The Double Inhibition of Endogenously Produced BMP and Wnt Factors Synergistically Triggers Dorsal Telencephalic Differentiation of Mouse ES Cells

Michele Bertacchi,[1] Luca Pandolfini,[1] Mara D'Onofrio,[2,3] Rossella Brandi,[2,3] Federico Cremisi[1,4]

[1] Scuola Normale Superiore, Classe di Scienze, Pisa, Italy

[2] Genomics Facility, European Brain Research Institute, Roma, Italy

[3] Istituto di Farmacologia Traslazionale, Consiglio Nazionale dell Ricerche, Roma, Italy

[4] Istituto di Tecnologie Biomediche, Consiglio Nazionale delle Ricerche, Pisa, Italy

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ABSTRACT: **Embryonic stem (ES) cells are becoming a popular model of *in vitro* neurogenesis, as they display intrinsic capability to generate neural progenitors that undergo the known steps of *in vivo* neural development. These include the acquisition of distinct regional fates, which depend on growth factors and signals that are present in the culture medium. The control of the intracellular signaling that is active at different steps of ES cell neuralization, even when cells are cultured in chemically defined medium, is complicated by the endogenous production of growth factors. However, this endogenous production has been poorly investigated so far. To address this point, we performed a high-throughput analysis of the expression of morphogens during mouse ES cell neuralization in minimal medium. We found that during their neuralization, ES cells increased the expression of members of Wnt, Fibroblast Growth Factor (FGF), and BMP families. Conversely, the expression of Activin/Nodal and Shh ligands was low in early steps of neuralization. In this experimental condition, neural progenitors and neurons generated by ES cells expressed a gene expression profile that was consistent with a midbrain identity. We found that endogenous BMP and Wnt signaling, but not FGF signaling, synergistically affected ES cell neural patterning, by turning off a profile of dorsal/telencephalic gene expression. Double BMP and Wnt inhibition allowed neuralized ES cells to sequentially activate key genes of cortical differentiation. Our findings are consistent with a novel synergistic effect of Wnt and BMP endogenous signaling of ES cells in inhibiting a cortical differentiation program.** © 2014 Wiley Periodicals, Inc. Develop Neurobiol 00: 000–000, 2014

Keywords: **Wnt; BMP; mouse embryonic stem cells; cortical fate; *in vitro* neurogenesis**

INTRODUCTION

The dissection of diffusible signals that orchestrate neural induction and regulate regional brain patterning has recently been made easier by the study of embryonic stem (ES) cell *in vitro* differentiation. Mouse and human ES cells acquire neural identity in the absence of external signals (Tropepe et al., 2001; Muñoz-Sanjuán and Brivanlou, 2002; Bouhon et al.,

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Correspondence to: F. Cremisi (f.cremisi@sns.it).

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2005; Smukler et al., 2006). ES cell neurogenesis *in vitro* in chemically defined culture media allows to perform either gain or loss of function assays with many molecules at different times, which is currently not feasible or extremely cumbersome with *in vivo* approaches. Thus, ES cell neuralization is becoming a useful tool to functionally assay the role of growth factors and morphogens that have distinct roles in specific regions of the embryonic brain at precise times of development.

According to the "Activation-Transformation" model, ectodermal cells initially acquire an anterior fate on neural induction (Activation), but they can subsequently be transformed into more caudal cell fates (Transformation) to obtain all of the different subdomains along the A/P axis, a process referred to as caudalization (Wilson and Houart, 2004). In many assays, whenever neural tissue is induced, it expresses transcripts that are later restricted to forebrain and midbrain territories. Expression of these "anterior markers" raises the possibility of an obligate link between induction of neural identity and acquisition of anterior character (Foley and Stern, 2001). Wnts, Retinoic acid (RA), and Fibroblast Growth Factors (FGFs) are thought to convert neuroepithelial cells from an anterior default state to a more posterior identity along the neuraxis.

The patterning events that define A–P and D–V identities *in vivo* can be mimicked performing ES cell differentiation in a minimal medium devoid of growth factors and morphogens. The effects of RA, BMPs, Wnts, FGFs, and Sonic Hedgehog (SHH) on the identity of neurons generated by ES cells have been described (Watanabe et al., 2005; Eiraku et al., 2008; Gaspard et al., 2008; Chatzi et al., 2011). Addition of caudalizing signals, such as RA or FGF2 can promote a spinal cord identity and subsequent production of motor neurons (Wichterle et al., 2002), whereas addition of Wnt and Nodal antagonists promotes production of telencephalic progenitors (Watanabe et al., 2005). These observations are consistent with a close parallelism between *in vivo* and *in vitro* molecular mechanisms of neural patterning and support the advantage of ES cells as *in vitro* model for the dissection of signaling involved in neural patterning.

Although the effect of growth factors and morphogens on ES neural patterning can be anticipated by their known role *in vivo*, the use of ES cultures for studies aimed to steer their neural patterning toward distinct fates, or to investigate *in vitro* the signaling controlling neuronal fate acquisition, is complicated by the possible production of growth factors and morphogens inside the neuralizing culture. Many studies reported that natural or chemical inhibitors/antagonists of Wnts (Bouhon et al., 2006; Kirkeby et al., 2012), BMPs (Surmacz et al., 2012; Lupo et al., 2013; Ozair et al., 2013), FGFs (Chambers et al., 2009; Kriks et al., 2011; Lupo et al., 2013), Activin/Nodal (Lupo et al., 2013), or Shh (Gaspard et al., 2008; Li et al., 2009; Nicoleau et al., 2013) affect the differentiation fate of neurons cultured in chemically defined media. These studies implicate that such factors were endogenously produced by ES differentiating cells. However, few of them went into the detail of the endogenous expression of these signals and their role in ES neural patterning has been somehow neglected. This approach limited stem cell studies, especially when addressing the concept of default ES cell differentiation fate and of the exact role of distinct morphogens during neuralization.

We have recently established a protocol of mouse ES neuralization in chemically defined minimal medium (CDMM) in which the generated neurons acquire a dorsal midbrain identity. We found that neuralizing ES cells produce BMPs and that inhibiting BMP signaling turns on a global profile of gene expression that is consistent with a dorsal telencephalic identity (Bertacchi et al., 2013). We hypothesized that in our protocol other signals than BMPs could be produced. To identify them, we performed a global analysis of gene expression at different times of ES neuralization. Here, we show that different forms of BMP, Wnt, and FGF were expressed by ES neural progenitors, whereas the expression level of Activin/Nodal and Shh ligands was low until late stages of neuronal differentiation in our protocol. Although FGF signaling inhibition had no gross effects on neural patterning, Wnt signaling inhibition induced massive expression of dorsal telencephalic markers. This effect was higher than the effect obtained by BMP inhibition. BMP/Wnt double inhibition exerted a synergistic effect resulting in time-specific and robust expression of key genes of corticogenesis.

METHODS

ES Cell Neuralization

Murine ES cell lines E14Tg2A (passages 25–38) and 46C (transgenic Sox1-GFP ES cells, kindly provided by A. Smith, University of Cambridge, UK; passages 33–39) were cultured on gelatin-coated tissue culture dishes at a density of 40,000 cells/cm^2. ES cell medium, which was changed daily, contained GMEM (Sigma), 10% Fetal Calf Serum, 2 m*M* Glutamine, 1 m*M* sodium Pyruvate, 1 m*M* nonessential amino acids, 0.05 m*M* β-mercaptoethanol, 100 U/mL Penicillin/Streptomycin, and 1000 U/mL recombinant mouse LIF (Invitrogen).

CDMM for neural induction consisted of DMEM/F12 (Invitrogen), 2 m*M* Glutamine, 1 m*M* sodium Pyruvate, 0.1 m*M* nonessential amino acids, 0.05 m*M* β-mercaptoethanol, 100 U/mL Penicillin/Streptomycin supplemented with N2/B27 (no vitamin A; Invitrogen). The protocol of ES neuralization consisted of three steps. In Step-I, dissociated ES cells were washed with DMEM/F12, aggregated in agar-coated culture dishes (65,000 cells per cm^2), and cultured as floating aggregates in CDMM for 2 days. The second day, 75% of CDMM was renewed. In Step-II, ES cell aggregates were dissociated and cultured in adhesion (65,000 cells per cm^2) on Poly-ornithine (Sigma; 20 μg/mL in sterile water, 24 h coating at 37°C) and natural mouse Laminin (Invitrogen; 2.5 μg/mL in PBS, 24 h coating at 37°C) for 4 days, changing CDMM daily. In Step-III, after a second dissociation, ES cells were cultured four additional days in CDMM devoid of B27 supplement to drive terminal differentiation, using the same type of seeding density and coated surface described for Step-II. Serum used for Trypsin inactivation was carefully removed by several washes in DMEM/F12.

Growth Factor and Morphogen Treatments

The following factors were tested by addition during Step-II: Dorsomorphin (BMP signaling inhibitor; Sigma-Aldrich; 5 μM), Shh agonist (SAG; Santa Cruz Biotechnology; 100/150 n*M*), IWR-1-Endo (Wnt inhibitor; Calbiochem; 5/10 μM), PD0325901 (MEK/ERK-inhibitor; Calbiochem; 10–100 n*M*), PD173074 (FGF-receptor -inhibitor; Calbiochem; 200 n*M*).

Semiquantitative Real-Time PCR

Total RNA was extracted from ES cell or tissue samples with NucleoSpin RNA II columns (Macherey-Nagel). ES cells from at least two-three different wells of 24-well plates were always pooled together to compensate for variability in cell seeding. RNA quantity and RNA quality were assessed with Nanodrop and gel electrophoresis. For each sample, 200/500 ng of total RNA were reverse-transcribed. Amplified cDNA was quantified using GoTaq qPCR Master Mix (Promega) on Rotor-Gene 6000 (Corbett). Amplification take-off values were evaluated using the built-in Rotor-Gene 6000 relative quantitation analysis function, and relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method, normalizing to the housekeeping gene β-Actin. Standard errors were obtained from the error propagation formula as described in (Nordgård et al., 2006) and statistical significance was probed with randomization test, taking advantage of REST Software (Pfaffl et al., 2002).

Immunocytodetection

Cells prepared for immunocytodetection experiments were cultured on Poly-ornithine/Laminin coated round glass coverslips. Cells were fixed using 2% paraformaldehyde for 10–15 min, washed twice with PBS, permeabilized using 0.1% Triton X100 in PBS, and blocked using 0.5% BSA in PBS. Primary antibodies used for microscopy included Oct3/4 (1:200; Santa Cruz DBA), Nanog (1:300; Novus Biologicals), acetylated N-Tubulin (1:500; Sigma), Neuronal Class III β-Tubulin (1:500; Covance), Musashi-1 (1:200; Cell Signaling), Nestin (1:200; Millipore), FoxG1 (1:200; Abcam), Tbr1 (1:400; Millipore), Satb2 (1:200; Abcam), Ctip2 (1:400; Abcam), vGlut2 (1:300; Abcam), GAD65 (1:500; Chemicon), Pax6 (1:400; Covance), and Nkx2.1 (1:400; Abcam). Primary antibodies were incubated 2 h at room temperature; cells were then washed three times with PBS (10′ each). Alexa Fluor 488 and Alexa Fluor 568 anti-mouse, anti-rabbit or anti-chicken IgG conjugates (Molecular Probes, 1:500) were incubated 1 h at RT in PBS containing 0.1% Triton X100 and 0.5% BSA for primary antibody detection, followed by three PBS washes (10′ each). Nuclear staining was obtained with DAPI. The protocol varied for Tbr1, Satb2, Ctip2, and FoxG1, which antibodies were incubated over-night at 4°C using 0.3% Triton X100.

Microarray Hybridization and Data Analysis

Total RNA was extracted with NucleoSpin RNA II columns (Macherey-Nagel). RNA from three different sets of experiments/embryos was pooled. RNA quality was assessed with Agilent Bioanalyzer RNA 6000 Nano kit; 200 ng of RNA were labeled with One Color (Agilent), purified and hybridized overnight onto an Agilent Mouse Gene Expression Microarray chip (8 × 60K, grid ID 028005), according to the manufacturer's instructions. Agilent Microarray scanner G2564C was used for slide acquisition and spot analysis was performed with Feature Extraction software (Agilent). Data were background-corrected and normalized between arrays by means of Bioconductor package limma (Smyth, 2005). Principal component analysis (PCA) was performed on normalized whole-gene expression dataset using software Cluster 3 (Eisen et al., 1998).

RESULTS

Mouse ES Cells Quickly Neuralize in Minimal Culture Medium

We followed a protocol of ES neuralization in three steps [Fig. 1(A)], minimizing the activation of morphogen signaling by the use of CDMM, see Methods; (Bertacchi et al., 2013). Undifferentiated mouse ES cells (E14 Tg2A ES cell line) were positive for pluripotency markers Oct4 and Nanog when expanded in the presence of LIF and serum before neuralization (Supporting Information Fig. 1A). Priming to neuralization was initiated by a first step in aggregation on agar-coated surface (Step-I, 2 days);

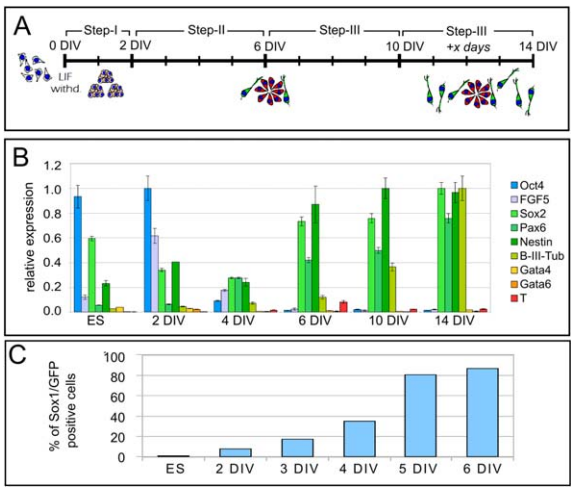

Figure 1 Time course of ES cells neural conversion. (A) scheme of the differentiation steps. (B) Expression of mRNAs coding for markers of pluripotency (Oct4, Sox2), priming to differentiation (FGF5), early neural progenitors (Nestin, Pax6, Sox2), postmitotic neurons (B-III-Tub), and endomesoderm (Gata4–6; Brachyury, T). Expression was evaluated by RT-PCR as relative to Beta-actin. Values were normalized to maximum expression, except those of Gata4–6 and T, which were normalized to the average relative expression of the other markers. (C) flow-cytometry analysis of Sox1-GFP ES cells at different DIV as indicated. Error bars show standard error. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

at the end of this first step (2 days of *in vitro* differentiation, 2 DIV) cells already turned off most of Nanog expression (Supporting Information Fig. 1B). At 2 DIV, aggregates were dissociated and cells were left to spontaneously neuralize in a second step as adherent cultures (Step-II, 4 days). At 4 DIV, cells had turned off Oct4 expression (not shown), already expressed the early neural progenitor marker nestin and formed rosette-like structures typical of neural progenitors (Zhang et al., 2001; Supporting Information Fig. 1C). Nestin expression increased and few B-III-tubulin neurons appeared at 6 DIV (Supporting Information Fig. 1D). The ratio of B-III-Tubulin-positive and N-Tubulin-positive neurons increased at DIV 10 (Supporting Information Fig. 1E) and became considerable at DIV14 (Supporting Information Fig. 1F). Gene expression analysis by RT-PCR confirmed the immunocytodetection analysis. Moreover, cells showed very low expression of endomesodermal markers Gata4, Gata6, and Brachyury (T), which was consistent with a high degree of neuralization [Fig. 1(B)]. To validate the occurrence of robust neuralization, we used a transgenic Sox1-GFP ES cell line (46C; Ying et al., 2003), in which GFP expression identifies early Sox1-positive neural progenitors. As determined by flow cytometry, neural progenitor ratio rapidly increased over the first days of neuralization, reaching 87% at 6 DIV [Fig. 1(C)]. A progressive process of ES cell differentiation through known steps of embryonic development was suggested also by the analysis of markers of pluripotency, priming to differentiation, neuralization, and differentiated neurons, using gene expression arrays (Supporting Information Fig. 2).

Mouse ES Cells Express Wnts, BMPs, and FGFs During *In Vitro* Neural Differentiation

To investigate the endogenous production of ligands of growth factor and morphogen families that are known to orchestrate neural patterning *in vivo*, we performed a high-throughput analysis of gene expression by microarray hybridization at different steps of ES cell neuralization.

In neuralizing cells at Step-II (6 DIV, Fig. 2), distinct members of Wnt, BMP and FGF families were highly expressed as compared to members of TGF-beta and Shh families. Wnt7b, Wnt8b, Wnt9a, Wnt1, BMP4, BMP7, and FGF23 showed the highest levels of expression. Notably, during Step-II neural progenitors are formed and previous studies indicated that they are competent to be patterned by BMP, RA, or Shh signaling (Bertacchi et al., 2013). According to the literature, FGF4, which is known to be expressed by mouse ES cells (Ying et al., 2008), showed the highest expression level at 0 DIV, whereas the expression of FGF5, which is a marker of priming to differentiation (Nichols and Smith, 2009), peaked at 2 DIV. The FGF member with the highest expression at 6 DIV was FGF23, whose expression reached its maximum at 10 DIV. Compared to Wnt, BMP, and FGF, members of TGF-beta and Shh familes were poorly expressed throughout all ES cell differentiation. In particular, Shh expression was very low until 10 DIV.

To get insights into the cell type that was responsible for the expression of ligands, we performed Aracytin (AraC) treatment, which is known to kill cycling cells (Orr and Smith, 1988). AraC treatment from 12 DIV to 14 DIV [Fig. 3(A)] negatively selected nestin-positive progenitor cells, leaving viable postmitotic neurons [Fig. 3(B–D)]; this diminished the level of expression of most members of Wnt, BMP, and FGF families, suggesting that cycling neural progenitors were responsible for the expression of these factors. BMP7, Wnt4, FGF5, and FGF7 were the exception, because their expression increased after AraC treatments, implying that they were produced preferentially by postmitotic neurons [Fig. 3(E–G)].

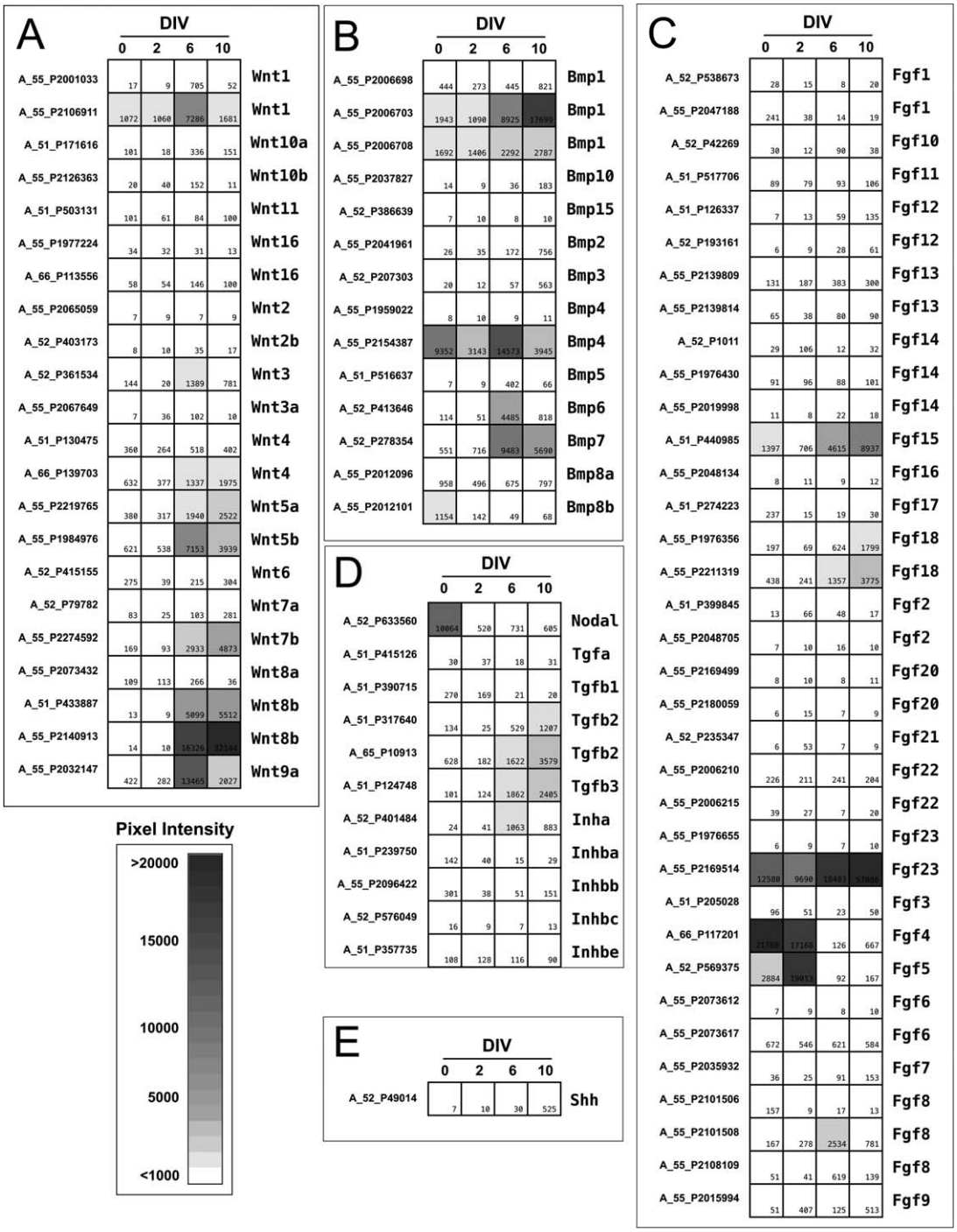

Figure 2 Endogenous signaling of neuralizing ES cells. (A–E) expression of different members of growth factor and morphogen families at different DIV: Wnts (A), BMPs (B), FGFs (C), Activin/Nodal (D), Shh (E). Values are expression levels in linear scale, obtained by the analysis of Agilent gene expression microarrays. Gray-scale indicates intensity degree, from minimum (white) to maximum (dark gray). Agilent probe annotation and gene name are indicated in the most left and right column of each panel, respectively.

Taken all together, these data suggest that mouse ES cells express different morphogens during neural differentiation *in vitro*, especially BMPs, Wnts, and FGFs. Thus, the concept of CDMM must be carefully addressed: even when using a morphogen-free culture medium, differentiating cells might be expressing

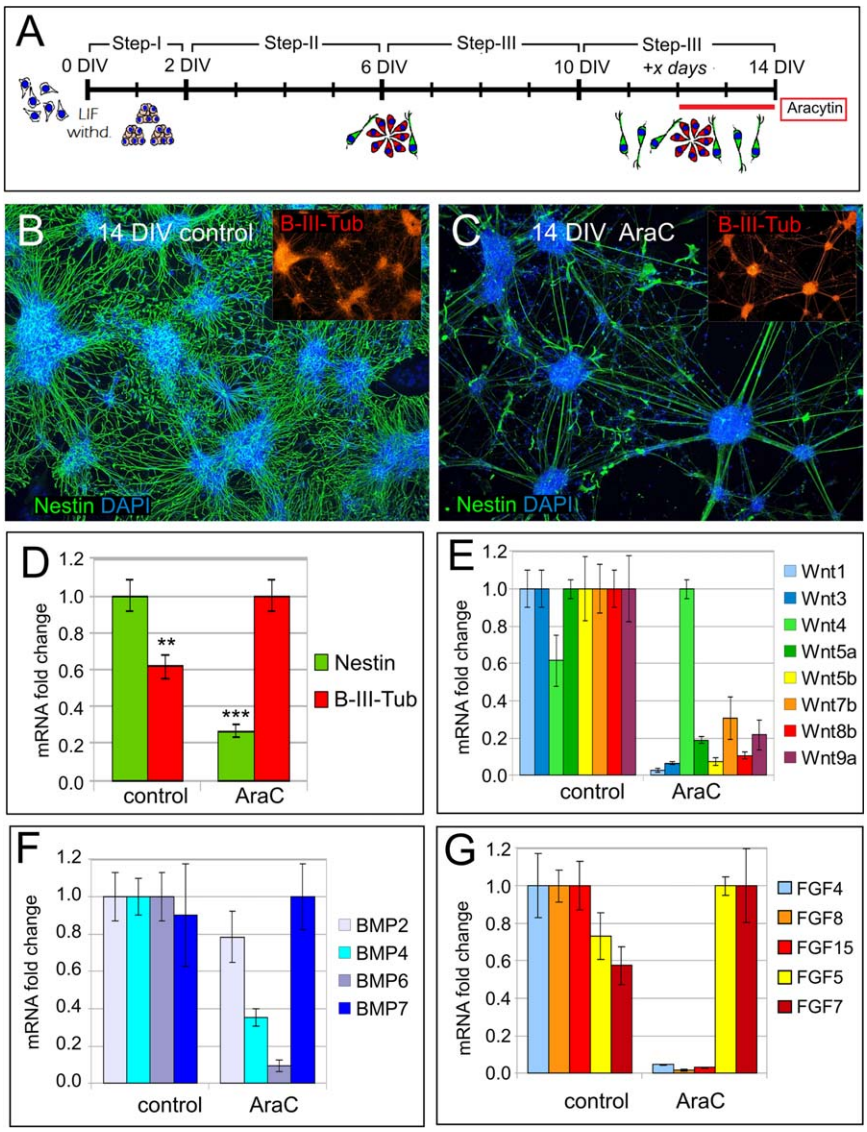

Figure 3 Effect of AraC treatment on the morphogen production of neuralizing ES cells. (A) experimental protocol of Aracytin (AraC) treatment. (B,C) Nestin and Beta-III-Tubulin (B-III-Tub) immunocytodetection in control (B) and AraC-treated (C) cells. (D) RT-PCR analysis of Nestin and Beta-III-Tubulin. (E–G) RT-PCR analysis of Wnts (E) BMPs (F) and FGFs (G) in control or AraC-treated (Ara C) cultures. Error bars show standard error; **p\0.01, ***p\0.001 (two-tailed Student's t test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

by themselves high amounts of different active molecules.

We postulated that endogenous Wnt, BMP, and FGF could affect the positional identity of progenitors generated by ES cells in CDMM.

Effect of BMP, Wnt, and FGF Inhibition on ES Cell Neural Patterning

BMPs produced by neuralizing ES cells were already proved to inhibit telencephalic identity in neural progenitors (Bertacchi et al., 2013). We, thus, assayed if also endogenous Wnts and FGF affected the patterning of ES-derived progenitors. ES cells neuralized in CDMM express midbrain markers starting from Step-II (Bertacchi et al., 2013). When Wnt signaling was inhibited by IWR-1-Endo treatment during Step-II [10 μM, Fig. 4(A)], the midbrain markers En1, Irx3, and Otx2 were downregulated and the forebrain and cortical markers Emx2, Six3, FoxG1 were upregulated at 6 DIV (not shown). We tested whether a double inhibition of BMP and Wnt pathways could

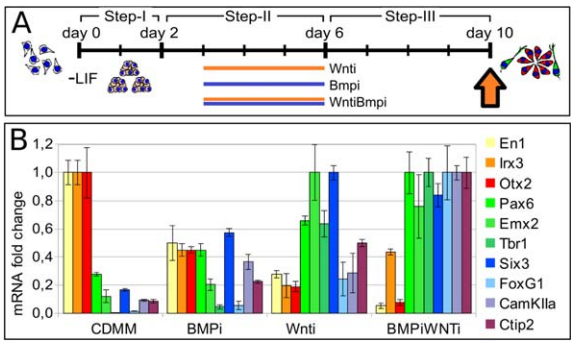

Figure 4 Effect of BMP and Wnt inhibition on ES cell neural patterning. (A) scheme of treatments. Wnti, Wnt inhibition; BMPi, BMP inhibition. The arrow shows the time of analysis. B, RT-PCR analysis of markers of dorsal/neural differentiation (Pax6) or anterior/posterior patterning. Forebrain markers: Emx2 (Simeone et al., 1992b), Tbr1 (Bulfone et al., 1995), Six3 (Oliver et al., 1995), FoxG1 (Xuan et al., 1995), CamKIIa (Arimatsu et al., 1999), Ctip2 (Arlotta et al., 2005). Midbrain markers: En1 (Davis et al., 1988), Irx3 (Bellefroid, 1998), and Otx2 (Simeone et al., 1992a).Values report fold changes. Error bars show standard error. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

strengthen the expression of forebrain markers. Cells were treated during Step-II with antagonist of BMP (Dorsomorphin, 5 μM; Bertacchi et al., 2013), of Wnt (IWR-1-Endo,10 μM), or with a combination of both. Wnt-inhibition was able to upregulate anterior/cortical markers (Emx2, Six3, FoxG1, CamKII-α, Tbr1,and Ctip2) more efficiently than BMP-inhibition, while the double inhibition showed a synergistic effect on the expression of A/P markers at Step-III [10 DIV; Fig. 4(B)]. Notably, positional memory of these changes was further maintained when prolonging the culture without treatment (14 DIV; Supporting Information Fig. 3A). The majority of Wnt/BMP double inhibited cells at 10 DIV were positive for the dorsal pan-neuronal marker Pax6 (66.4%,) and the telencephalic marker FoxG1 [69%, Fig. 5(A–F)], whereas the ratio of double BMP-Wnt inhibited cells expressing the ventral marker Nkx2.1 was very low (0.2%, Supporting Information Fig. 3B), according to the modest expression of Shh (see earlier). However, BMP/Wnt inhibited cells were competent to be ventralized by Shh signaling, as treatment with a SAG (150nM at Step-II) induced Nkx2.1 expression in 84% of cells (Supporting Information Fig. 3C).

The induction of a telencephalic identity was quite rapid, since at 6 DIV 55% of cells were already FoxG1 positive [Fig. 5(G)]. Treatments at Step-I or Step-III, either by Wnt-inhibition alone, or by double Wnt/BMP inhibition, were much less effective than treatments at Step-II on the expression of early markers of A/P patterning (Supporting Information Fig. 4). This is consistent with previous results obtained by BMP inhibition (Bertacchi et al., 2013) and indicates that Step-II is the time when early A/P patterning occurs in our *in vitro* protocol of ES cell differentiation.

To investigate the specificity of action of the treatments, we quantified by RT-PCR the expression of ID1, a downstream effector of the BMP-responsive pathway, and the expression of LEF1, a *bona fide* marker of the canonical Wnt pathway activity. Cells were treated during Step-II (starting at the beginning of day 2) and analyzed 72 h after, at 6 DIV. Dorsomorphin-mediated BMP-inhibition, both in single and in double treatment, resulted in a strong decrease of ID1 expression, compared to control cells. Similarly, LEF1 expression decreased when cells were treated with IWR-1-Endo (both in single Wnt-inhibition and in double BMP/Wnt-inhibition). Surprisingly, LEF1 expression showed a marked decrease also in Dorsomorphin-treated cells, [Fig. 5(H)]. This suggested a cross-talk mechanism between the two pathways. However, when culturing ES cells until 6 DIV and then performing an acute treatment of 12 h with chemical drugs, LEF1 and ID1 levels decreased in cells treated with IWR-1-Endo and Dorsomorphin, respectively, but LEF1 expression level in Dorsomorphin-treated cells was comparable to that of control cells [Fig. 5(I)]. Thus, the acute treatment demonstrated the specificity of the two antagonists on their respective pathways. We concluded that Dorsomorphin affects BMP signaling but is able to inhibit the Wnt canonical signaling as a secondary/late effect.

We investigated the effect of endogenous FGFs by treating the cell culture with PD0325901 (10 to 100 nM during Step-II or during Step-III), which blocks FGF signaling pathway by inhibiting MEK/ERK kinases. At each of the concentrations used, FGF depletion caused high level of cell death (not shown). This effect was likely due to a strict requirement of FGF signaling for cell survival during Step-II, or to high toxicity following MEK/ERK-inhibition. The lack of viability impeded an analysis of the patterning of neural progenitors. Better results were obtained with a specific inhibitor of FGF-receptor, PD173074 (200 nM during Step-II), which slowed-down cell proliferation (not shown) but allowed neural conversion (Supporting Information Fig. 5A–C). FGF-activation by exogenous FGF8b at Step-II significantly induced FoxG1 expression, while treatment at Step-III induced Gbx2 and En2. FGF-inhibition left

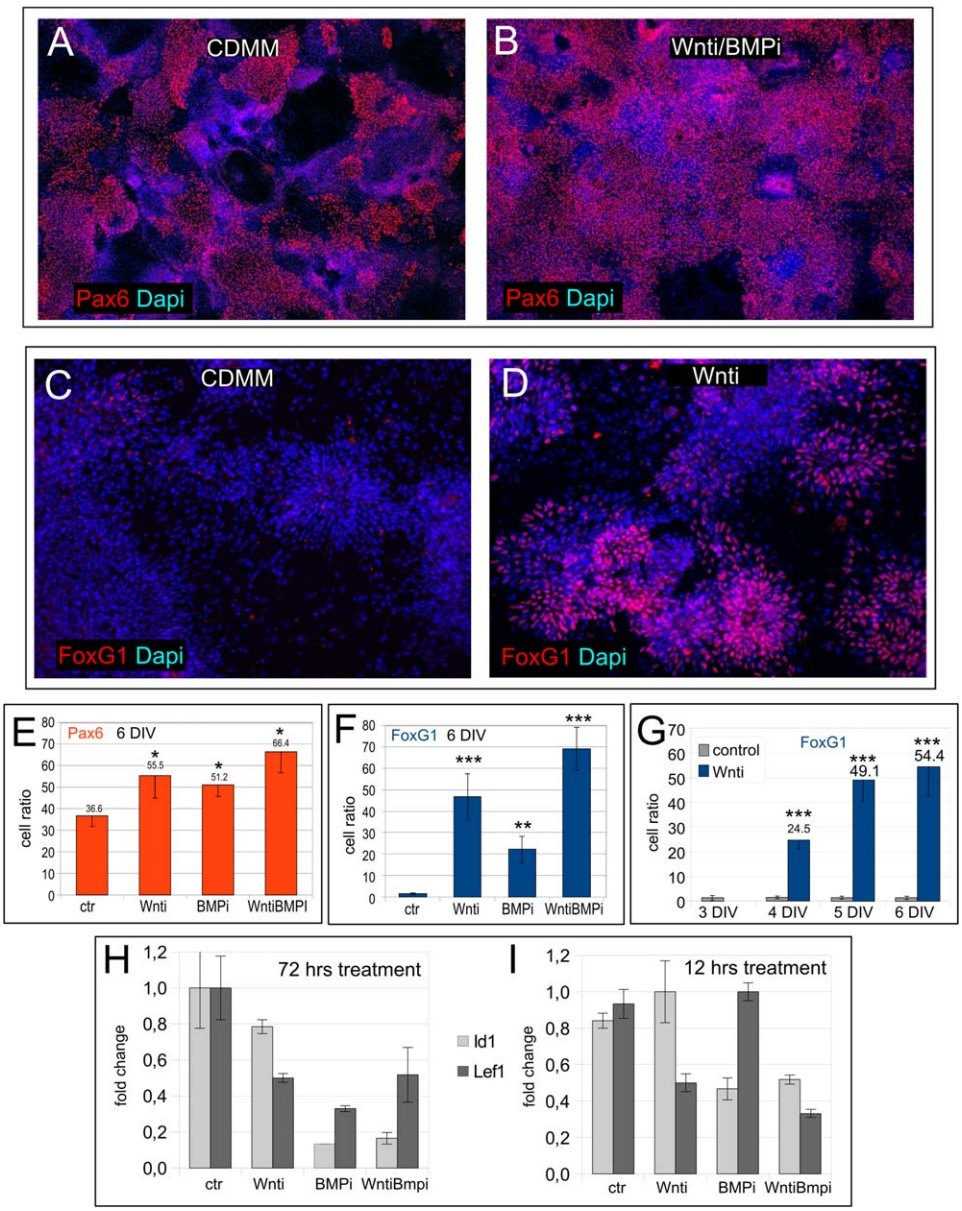

Figure 5 Effects of Wnt/BMP double inhibition of neuronal cell identity. (A–D) immunocytodetection of dorsal neural marker Pax6 (A,B) and forebrain marker FoxG1(C,D) in control (CDMM) or Wnt/BMP double inhibited ES cultures at 10 DIV. E,F, Ratio of Pax6 (E) and FoxG1 (F) positive cells at 10 DIV. (G) ratio of FoxG1positive cells at different DIV. H,I, RT-PCR analysis of Lef1 and ID1, reporters of Wnt and BMP signaling pathways, respectively, after 72 h (H) or 12 h (I) of inhibition of Wnt and/or BMP signaling. Values are expressed as fold change. Error bars show standard error; **p*\0.05, ***p*\0.01, ****p*\0.001 (two-tailed Student's *t* test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the expression level of A/P markers almost unaffected (Supporting Information Fig. 5D). The double nature of FGF8b as a modulator of both anterior/telencephalic and midbrain markers is consistent with its expression pattern *in vivo*, as FGF8b can be found in the anterior telencephalon and in the midbrain/hindbrain boundary (Rhinn and Brand, 2001; Shimogori et al., 2004). As a control of specificity of our treatments, FGF depletion/activation was confirmed by the regulation of the FGF signaling targets Spry2 and Sp8 (Supporting Information Fig. 5E). As little or no difference in the expression level of A/P

markers was found by comparing control cells with FGF-depleted cells, we concluded that the endogenous FGF signaling of neural progenitors during Step-II is necessary to neural differentiation and cell survival but it is not affecting in a relevant way the A/P identity of neural precursors in this window of treatments. However, we cannot exclude that the activation/inhibition of specific subsets of receptors by different FGF ligands could exert discordant effects on patterning.

Double Wnt/BMP Inhibition Turns on a Gene Expression Program that is Comparable to that of Cortical Progenitors

BMP and Wnt inhibition increased the ratio of cells expressing Tbr1, which specifically identifies a subset of early-generated cortical neurons, and Ctip2, which labels medium/early-generated cortical neurons of cortical layer 5, with the double inhibition exerting a synergistic effect [Fig. 6(A–E); Leone et al., 2008). We asked if the BMP-Wnt double inhibition could induce a neurogenic program similar to that of *in vivo* telencephalic development. We, thus, assayed if the expression of the markers of early, medium-late and late projecting neurons (Tbr1, Ctip2, and Satb2, respectively), was activated following a temporal sequence as during *in vivo* corticogenesis. The analysis of cells expressing the three markers [Fig. 6(F,G)] showed that a sequential onset of Tbr1-Ctip2-Satb2 expression *in vitro* occurs similarly to embryonic cortical development (Leone et al., 2008). Moreover, BMP/Wnt inhibited ES cultures at 18 DIV showed a robust labeling with B-III-tubulin and vGlut2 (87%), but were poorly labeled by GAD2 (8%; Supporting Information Fig. 6A–I), which was instead induced by the activation of Shh signaling with SAG (Supporting Information Fig. 6H,I), These observations suggest that BMP/Wnt inhibition induced a glutamatergic fate and are consistent with a cortical identity of the neurons generated by ES cells.

To better characterize neuronal identity, we compared the global gene expression profile of control or BMP/Wnt inhibited ES cultures at 15 DIV to the profile of isocortex, hippocampus, ventral telencephalon, or midbrain dissected from E15 embryo. We first compared gene expression profiles by PCA (see Methods). The first component, which accounts for 29.8% of variability, separated embryonic tissues from ES cell cultures. The second and third components, accounting for 19.4% and 17.6% of variability, respectively, distinguished the different embryonic brain regions. A 3D plot of these first three components, accounting in total for 66.8% of gene expression variability, is shown in Figure 7(A). Compared to control cells differentiated in CDMM, Wnt/BMP-inhibited cells (WiBi) are closer to isocortex and hippocampus than to midbrain or ventral telencephalon, indicating that Wnt/BMP-inhibition steers ES cell identity toward a general telencephalic identity. This observation is consistent with the results obtained when comparing the gene expression fold change between WiBi and CDMM to the gene expression fold change between isocortex and midbrain [Fig. 7(B)], isocortex and hippocampus [Fig. 7(C)] or isocortex and ventral telencephalon [Fig. 7(D)]. In fact, there is a pretty high coherence between WiBi/CDMM gene expression fold-change (gray bar plot) and Isocortex/Midbrain or Isocortex/Ventral Telencephalon gene expression fold change (black dot plot). All in all, these data support the ability of Wnt/BMP double inhibition to steer the identity of ES cells toward a dorsal/telencephalic identity rather than toward the identity of other brain regions.

DISCUSSION

Endogenous Production of Growth Factors

By adopting an *in vitro* neuralization protocol of mouse ES cells that makes use of minimal medium, we were able to investigate the endogenous activation of BMP, Wnt, and FGF signaling and their effects on neural patterning. In a previous work, we demonstrated that neuralizing ES cell cultures produce and are sensitive to BMPs, and that BMP signaling inhibition was permissive to the activation of a gene expression program consistent with a dorsal telencephalic identity (Bertacchi et al., 2013). In this study, we performed a high-throughput analysis to characterize, at distinct times of our neuralization protocol, the expression of ligands of the main growth factor and morphogen pathways known to act during early neural development. We found that members of BMP, Wnt and FGF, but not of Shh and TGFb families, were endogenously produced at high level in early neuralized ES cultures. Many previous studies described the effects of growth factor and morphogen antagonists on ES cells neuralization and patterning (see Introduction). This indirectly supported evidence of endogenous production of growth factors and morphogens, but no particular attention was addressed to characterize when and which particular ligand was produced. With our observations, we contributed to fill in this gap and highlighted the

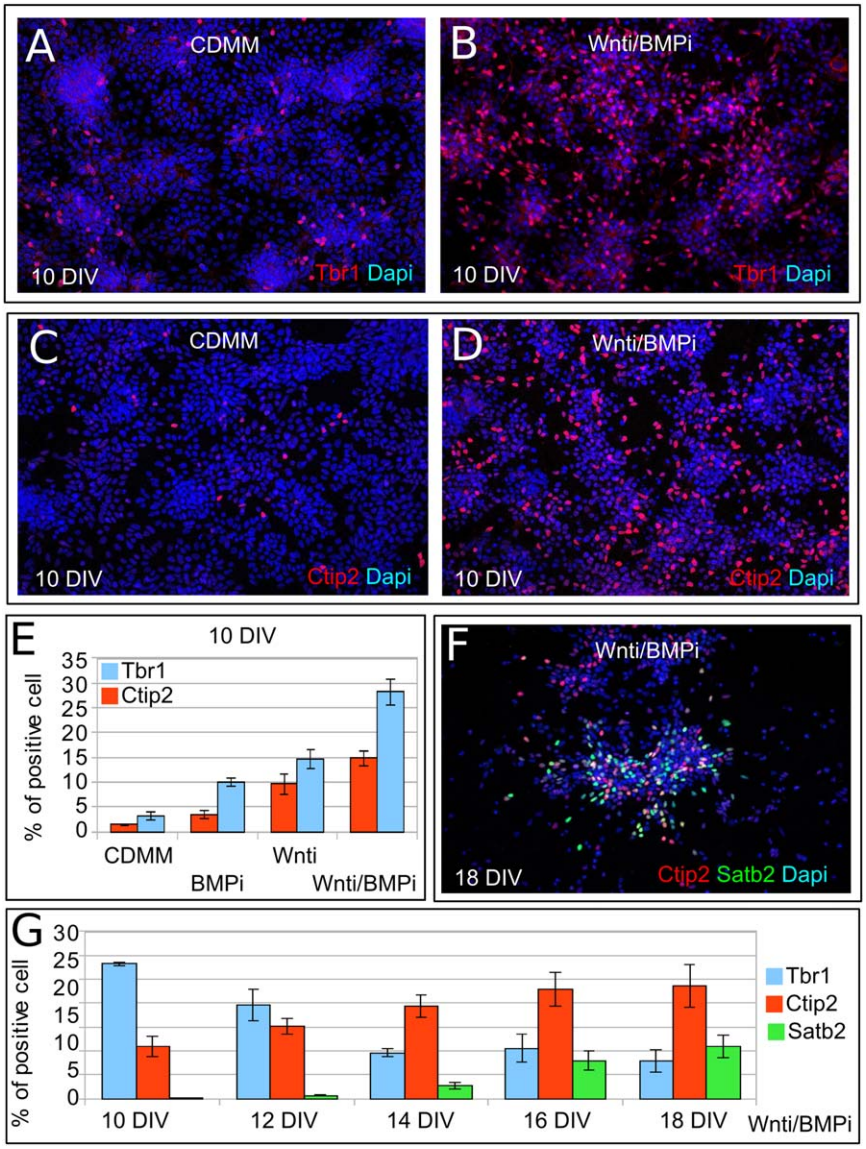

Figure 6 ES cells corticalization by double Wnt/BMP inhibition. (A–D,F) Tbr1, Ctip2, and Satb2 immunocytodetections at the indicated DIV in control cells (CDMM) or in cells treated by Wnt/BMP double inhibition (Wnti/BMPi). (E) ratio of cells positive for Tbr1 and Ctip2 at 10 DIV. (G) ratio of Wnt/BMP double inhibited cells positive to Tbr1, Ctip2, and Satb2 at different DIV as indicated. Error bars show standard error. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

importance of knowing which is the endogenous contribution of signaling when steering the neural patterning of ES cultures. The novelty of our approach is to relate the final fate of ES-derived neurons with the role of specific morphogens, whose endogenous production in neuralizing ES cells was characterized by global gene expression profiling.

We found that endogenous FGF signaling acted mainly on cell viability and proliferation, whereas both endogenous Wnts and BMPs worked as posteriorizing signals, inhibiting the expression of forebrain markers and supporting midbrain identity. Comparing to BMP inhibition, whose effect on ES cells neural patterning was previously described (Bertacchi et al., 2013), Wnt inhibition was more effective in inducing the expression of dorsal/telencephalic genes. We found that BMP inhibition was able to inhibit also Wnt signaling as a secondary, later

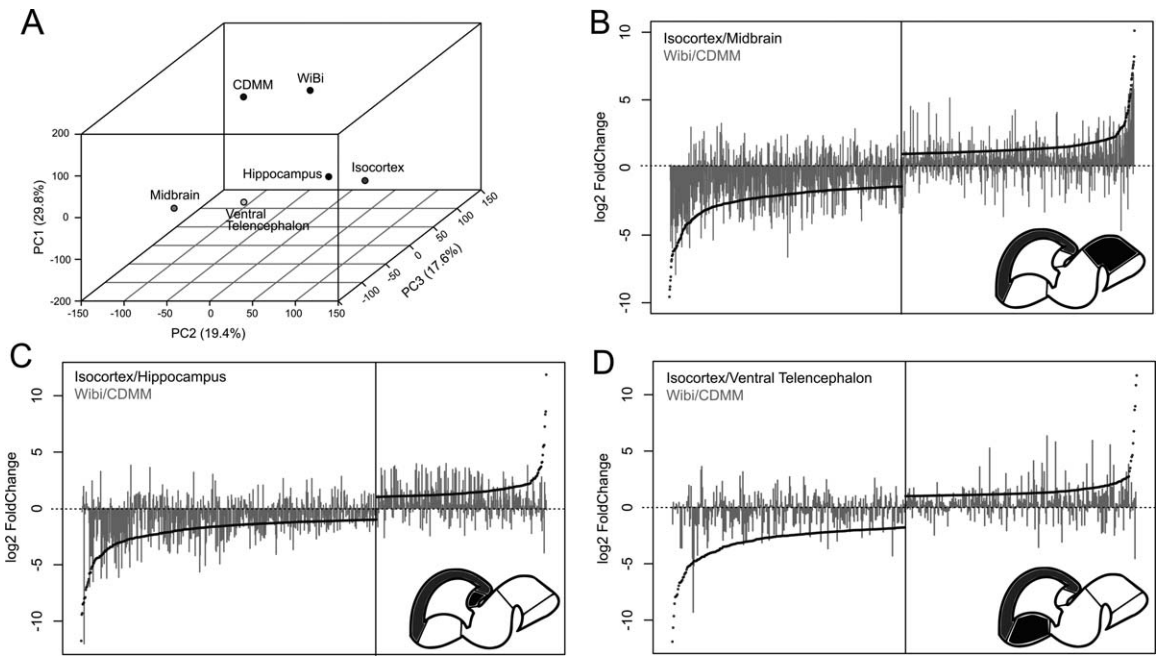

Figure 7 Global gene expression profiles of neuralized ES cells and embryonic brain regions. (A) PCA of the gene expression profiles of 15 DIV ES cells and E15 brain regions. The first three components, whose values are indicated in brackets, were plotted in *X*, *Y*, and *Z* axis, respectively. (B–D) show the gene expression fold-change between cell culture conditions (gray bars) or between brain regions (black dots), as indicated in labels and in schematic drawings. Comprehensive lists of genes showing fold-change higher than 2 in the different brain regions are reported in Supporting Information Table 1.

effect [see Fig. 5(H,I)]. The exact mechanism by which the two signals act in a cooperative manner on the genetic program of telencephalic specification remains to be elucidated.

Identitity of Neurons Generated by ES Cells

BMP/Wnt double inhibition was capable to induce massive anteriorization of ES-derived neural progenitors, since 69% of cells were FoxG1 positive at 10 DIV [Fig. 5(F)]. The nature of these telencephalic cells was mainly dorsal, as 66.4% of them were Pax6-positive [Fig. 5(E)] and 87% of them differentiated at 18 DIV as glutamatergic neurons labeled by vGlut2 (Supporting Information Fig. 5), which *in vivo* derive from the dorsal telencephalic aspect. Accordingly, ventral Nkx2.1-positive cells at 10 DIV were almost absent (0.2%, Supporting Information Fig. 3B) and the ratio of GAD2-positive neurons at 18 DIV, which *in vivo* originate from the ventral developing pallium, was low (8%, Supporting Information Fig. 6). These observations are consistent with very low expression of Shh until 10 DIV (Fig. 2) and suggest a dorsal default identity of ES-generated neural progenitors. Nonetheless, progenitors were competent to respond to Shh signaling, as SAG treatment at Step-II induced Nkx2.1 expression in 84% of cells (Supporting Information Fig. 3C). It is still to be established why 8% of cells became GAD2-positive GABAergic neurons. We speculate that this fraction derived from late neurogenesis occurring after 10 DIV and induced to a ventral identity by endogenous Shh signaling, which starts at 10 DIV (Fig. 2). In fact, after 10 DIV a fraction of cycling progenitors still remains in our cultures [see Fig. 3(B)], which might have been responsible for the generation of GAD2-positive neurons due to the effect of late Shh signaling.

Ventral default identity of ES-derived neural progenitors was described by Gaspard et al. (Gaspard et al., 2008). In their work, mouse ES cells cultured at low density in a minimal medium spontaneously neuralized and turned on ventral telencephalic markers. The inhibition of Shh signaling by Cyclopamine treatment was sufficient to shift the expression from ventral to dorsal markers and induced a cortical fate. The acquisition of a telencephalic identity is in line with similar reports of monolayer cultures at low

density (Tropepe et al., 2001; Smukler et al., 2006). Moreover, it may be still consistent with our findings, if we consider that the dilution of posteriorizing ligands such as BMP and Wnt, due to low plating density in minimal culture medium used by Gaspard et al., might have highly weakened their signaling. However, the need of Shh signaling inhibition to support dorsal identity is apparently inconsistent with our observations. We assume that this divergence is due to different culture conditions. In fact, compared to our protocol, the protocol of neuralization followed by Gaspard et al. used a low seeding density of the initial ES cell plating (5000 vs. 40,000 cells per square cm), which might be responsible of a higher and earlier Shh expression. We thus hypothesize that in the protocol of Gaspard et al. most of neural patterning occurred in the presence of high endogenous Shh signaling, thus resulting in ventral identity.

All in all, our observations suggest that inhibiting BMP and Wnt signaling in mouse ES cells neuralizing in minimal medium is sufficient to initiate a program of dorsal /telencephalic differentiation. This is consistent with the "Activation-Transformation" model in which, during neural induction (Activation), cells would acquire an anterior fate that would be maintained in the absence of signals inducing posterior Transformation (see Introduction). In addition, we propose that Activation would specify dorsal identity as default, unless ventralizing signals such as Shh are expressed (see earlier). This view is coherent with a genuine parallelism between early steps of ES cells neuralization *in vitro* and neural patterning *in vivo*.

In Vitro Corticogenesis

To induce cortical fate, some procedures made use of a feeder layer of stromal cells (Ideguchi et al., 2010), or cell aggregation (Eiraku et al., 2008). In these studies, the factors that were endogenously produced by cells in culture and that might have influenced ES cell differentiation were not identified. In fact, the multicellular and multidimensional nature of these cultures might complicate the study of the signals involved in the acquisition of a particular neuronal identity. Gaspard et al. showed that ES cell cultured in minimal medium at low density in the presence of Shh inhibitor Cyclopamine generated cells with morphological, electrophysiological and molecular features of cortical projection neurons (Gaspard et al., 2008). Notably, Gaspard et al. showed that the sequential activation of key transcription factors specifying early neurons of deep layers first (Tbr1 and Ctip2), and then late neurons of superficial layers (Cux1 and Satb2), occurs as observed *in vivo*. This result suggested that neural progenitors specified toward a cortical identity require neither a tridimensional environment nor spatially organized signaling to orchestrate the production of distinct types of cortical cells. The same conclusion came from pivotal works demonstrating that isolated cortical progenitors seeded at clonal density in primary cultures generated lineages whose composition, in terms of different types of cells and their respective birthdates, were mimicking those of cortical lineages *in vivo* (Qian et al., 2000; Shen et al., 2006; Slater et al., 2009).

Working with monolayer cultures of ES cells at subconfluent density, we confirmed the capability of ES-derived progenitors with a dorsal/telencephalic identity to express *in vitro* markers of cortical development. In fact, Wnt-BMP double-inhibited ES cells undergo sequential activation of Tbr1-Ctip2-Satb2 transcription factors [Fig. 6(G)]. Moreover, they differentiate predominantly as vGlut2-positive projection neurons. Global gene expression profiling of 15 DIV neurons derived from ES cells helped to investigate cell identity more in deep. PCA analysis indicated that the profile of neurons derived from ES cell cultures is quite distant from the profiles of the embryonic regions. We think that this is an expected result for two reasons: (i) neuronal cultures are much less complex cell populations than embryonic brain tissues; (ii) as it is difficult to know the exact correspondence between cell cultures and embryonic tissues in terms of developmental time, a gross part of gene expression diversity between them might by due to difference in proliferation, terminal differentiation, and maturation of differentiated cells. Nonetheless, PCA indicates that Wnt-BMP double inhibition steers ES cell identity more toward isocortex and hippocampus than toward ventral telencephalon or midbrain. Moreover, the analysis of gene expression fold-change shows a robust coherence between Wnt-BMP double inhibited cells and isocortex. Although an exact isocortical or hippocampal identity cannot be ascertained, our findings suggest a general cortical identity of Wnt-BMP double inhibited cells.

Further experiments, including assays of neuronal activity and *in vivo* grafting, are required to confirm real identity and potentials of the neurons generated by ES cells after BMP/Wnt double inhibition. However, due to the shorter time required for differentiation and the higher amount of neurons produced compared to the approaches discussed earlier, our method might be preferable for studies aimed to elucidate the molecular mechanisms regulating the production of different types of cortical projection neurons, and to produce cortical projection neurons suited for experiments of cell therapy.

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