

AP2 γ 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 AP2 γ , a transcription factor activator protein (AP)-2 family member, is upregulated by BMP4 during neural differentiation of pluripotent stem cells. Knockdown of AP2 γ facilitates mouse embryonic stem cell (ESC) neural fate determination and impairs epidermal differentiation, whereas AP2 γ overexpression inhibits neural conversion and promotes epidermal commitment. In the early chick embryo, AP2 γ 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 AP2 γ inhibits excessive neural expansion and it also promotes epidermal development in the surface ectoderm. Moreover, AP2 γ knockdown in ESCs and chick embryos partially rescued the neural inhibition and epidermal induction effects of BMP4. Mechanistic studies showed that BMP4 directly regulates AP2 γ expression through Smad1 binding to the AP2 γ promoter. Taken together, we propose that during the early stages of ectodermal patterning in the chick embryo, AP2 γ 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: AP2 γ ; 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 Δ Np63 [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.

AP2 γ (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, AP2 α , AP2 β and AP2 γ 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 AP2 α downregulation from embryonic day 9.5 (E9.5) in mouse embryo [29]. AP2 α also regulates neural border specification during the neuralization stage in *Xenopus* and lamprey [30, 31]. Mouse AP2 γ 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 AP2 γ leads to mouse embryonic lethality at approximately E7.5, showing extraembryonic cell defects and abnormal embryonic gastrulation [36]. However, it is unclear whether AP2 γ is involved in ectodermal patterning at earlier stages of embryonic development and what is the relationship between AP2 γ and BMP signaling.

Here we show that AP2 γ is upregulated by BMP4 during pluripotent stem cell differentiation and that AP2 γ partially mediates the BMP4 functions of neural inhibition and epidermal promotion. *In vivo*, chick AP2 γ (cAP2 γ) is expressed in the epiblast before HH stage 3 and gradually shifts to the peripheral ectoderm during HH stage 4, and AP2 γ acts downstream of BMP signal-

ing to regulate neural and epidermal development in different regions of the epiblast in the early chick embryo.

Results

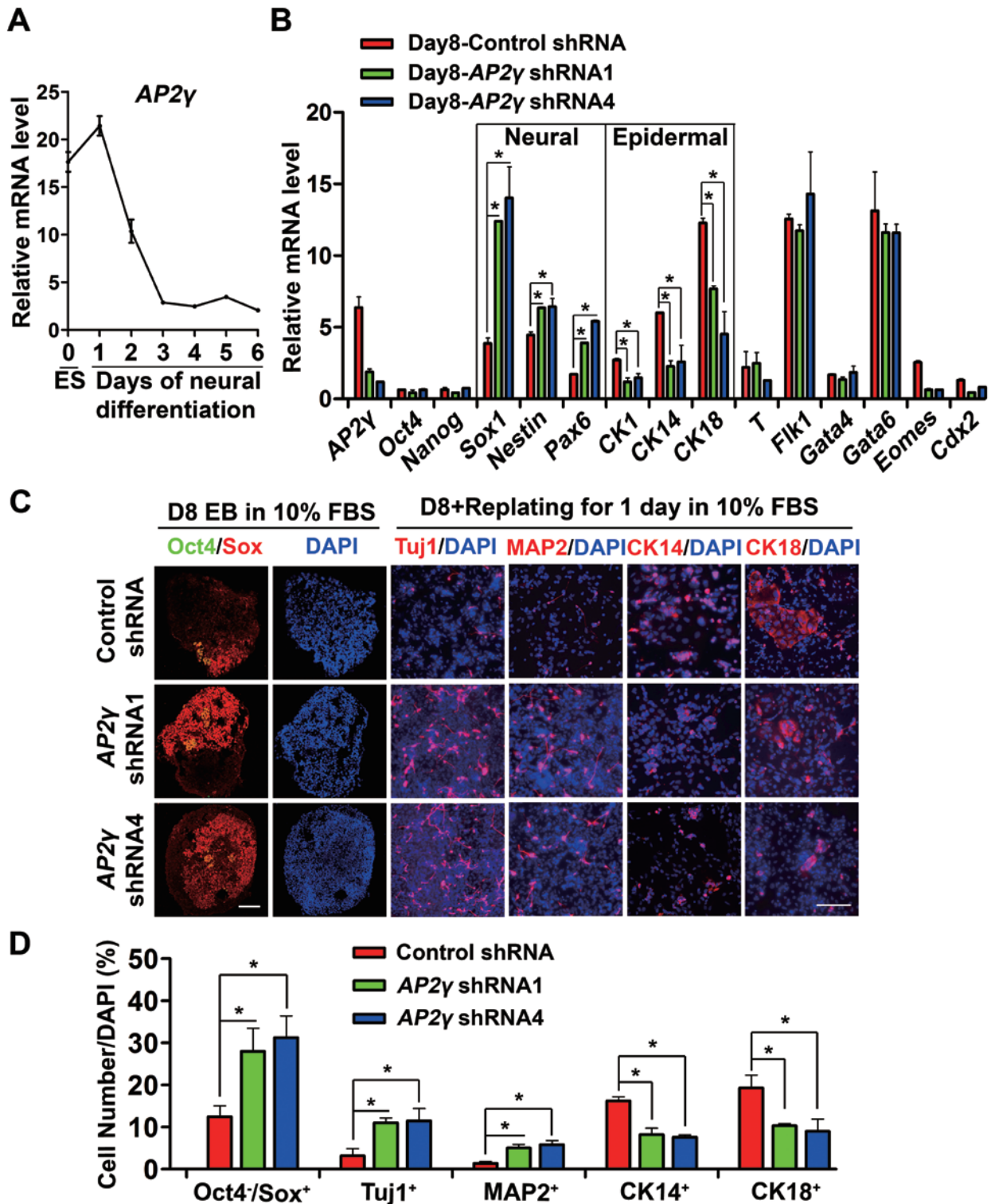
AP2 γ knockdown facilitates neural differentiation and impairs epidermal commitment of ESCs

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, AP2 γ 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 AP2 γ function in early embryonic development. We found that AP2 γ mRNA was expressed in undifferentiated mouse ESCs and that its level gradually decreased with progressing neural conversion (Figure 1A), suggesting that AP2 γ might be involved in the neural differentiation of ESCs.

To test this hypothesis, shRNAs specifically targeted to AP2 γ were introduced into ESCs using lentivirus, and two shRNAs (shRNA1 and shRNA4) could efficiently knock down AP2 γ 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 AP2 γ knockdown upregulated the expression of NPC markers *Sox1*, *Pax6* and *Nestin*

Figure 1 AP2 γ knockdown facilitates neural commitment and impairs epidermal fate determination during ESC differentiation. **(A)** qRT-PCR analysis of AP2 γ 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 AP2 γ -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 μ m.



(Figure 1B). Immunostaining of day 8 EBs confirmed that control shRNA-expressing ESCs produced approximately 15% Oct4⁻/Sox⁺ NPCs, whereas ESCs with AP2 γ 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 AP2 γ 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-

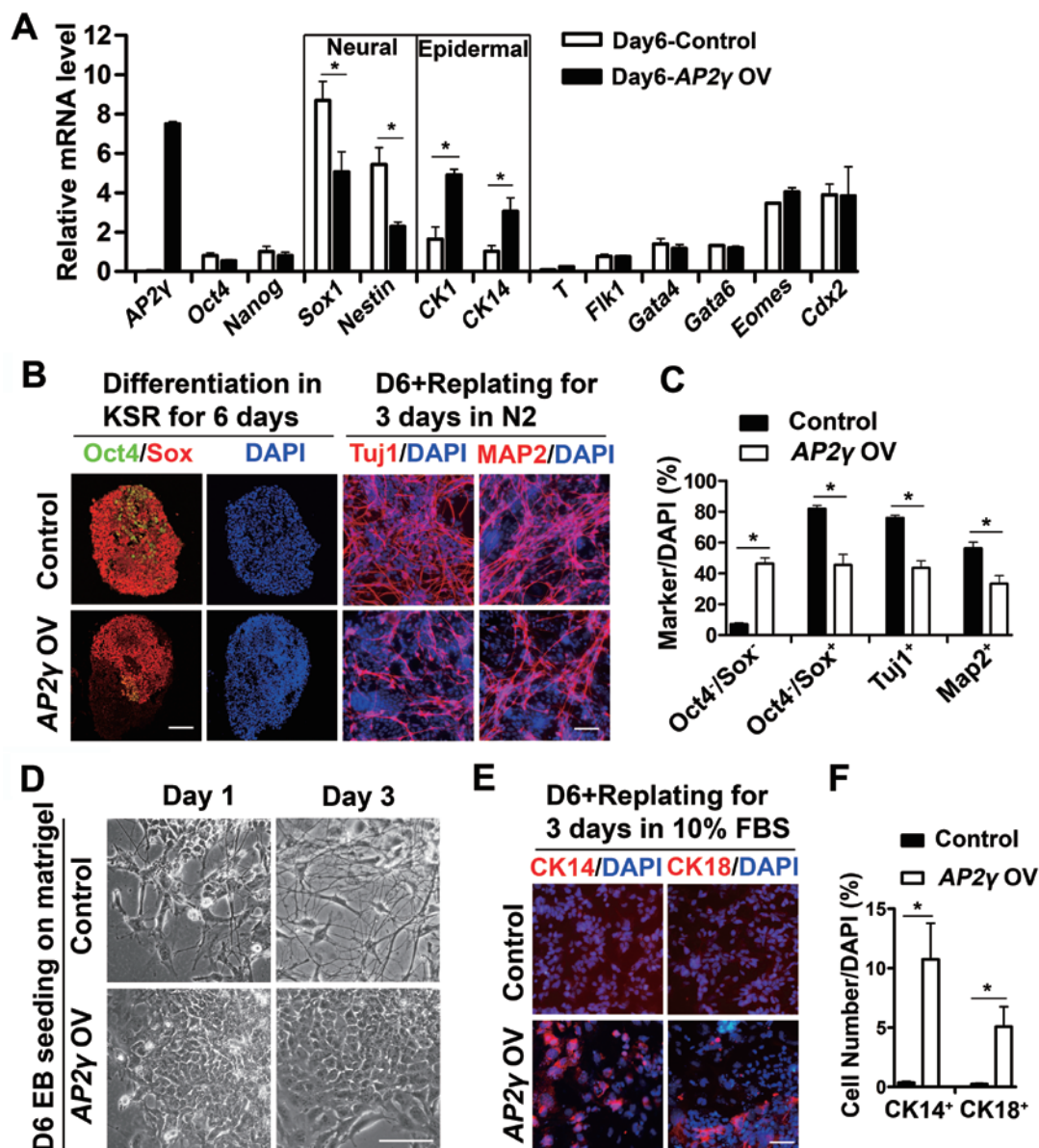


Figure 2 *AP2γ* overexpression suppresses neural commitment and promotes epidermal marker expression during ESC neural differentiation. **(A)** The expression of neural and epidermal markers was analyzed by qRT-PCR in day 6 EBs derived from control or *AP2γ*-overexpressing (OV) ESCs in 8% KSR medium. **(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 AP2 γ knock-down (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 AP2 γ shRNAs (Figure 1B). Together, these data suggest that AP2 γ might be necessary for epidermal commitment and be a negative regulator of neural specification during ESC differentiation.

AP2 γ overexpression inhibits neural conversion and promotes epidermal differentiation of ESCs

To examine whether AP2 γ is sufficient for neural inhibition and epidermal induction, AP2 γ was overexpressed in ESCs by lentivirus. Similarly to the AP2 γ -knockdown ESCs, AP2 γ -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, AP2 γ -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 AP2 γ -overexpressing cells (Figure 2B and 2C). Subsequent neuronal differentiation was also inhibited by AP2 γ 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 AP2 γ overexpression (Figure 2A). Replated cells from AP2 γ -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 AP2 γ -knockdown cells, the expression of other germ layer markers was not affected by AP2 γ overexpression (Figure 2A). Taken together, these results suggest that AP2 γ inhibits neural conversion and promotes epidermal differentiation of ESCs.

AP2 γ partially mediates BMP4 functions during ESC differentiation

Given that AP2 γ phenocopies BMP's effects of promoting epidermal conversion and inhibiting neural commitment during ESC differentiation [40], and that AP2 γ expression is upregulated by BMP4 (Supplementary information, Figure S2), we speculated that AP2 γ mediates BMP functions in neural and epidermal differentiation.

Previously, we showed that BMP4 had different ef-

fects 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 AP2 γ 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 AP2 γ 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 AP2 γ upregulation at day 2-3 was confirmed at the protein level by western blot (Figure 3B). Given that AP2 γ responsiveness to BMP4 correlates with the neural inhibition and epidermal induction by BMP in ESC differentiation, we further propose that AP2 γ might mediate BMP functions during the BMP4-sensitive window.

To test this hypothesis, we cultured AP2 γ 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 AP2 γ -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 AP2 γ 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 AP2 γ 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 AP2 γ -knockdown EBs were treated with BMP4 at day 2-3 (Supplementary information, Figure S4C), suggesting that AP2 γ may not mediate BMP functions during mesendodermal specification.

Together, these data suggest that AP2 γ partially mediates the BMP4 functions of neural inhibition and epidermal induction during the BMP4-sensitive window in ESC neural differentiation.

AP2 γ expression shifts from the epiblast to the putative epidermal ectoderm of the early chick embryo

To explore the biological functions of AP2 γ *in vivo*,

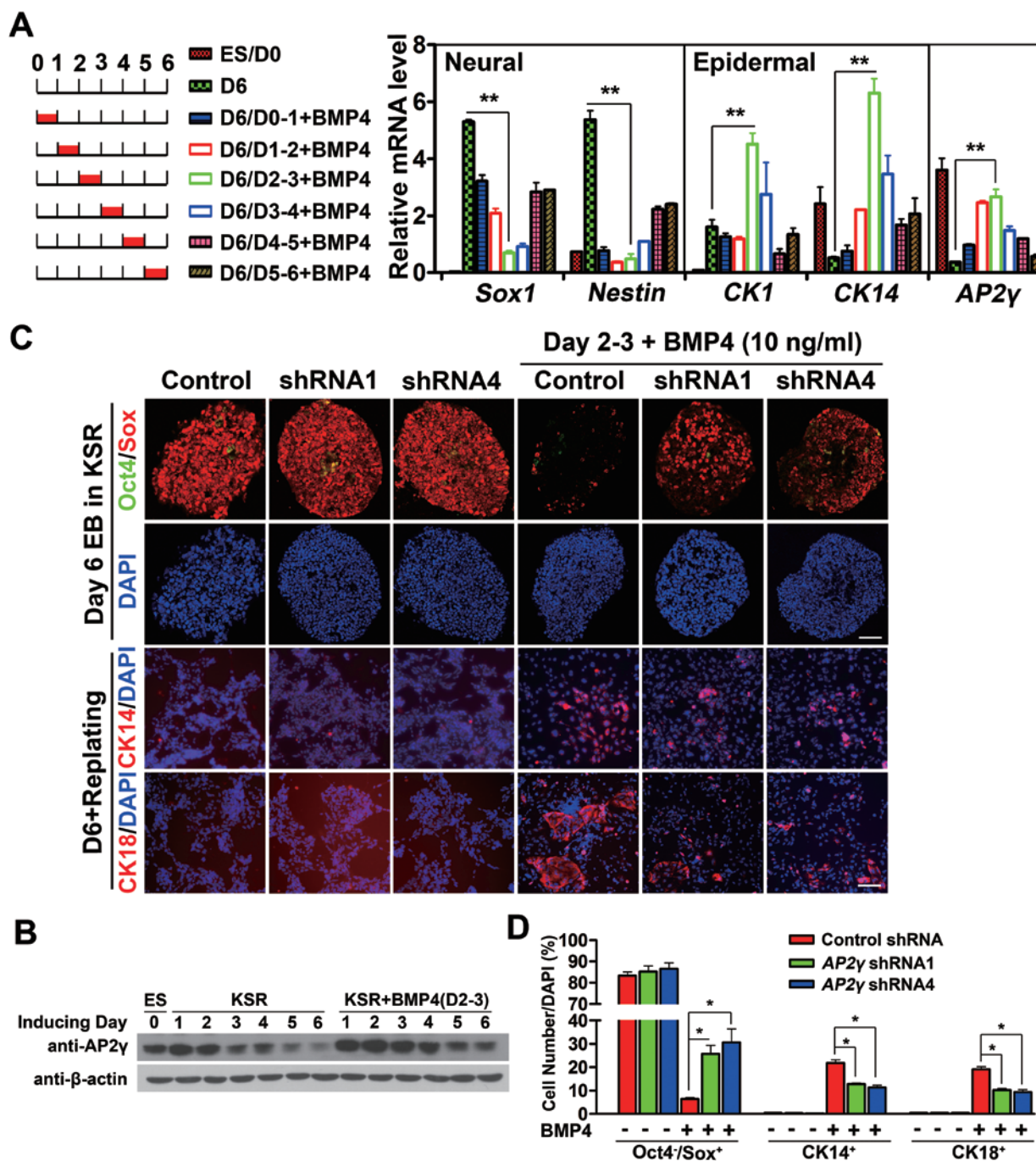


Figure 3 *AP2 γ* partially mediates BMP4 functions in ESC neural differentiation. **(A)** qRT-PCR analysis of ectoderm markers and *AP2 γ* expression in EBs following culture in KSR medium for 6 days with or without BMP4 (10 ng/ml) treatment. BMP4 was added to the medium on the indicated days for 24 h. Undifferentiated ESCs without feeders served as a negative control and normally-induced day 6 EBs served as a positive control. **(B)** *AP2 γ* 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 *AP2 γ* 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 μ m.

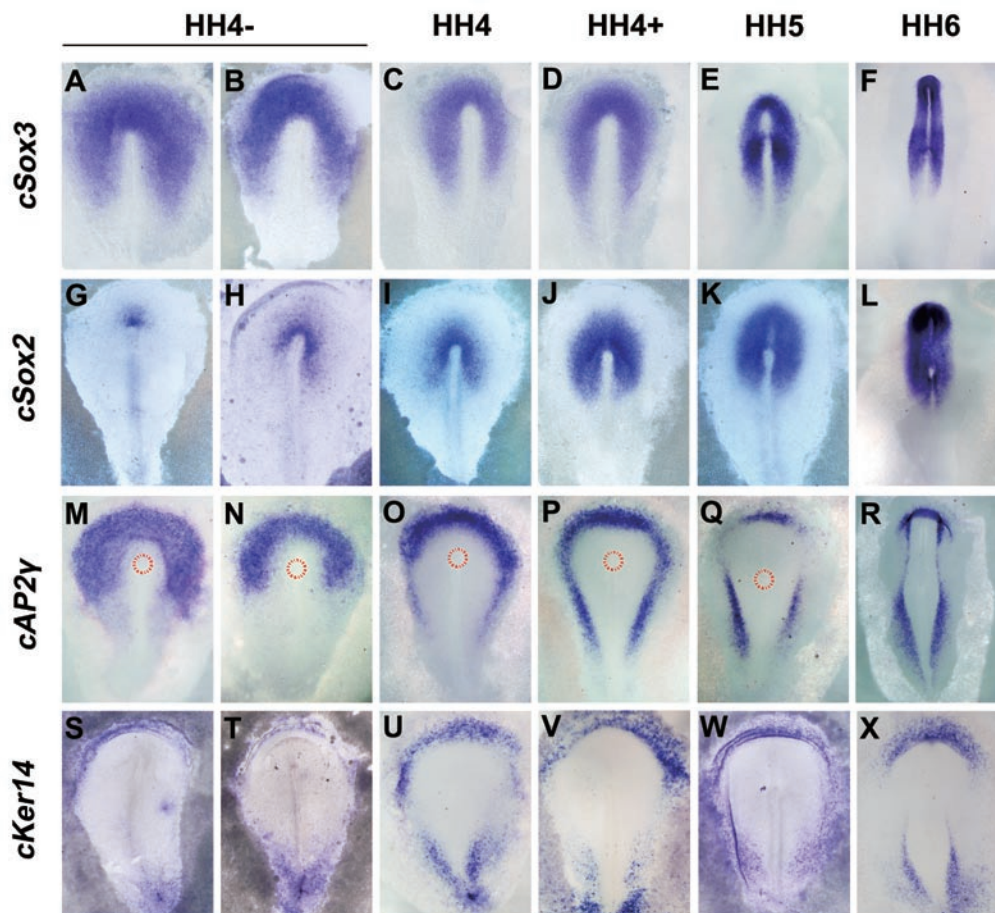


Figure 4 *AP2 γ* shifts from the epiblast to the peripheral ectoderm concurrent with neural plate expansion in the chick embryo. Expression of *cSox3* (A-F), *cSox2* (G-L), *cAP2 γ* (M-R) and *cKer14* (S-X) is shown by ISH using digoxigenin-labeled riboprobes in HH stage 4-6 chick embryos. The broken red line indicates the area (M-Q) of Hensen's node.

the spatial-temporal relationship between *AP2 γ* 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*, *cAP2 γ* was expressed throughout the epiblast marked by the early epiblast marker *cSox3* [41] and *cAP2 γ* 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), *cAP2 γ* expression progressively shifted from the epiblast to the peripheral ectoderm at HH stage 4 (Figure 4M-4P). Double ISH showed that *cAP2 γ* 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+ *cAP2 γ* 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), *cAP2 γ* 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 *cAP2 γ* 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 *cAP2 γ* expression shifts from the epiblast to the putative epidermal ectoderm during neural plate formation at HH stage 4, suggesting that *cAP2 γ* 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 electropo-

rated 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-

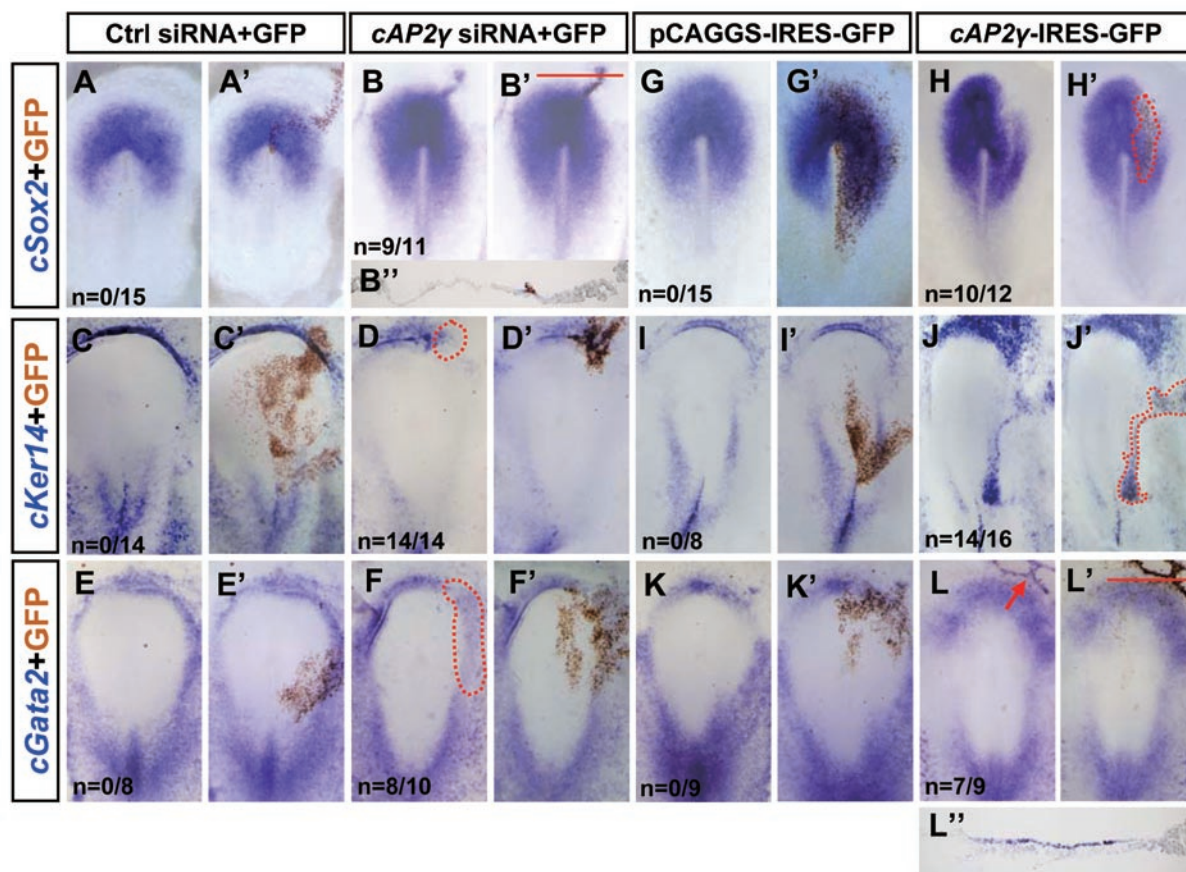


Figure 5 *AP2γ* inhibits neural expansion and promotes epidermal commitment at different regions of epiblast. Whole-mount ISH (blue) was performed in chick embryos that had been previously electroporated in the epiblast layer at HH stage 3. The same embryo was stained for GFP expression (brown), which 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 AP2 γ (mAP2 γ) (Supplementary information, Figure S7D). To determine whether AP2 γ is sufficient for epidermal induction, we overexpressed *cAP2 γ* in the peripheral epiblast and found that *cAP2 γ* could efficiently induce the ectopic expression of *cKer14*

and *cGata2* (Figure 5I-5L',5L''). *cSox3* expression was not affected by *AP2 γ* overexpression or knockdown (Supplementary information, Figure S7F), similarly to BMP4 misexpression's effects on the early expression of *Sox3* [13]. Collectively, AP2 γ restricts excessive neural

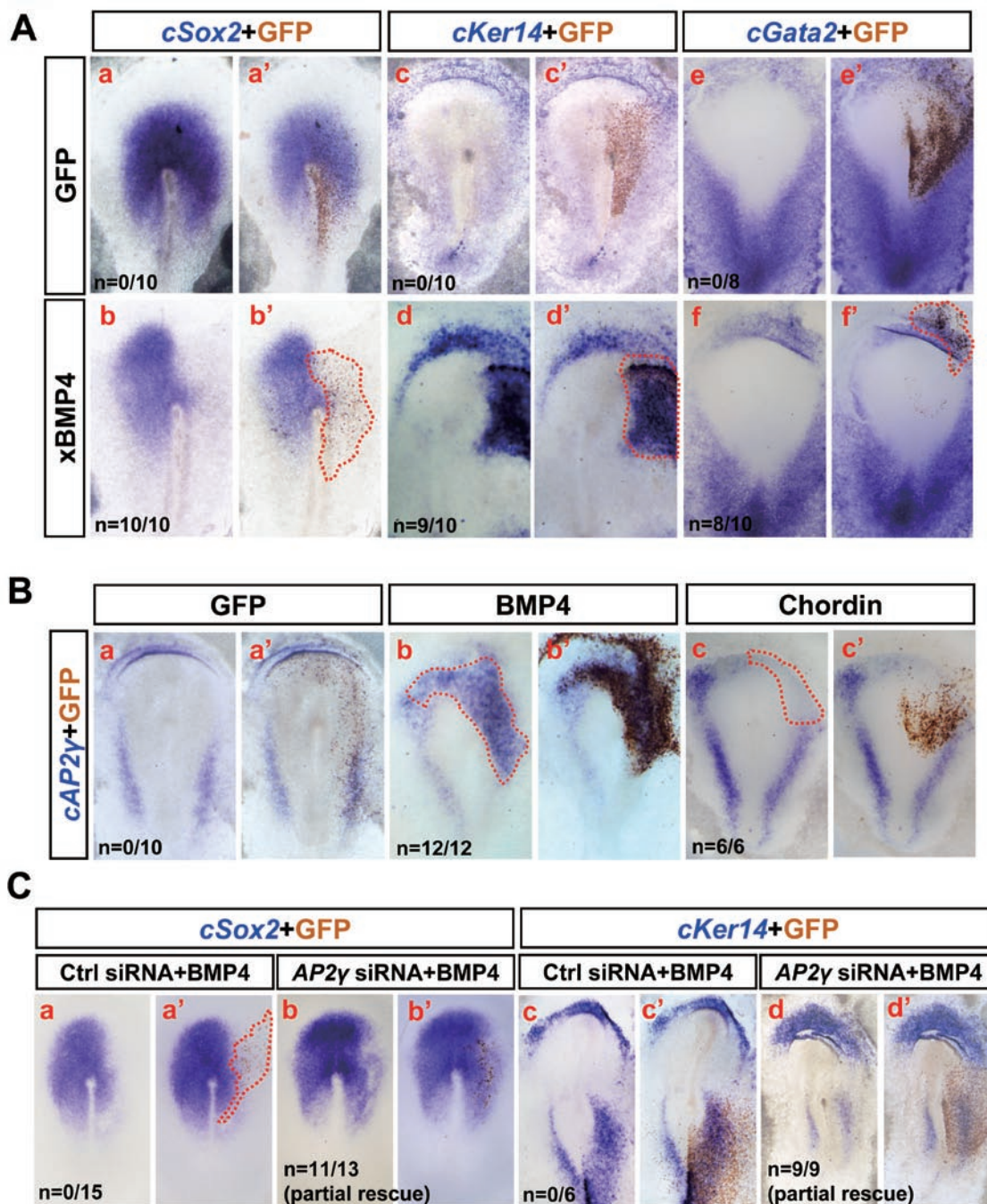


Figure 6 AP2 γ partially mediates BMP4 functions in ectodermal patterning. **(A)** Whole-mount ISH analysis of *cSox2*, *cKer14* and *cGata2* in control GFP- or xBMP4-electroporated chick embryos at HH stage 5. **(B)** Whole-mount ISH analysis of *cAP2 γ* in control GFP-, xBMP4- or Chordin-electroporated chick embryos. **(C)** Control or AP2 γ siRNA (50 ng/ μ l) and xBMP4 (0.3 μ g/ μ l) 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 (Δ P) 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 Δ P 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 Δ P fragment had no such activity (Figure 7E, WT: 6/9, Δ P: 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-

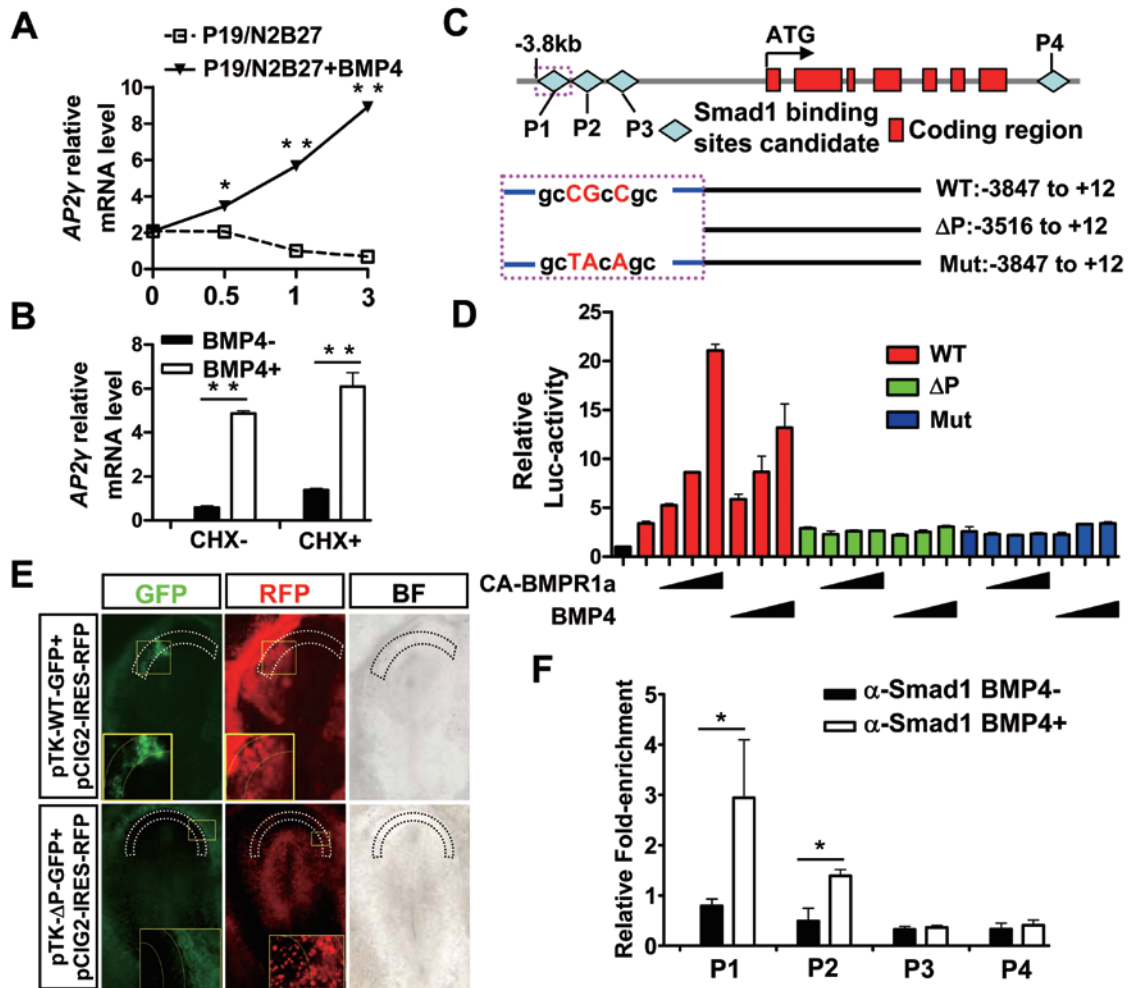


Figure 7 AP2 γ is directly regulated by BMP4. **(A)** AP2 γ expression was examined using qRT-PCR in N2B27 serum-free medium-induced P19 cells with or without BMP4 (10 ng/ml) treatment for 0.5, 1 or 3 h. **(B)** qRT-PCR analysis of AP2 γ expression with or without BMP4 (10 ng/ml) in the presence of CHX (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 AP2 γ transcribed region. The 3.8-kb (WT: -3 847 to +12) fragment, 3.5-kb (Δ P: -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), Δ P-Luc (50 ng) or Mut-Luc (50 ng) with increasing amounts of CA-BMPR1a (0.1, 0.2 or 0.4 μ g) 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 Δ P fragment was constructed into the pTK-GFP vectors and co-electroporated with pCIG2-IRES-RFP into HH stage 3 embryos. The presumptive AP2 γ -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 AP2 γ 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 *cAP2 γ* suggests that AP2 γ

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 *cAP2 γ* and *cSox2*

are complementary to each other, and *cAP2 γ* acts as a gatekeeper to restrict excessive neural expansion. When *cAP2 γ* 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 *cAP2 γ* restricts normal neural expansion (Figure 5). These results indicate that *cAP2 γ* is sufficient and necessary for the inhibition of excessive neural plate expansion during early neuralization. This raises the question of how *cAP2 γ* inhibits *cSox2* extension. There are two possibilities: (1) *AP2 γ* functions as a transcriptional repressor through direct binding to the *cSox2* enhancer N2, which possesses a characterized AP2-binding site [22, 50, 51]; (2) *AP2 γ* biases the epiblast to differentiate into non-neural tissues [52]. At HH stage 4+, the expression of *cAP2 γ* 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 *AP2 γ* in the nascent neural plate.

cAP2 γ 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. *AP2 γ* 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 *AP2 γ* is involved in the initial epidermal commitment. We show that *cKer14* expression generally overlapped with *cAP2 γ* (Figure 4) and that *AP2 γ* 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 *AP2 γ* 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 *cAP2 γ* (Figures 4-6). It is possible that *Gata2* induction occurs in parallel with primary epidermal commitment. TAp63 α 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 *AP2 γ* triggers the initial step of epidermal development in the surface ectoderm. Thereafter when *p63* and *AP2 γ* are expressed in the committed surface ectoderm after HH stage 6, *AP2 γ* might act downstream of *p63* in the stratification program [52] and combine with other epidermal factors such as *p63*, *Msx1* and *AP2 α* to regulate the subsequent epidermal maturation at later stages [19, 26, 34, 54, 60, 61].

During the *AP2 γ* expression shift from the epiblast to the surface ectoderm, *AP2 γ* transitionally occupies the gap between the peripheral epidermis and the neural plate named pre-placodal region. We observed that BMP4 and *AP2 γ* could inhibit the expression of pre-placodal markers *cEya2* and *cSix1* (data not shown), suggesting that *AP2 γ* might be also involved in pre-placode development [62]. Furthermore, *AP2 γ* has redundant activities with *AP2 α* in neural crest induction and non-neural ectoderm derivative development [26]. Other factors such as *Pax7*, *Msx1*, *Zic1*, *Dlx5* and *AP2 α* 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 *AP2 α*) 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 *AP2 γ* , its expression consistently surrounds the expanding neural plate in the anterior and lateral region. At the onset of neuralization, *AP2 γ* -expressing cells in the future neural plate have the competence to differentiate into neural cells and the excessive expansion of *cSox2* is restricted by *AP2 γ* . Only when *AP2 γ* is cleared from neighboring cells around the *cSox2*-expressing region can *cSox2* expand moderately. *AP2 γ* 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 *AP2 γ* -expressing epiblast at HH stage 3+/4- may differentiate into one of three directions: neural plate (the epiblast around Hensen’s node gradually loses *AP2 γ* expression and expresses *cSox2*), neural crest or pre-placode (*AP2 γ* might collaborate with some neural border factors to specify the neural crest or pre-placodal ectoderm [26, 62]) or epidermis (*AP2 γ* 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 *AP2 γ* as a novel BMP downstream target that is directly regulated by BMP4 through Smad1 binding to the *AP2 γ* 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, *cAP2 γ* can be ectopically induced by BMP4 and suppressed by chordin (Figure 6), suggesting that clearance of *AP2 γ* 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 *AP2 γ* 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]. *AP2 γ* 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, *AP2 γ* partially mediates the BMP4 function of epidermal induction in the peripheral ectoderm. Further investigation is needed to determine whether *AP2 γ* mediates BMP functions in pre-placodal development and neural crest specification, during which *AP2 α* might play important roles downstream of BMP signaling [29, 30].

To summarize, we propose a model for *AP2 γ* 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. *AP2 γ* 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, *AP2 γ* might mediate dual roles of BMP in ectodermal patterning: *AP2 γ* 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 μ M, Sigma), Dorsomorphin (0.5–20 μ M, Enzo life sciences) were used.

Gene overexpression and knockdown in ESCs

Mouse *AP2 γ* 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 *AP2 γ* knockdown in ESCs. The control and *AP2 γ* 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-*AP2 γ* (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 μ g/ μ l) or siRNA reagents with Fast Green and Sucrose were electroporated at Hamburger and Hamilton (HH) 3 as previously described [80]. *cAP2 γ* (NCBI: XM_417497) that displays 72% identity to *mAP2 γ* 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: *cAP2 γ* , *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.

cAP2 γ and control siRNA sequences were listed in Supplementary information, Table S1. siRNAs (0.5 $\mu\text{g}/\mu\text{l}$) were electroporated into the epiblast of early chick embryo and 0.15 $\mu\text{g}/\mu\text{l}$ 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-AP2 γ 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 \pm 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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