and noggin are sequentially expressed in node or notochord [63, 73], resulting in medial to lateral clearance of BMP activity. *AP2γ* expression shifts progressively from the entire epiblast to the non-neural ectoderm under the regulation of BMP and its antagonists (Figure 6B). During that process, AP2γ might mediate dual roles of BMP in ectodermal patterning: AP2γ inhibits excessive neural expansion in the young neural plate to prevent precocious neuralization and also initiates epidermal commitment in the surface ectoderm.

## Materials and Methods

*Cell culture and treatment*

P19 cells were cultured as previously described [75]. Mouse ESC lines R1 was used. ESCs were maintained on mitomycin C-treated mouse embryonic fibroblasts (feeders) in standard medium. P19 cells and ESC neural differentiation was performed as described previously [37, 39]. The factors and inhibitors, such as BMP4 (10 ng/ml; R&D Systems, Minneapolis, MN, USA), Noggin (100 ng/ml; Peprotech, Rocky Hill, NJ, USA), SB431542 (10 μM, Sigma), Dorsomorphin (0.5-20 μM, Enzo life sciences) were used.

*Gene overexpression and knockdown in ESCs*

Mouse *AP2γ* was cloned from ESC/R1 cDNA and constructed into lentiviral vector pFUGW-IRES-EGFP for overexpression in ES cells. The empty lentiviral expression vector pFUGW-EGFP was used as a negative control. Lentiviral vector pLentiLox 3.7 expressing shRNA was used for *AP2γ* knockdown in ESCs. The control and *AP2γ* shRNA sequences were listed in Supplementary information, Table S1. Lentiviral packaging and lentiviral transfection were performed as described [76]. GFP-positive cells were sorted with FACS Aria cell sorter (BD Biosciences) and were used for analysis according to the diagram in Supplementary information, Figure S11.

*Immunostaining*

Immunostaining was performed as described previously [77]. The following primary antibodies were used. Mouse monoclonal antibodies included: anti-GFP (1:400, MP Biomedicals), anti-Oct4 (1:200, Santa Cruz Biotechnology), anti-nestin (1:200, Upstate), anti-Tuj1 (1:500, Sigma), anti-MAP2 (1:500, Sigma), anti-AP2γ (1:500, Abcam), anti-cytokeratin18 (1:200, Abcam) and anti-cytokeratin14 (1:50, Abcam). Rabbit polyclonal antibodies were anti-GFP (1:400, Santa Cruz Biotechnology), an anti-Sox1/(2)/3 (1:100) that has a preference for Sox1 and Sox3 over Sox2 [78, 79]. Fluorescein isothiocyanate- and Cy3-conjugated secondary antibodies were used (1:200 and 1:500, Jackson ImmunoResearch).

*Early chick embryo electroporation*

Fertilized eggs (Shanghai Academy of Agricultural Sciences, Shanghai, China) were incubated at 38 °C to the desired stages. Plasmids (0.1-2 μg/μl) or siRNA reagents with Fast Green and Sucrose were electroporated at Hamburger and Hamilton (HH) 3 as previously described [80]. *cAP2γ* (NCBI: XM_417497) that displays 72% identity to *mAP2γ* was cloned. The C-terminal region of isolated cDNA was subcloned for probe preparation. The primers used for cloning were listed in Supplementary information, Table S2.

*Whole-mount ISH and sectioning of embryos following ISH*

Whole-mount ISH of the early chick embryo, double ISH and sectioning of embryos following whole-mount ISH were performed as described previously [41, 81-83]. Detailed protocols are available upon request. The following probes were used: *cAP2γ*, *cKeratin14*, *cSox2*, *cSox3*, *cGata2, cGata3* and *cDlx5*.

*RNA preparation and qRT-PCR analysis*

Total RNA was extracted from cultured cells or chick embryos using TRIzol reagent. Reverse transcription and qRT-PCR were performed as described previously [75, 84]. The primers are listed in Supplementary information, Table S3.

*RNA interference in chick embryo*

The siRNAs used for knockdown experiments in chick embryo were chemically synthesized by Shanghai GeneChem Co., Ltd.

cAP2γ and control siRNA sequences were listed in Supplementary information, Table S1. siRNAs (0.5 μg/μl) were electroporated into the epiblast of early chick embryo and 0.15 μg/μl GFP plasmid was co-electroporated as a tracer.

*Luciferase assay*

The luciferase assay was performed as described previously [77, 84] according to the manufacturer's instructions (Promega). During N2B27 aggregation induction, P19 cells were transiently transfected with pRL-TK and pGL3-basic-AP2γ promoter plasmids. CA-BMPR1a was co-transfected or BMP4 was added to the medium after transfection for 12 h. Luciferase activity was determined after treatment for 24 h.

*ChIP*

ChIP assay was performed according to the method described [75, 84]. Mouse monoclonal anti-Smad1 antibody (10 μg per sample, CST) and normal mouse IgG in control groups were used for immunoprecipitation. Primers for ChIP assay are listed in Supplementary information, Table S4.

*Statistics*

In each experiment, 50-100 cell aggregates were examined. Each experiment was repeated at least three times, and similar results were obtained. Data were presented as mean ± SD. Student's *t* tests were used to compare the effects of all treatments. Differences were considered statistically significant at * $P < 0.05$, ** $P < 0.05$. For statistic analysis in chick embryo, the successfully electroporated embryos (GFP-expressing embryos) were calculated.

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## References

1 Wilson SI, Edlund T. Neural induction: toward a unifying mechanism. *Nat Neurosci* 2001; **4** Suppl:1161-1168.
2 Streit A, Berliner AJ, Papanayotou C, Sirulnik A, Stern CD. Initiation of neural induction by FGF signalling before gastrulation. *Nature* 2000; **406**:74-78.
3 Dias MS, Schoenwolf GC. Formation of ectopic neurepithelium in chick blastoderms: age-related capacities for induction and self-differentiation following transplantation of quail Hensen's nodes. *Anat Rec* 1990; **228**:437-448.
4 Streit A, Sockanathan S, Perez L, *et al.* Preventing the loss of competence for neural induction: HGF/SF, L5 and Sox-2. *Development* 1997; **124**:1191-1202.
5 Storey KG, Crossley JM, De Robertis EM, Norris WE, Stern CD. Neural induction and regionalisation in the chick embryo. *Development* 1992; **114**:729-741.
6 Storey KG, Selleck MA, Stern CD. Neural induction and regionalisation by different subpopulations of cells in Hensen's node. *Development* 1995; **121**:417-428.
7 Munoz-Sanjuan I, Brivanlou AH. Neural induction, the default model and embryonic stem cells. *Nat Rev Neurosci* 2002; **3**:271-280.
8 Hogan BL. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev* 1996; **10**:1580-1594.
9 Neave B, Holder N, Patient R. A graded response to BMP-4 spatially coordinates patterning of the mesoderm and ectoderm in the zebrafish. *Mech Dev* 1997; **62**:183-195.
10 Hemmati-Brivanlou A, Melton D. Vertebrate embryonic cells will become nerve cells unless told otherwise. *Cell* 1997; **88**:13-17.
11 Weinstein DC, Hemmati-Brivanlou A. Neural induction. *Annu Rev Cell Dev Biol* 1999; **15**:411-433.
12 Di-Gregorio A, Sancho M, Stuckey DW, *et al.* BMP signalling inhibits premature neural differentiation in the mouse embryo. *Development* 2007; **134**:3359-3369.
13 Linker C, Stern CD. Neural induction requires BMP inhibition only as a late step, and involves signals other than FGF and Wnt antagonists. *Development* 2004; **131**:5671-5681.
14 Wilson SI, Rydstrom A, Trimborn T, *et al.* The status of Wnt signalling regulates neural and epidermal fates in the chick embryo. *Nature* 2001; **411**:325-330.
15 Launay C, Fromentoux V, Shi DL, Boucaut JC. A truncated FGF receptor blocks neural induction by endogenous Xenopus inducers. *Development* 1996; **122**:869-880.
16 Mizuseki K, Kishi M, Matsui M, Nakanishi S, Sasai Y. Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* 1998; **125**:579-587.
17 Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 2003; **115**:281-292.
18 Miyama K, Yamada G, Yamamoto TS, *et al.* A BMP-inducible gene, dlx5, regulates osteoblast differentiation and mesoderm induction. *Dev Biol* 1999; **208**:123-133.
19 Suzuki A, Ueno N, Hemmati-Brivanlou A. Xenopus msx1 mediates epidermal induction and neural inhibition by BMP4. *Development* 1997; **124**:3037-3044.
20 Bakkers J, Hild M, Kramer C, Furutani-Seiki M, Hammerschmidt M. Zebrafish DeltaNp63 is a direct target of Bmp signaling and encodes a transcriptional repressor blocking neural specification in the ventral ectoderm. *Dev Cell* 2002; **2**:617-627.
21 Eckert D, Buhl S, Weber S, Jager R, Schorle H. The AP-2 family of transcription factors. *Genome Biol* 2005; **6**:246.
22 Hilger-Eversheim K, Moser M, Schorle H, Buettner R. Regulatory roles of AP-2 transcription factors in vertebrate development, apoptosis and cell-cycle control. *Gene* 2000; **260**:1-12.
23 Imagawa M, Chiu R, Karin M. Transcription factor AP-2 mediates induction by two different signal-transduction path-

ways: protein kinase C and cAMP. *Cell* 1987; **51**:251-260.
24 Byrne C, Tainsky M, Fuchs E. Programming gene expression in developing epidermis. *Development* 1994; **120**:2369-2383.
25 Schmidt M, Huber L, Majdazari A, Schutz G, Williams T, Rohrer H. The transcription factors AP-2beta and AP-2alpha are required for survival of sympathetic progenitors and differentiated sympathetic neurons. *Dev Biol* 2011; **355**:89-100.
26 Li W, Cornell RA. Redundant activities of Tfap2a and Tfap2c are required for neural crest induction and development of other non-neural ectoderm derivatives in zebrafish embryos. *Dev Biol* 2007; **304**:338-354.
27 Hoffman TL, Javier AL, Campeau SA, Knight RD, Schilling TF. Tfap2 transcription factors in zebrafish neural crest development and ectodermal evolution. *J Exp Zool B Mol Dev Evol* 2007; **308**:679-691.
28 Luo T, Lee YH, Saint-Jeannet JP, Sargent TD. Induction of neural crest in Xenopus by transcription factor AP2alpha. *Proc Natl Acad Sci USA* 2003; **100**:532-537.
29 Nie X, Deng CX, Wang Q, Jiao K. Disruption of Smad4 in neural crest cells leads to mid-gestation death with pharyngeal arch, craniofacial and cardiac defects. *Dev Biol* 2008; **316**:417-430.
30 De Croze N, Maczkowiak F, Monsoro-Burq AH. Reiterative AP2a activity controls sequential steps in the neural crest gene regulatory network. *Proc Natl Acad Sci USA* 2011; **108**:155-160.
31 Nikitina N, Sauka-Spengler T, Bronner-Fraser M. Dissecting early regulatory relationships in the lamprey neural crest gene network. *Proc Natl Acad Sci USA* 2008; **105**:20083-20088.
32 Kuckenberg P, Buhl S, Woynecki T, *et al*. The transcription factor TCFAP2C/AP-2gamma cooperates with CDX2 to maintain trophectoderm formation. *Mol Cell Biol* 2010; **30**:3310-3320.
33 Chazaud C, Oulad-Abdelghani M, Bouillet P, Decimo D, Chambon P, Dolle P. AP-2.2, a novel gene related to AP-2, is expressed in the forebrain, limbs and face during mouse embryogenesis. *Mech Dev* 1996; **54**:83-94.
34 Guttormsen J, Koster MI, Stevens JR, Roop DR, Williams T, Winger QA. Disruption of epidermal specific gene expression and delayed skin development in AP-2 gamma mutant mice. *Dev Biol* 2008; **317**:187-195.
35 Weber S, Eckert D, Nettersheim D, *et al.* Critical function of AP-2 gamma/TCFAP2C in mouse embryonic germ cell maintenance. *Biol Reprod* 2009; **82**:214-223.
36 Auman HJ, Nottoli T, Lakiza O, Winger Q, Donaldson S, Williams T. Transcription factor AP-2gamma is essential in the extra-embryonic lineages for early postimplantation development. *Development* 2002; **129**:2733-2747.
37 Xia C, Wang C, Zhang K, Qian C, Jing N. Induction of a high population of neural stem cells with anterior neuroectoderm characters from epiblast-like P19 embryonic carcinoma cells. *Differentiation* 2007; **75**:912-927.
38 Zhang K, Li L, Huang C, *et al.* Distinct functions of BMP4 during different stages of mouse ES cell neural commitment. *Development* 2010; **137**:2095-2105.
39 Watanabe K, Kamiya D, Nishiyama A, *et al.* Directed differentiation of telencephalic precursors from embryonic stem cells. *Nat Neurosci* 2005; **8**:288-296.
40 Wilson PA, Hemmati-Brivanlou A. Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* 1995; **376**:331-333.
41 Rex M, Orme A, Uwanogho D, *et al.* Dynamic expression of chicken Sox2 and Sox3 genes in ectoderm induced to form neural tissue. *Dev Dyn* 1997; **209**:323-332.
42 Pera E, Stein S, Kessel M. Ectodermal patterning in the avian embryo: epidermis versus neural plate. *Development* 1999; **126**:63-73.
43 Yu PB, Hong CC, Sachidanandan C, *et al.* Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nat Chem Biol* 2008; **4**:33-41.
44 Marazzi G, Wang Y, Sassoon D. Msx2 is a transcriptional regulator in the BMP4-mediated programmed cell death pathway. *Dev Biol* 1997; **186**:127-138.
45 Katagiri T, Imada M, Yanai T, Suda T, Takahashi N, Kamijo R. Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. *Genes Cells* 2002; **7**:949-960.
46 Zhang P, Li J, Tan Z, *et al.* Short-term BMP-4 treatment initiates mesoderm induction in human embryonic stem cells. *Blood* 2008; **111**:1933-1941.
47 Winger Q, Huang J, Auman HJ, Lewandoski M, Williams T. Analysis of transcription factor AP-2 expression and function during mouse preimplantation development. *Biol Reprod* 2006; **75**:324-333.
48 Cajal M, Lawson KA, Hill B, *et al.* Clonal and molecular analysis of the prospective anterior neural boundary in the mouse embryo. *Development* 2012; **139**:423-436.
49 Linker C, De Almeida I, Papanayotou C, *et al.* Cell communication with the neural plate is required for induction of neural markers by BMP inhibition: evidence for homeogenetic induction and implications for Xenopus animal cap and chick explant assays. *Dev Biol* 2009; **327**:478-486.
50 Mohibullah N, Donner A, Ippolito JA, Williams T. SELEX and missing phosphate contact analyses reveal flexibility within the AP-2[alpha] protein: DNA binding complex. *Nucleic Acids Res* 1999; **27**:2760-2769.
51 Zhang X, Leung YK, Ho SM. AP-2 regulates the transcription of estrogen receptor (ER)-beta by acting through a methylation hotspot of the 0N promoter in prostate cancer cells. *Oncogene* 2007; **26**:7346-7354.
52 Koster MI, Kim S, Huang J, Williams T, Roop DR. TAp63alpha induces AP-2gamma as an early event in epidermal morphogenesis. *Dev Biol* 2006; **289**:253-261.
53 Turksen K, Troy TC. Epidermal cell lineage. *Biochem Cell Biol* 1998; **76**:889-898.
54 Wang X, Pasolli HA, Williams T, Fuchs E. AP-2 factors act in concert with Notch to orchestrate terminal differentiation in skin epidermis. *J Cell Biol* 2008; **183**:37-48.
55 Sinha S, Degenstein L, Copenhaver C, Fuchs E. Defining the regulatory factors required for epidermal gene expression. *Mol Cell Biol* 2000; **20**:2543-2555.
56 Leask A, Byrne C, Fuchs E. Transcription factor AP2 and its role in epidermal-specific gene expression. *Proc Natl Acad Sci USA* 1991; **88**:7948-7952.
57 Koster MI, Kim S, Mills AA, DeMayo FJ, Roop DR. p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev* 2004; **18**:126-131.
58 Koster MI, Roop DR. The role of p63 in development and differentiation of the epidermis. *J Dermatol Sci* 2004; **34**:3-9.

59 Yasue A, Tao H, Nohno T, Moriyama K, Noji S, Ohuchi H. Cloning and expression of the chick p63 gene. *Mech Dev* 2001; **100**:105-108.
60 Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 1999; **398**:708-713.
61 Wanner R, Zhang J, Henz BM, Rosenbach T. AP-2 gene expression and modulation by retinoic acid during keratinocyte differentiation. *Biochem Biophys Res Commun* 1996; **223**:666-669.
62 Kwon HJ, Bhat N, Sweet EM, Cornell RA, Riley BB. Identification of early requirements for preplacodal ectoderm and sensory organ development. *PLoS Genet* 2010; **6**:pii: e1001133.
63 Streit A, Stern CD. Establishment and maintenance of the border of the neural plate in the chick: involvement of FGF and BMP activity. *Mech Dev* 1999; **82**:51-66.
64 Meulemans D, Bronner-Fraser M. Gene-regulatory interactions in neural crest evolution and development. *Dev Cell* 2004; **7**:291-299.
65 Monsoro-Burq AH, Wang E, Harland R. Msx1 and Pax3 cooperate to mediate FGF8 and WNT signals during Xenopus neural crest induction. *Dev Cell* 2005; **8**:167-178.
66 McLarren KW, Litsiou A, Streit A. DLX5 positions the neural crest and preplacode region at the border of the neural plate. *Dev Biol* 2003; **259**:34-47.
67 Sato T, Sasai N, Sasai Y. Neural crest determination by co-activation of Pax3 and Zic1 genes in *Xenopus* ectoderm. *Development* 2005; **132**:2355-2363.
68 Khudyakov J, Bronner-Fraser M. Comprehensive spatiotemporal analysis of early chick neural crest network genes. *Dev Dyn* 2009; **238**:716-723.
69 Luo T, Matsuo-Takasaki M, Thomas ML, Weeks DL, Sargent TD. Transcription factor AP-2 is an essential and direct regulator of epidermal development in *Xenopus*. *Dev Biol* 2002; **245**:136-144.
70 Tribulo C, Aybar MJ, Nguyen VH, Mullins MC, Mayor R. Regulation of Msx genes by a Bmp gradient is essential for neural crest specification. *Development* 2003; **130**:6441-6452.
71 Mishina Y, Starbuck MW, Gentile MA, *et al.* Bone morphogenetic protein type IA receptor signaling regulates postnatal osteoblast function and bone remodeling. *J Biol Chem* 2004; **279**:27560-27566.
72 Yoon BS, Ovchinnikov DA, Yoshii I, Mishina Y, Behringer RR, Lyons KM. Bmpr1a and Bmpr1b have overlapping functions and are essential for chondrogenesis *in vivo*. *Proc Natl Acad Sci USA* 2005; **102**:5062-5067.
73 Streit A, Lee KJ, Woo I, Roberts C, Jessell TM, Stern CD. Chordin regulates primitive streak development and the stability of induced neural cells, but is not sufficient for neural induction in the chick embryo. *Development* 1998; **125**:507-519.
74 Zhang Y, Luo T, Sargent TD. Expression of TFAP2beta and TFAP2gamma genes in *Xenopus laevis*. *Gene Expr Patterns* 2006; **6**:589-595.
75 Jin Z, Liu L, Bian W, *et al.* Different transcription factors regulate nestin gene expression during P19 cell neural differentiation and central nervous system development. *J Biol Chem* 2009; **284**:8160-8173.
76 Tiscornia G, Singer O, Verma IM. Production and purification of lentiviral vectors. *Nat Protoc* 2006; **1**:241-245.
77 Gao X, Bian W, Yang J, *et al.* A role of N-cadherin in neuronal differentiation of embryonic carcinoma P19 cells. *Biochem Biophys Res Commun* 2001; **284**:1098-1103.
78 Tanaka S, Kamachi Y, Tanouchi A, Hamada H, Jing N, Kondoh H. Interplay of SOX and POU factors in regulation of the *Nestin* gene in neural primordial cells. *Mol Cell Biol* 2004; **24**:8834-8846.
79 Okada Y, Shimazaki T, Sobue G, Okano H. Retinoic-acid-concentration-dependent acquisition of neural cell identity during *in vitro* differentiation of mouse embryonic stem cells. *Dev Biol* 2004; **275**:124-142.
80 Voiculescu O, Papanayotou C, Stern CD. Spatially and temporally controlled electroporation of early chick embryos. *Nat Protoc* 2008; **3**:419-426.
81 Nieto MA, Patel K, Wilkinson DG. *In situ* hybridization analysis of chick embryos in whole mount and tissue sections. *Methods Cell Biol* 1996; **51**:219-235.
82 Streit A, Stern CD. Combined whole-mount *in situ* hybridization and immunohistochemistry in avian embryos. *Methods* 2001; **23**:339-344.
83 Huang C, Chen J, Zhang T, *et al.* The dual histone demethylase KDM7A promotes neural induction in early chick embryos. *Dev Dyn* 2010; **239**:3350-3357.
84 Peng G, Han M, Du Y, *et al.* SIP30 is regulated by ERK in peripheral nerve injury-induced neuropathic pain. *J Biol Chem* 2009; **284**:30138-30147.

(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)