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STEM CELLS AND REGENERATION

RESEARCH REPORT

BMP4 patterns Smad activity and generates stereotyped cell fate organization in spinal organoids

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ABSTRACT

Bone morphogenetic proteins (BMPs) are secreted regulators of cell fate in several developing tissues. In the embryonic spinal cord, they control the emergence of the neural crest, roof plate and distinct subsets of dorsal interneurons. Although a gradient of BMP activity has been proposed to determine cell type identity *in vivo*, whether this is sufficient for pattern formation *in vitro* is unclear. Here, we demonstrate that exposure to BMP4 initiates distinct spatial dynamics of BMP signalling within the self-emerging epithelia of both mouse and human pluripotent stem cell-derived spinal organoids. The pattern of BMP signalling results in the stereotyped spatial arrangement of dorsal neural tube cell types, and concentration, timing and duration of BMP4 exposure modulate these patterns. Moreover, differences in the duration of competence time-windows between mouse and human account for the species-specific tempo of neural differentiation. Together, this study describes efficient methods for generating patterned subsets of dorsal interneurons in spinal organoids and supports the conclusion that graded BMP activity orchestrates the spatial organization of the dorsal neural tube cellular diversity in mouse and human.

KEY WORDS: Bone morphogenetic proteins, Patterning, Organoid, Relay and associating spinal dorsal interneurons, Pluripotent stem cells differentiation, Self-organization

INTRODUCTION

The rise of methods to generate pluripotent stem cell (PSC)-derived organoids containing multiple cellular subtypes arrayed in three-dimensional (3D) structures has opened new avenues to decipher the principles underlying the emergence of patterns of differentiation (Huch et al., 2017; Trujillo and Muotri, 2018). These patterns are likely to be established in response to chemical and mechanical cues applied through culture medium and generated by the endogenous cellular diversity. However, the mechanisms by which organoid cells interpret these cues and acquire specific cell fates largely remain to be determined.

To gain insight into these mechanisms, we focused on cellular subtypes generated in the dorsal part of the embryonic spinal cord. The spinal cord originates from the caudal lateral epiblast (CLE) that transits through a pre-neural (PNP) state before acquiring a neurogenic progenitor (NP) state (Henrique et al., 2015). Acquisition of the CLE and PNP states depends on the combined activity of fibroblast growth factor (FGF) and Wnt signalling, while the transition to the NP state is promoted by retinoic acid (RA) signalling. NPs are then directed towards specific neurogenic programmes, depending on their position along the dorso-ventral (D-V) axis of the neural tube (Fig. 1A) (Kalcheim, 2018; Lai et al., 2016). In the dorsal neural tube, NPs comprise six discrete cell types, named from dorsal to ventral as dp1 to dp6 NPs, as well as a group of very dorsal cells that gives rise to neural crest cells (NCCs) and the roof plate (RP) (Fig. 1A). dp4 to dp6 NPs differentiate into five associating interneuron (IN) subtypes: the early born dI4 to dI6 and the late born dILA and dILB. dp1 to dp3 NPs generate dI1 to dI3 relay INs. These neurons form functional circuits, in which associating INs transmit the information coming from NCC-derived peripheral sensory neurons to relay sensory spinal IN that in turn convey this information to the brain.

The commitment of NPs towards these dorsal fates is determined by specific combinations of transcription factors (TFs) that display stereotypic temporal and spatial expression profiles (Fig. 1A) (Kalcheim, 2018; Lai et al., 2016). The profiles of TFs that mark the NCCs, RP and dp1 to dp3 NPs are, in part, generated in response to gradients of diffusing bone morphogenetic proteins (BMPs) (Kalcheim, 2018; Le Dréau and Martí, 2013; Zagorski et al., 2017). Classical developmental studies led to the model in which the concentration, duration and timing of exposure to BMP are interpreted by PNP and NP cells, guiding them towards a specific fate. Exposing PNP cells explanted from chick embryos to increasing BMP4 concentrations or longer incubations triggers a progressive dorsalization of the generated cell types (Liem et al., 1995; Sasai et al., 2014; Tozer et al., 2013). The emergence of distinct cell fates through modulating BMP receptor activity within chick embryo NPs indicated that *in vivo* BMP signalling levels may also provide positional information (Timmer et al., 2002; Zechner et al., 2003). Similarly, inhibiting BMP signalling using the antagonist Smad6 at discrete developmental stages reinforced the idea that the duration of BMP signalling discriminates between dorsal cell types (Tozer et al., 2013). Moreover, PNP cells harbour time-regulated competence for dorsal fate specification in response to BMP: the ability of PNP cells to generate NCCs in response to BMP4 being limited in time (Nitzan et al., 2016; Sasai et al., 2014).

However, recent work based on PSC-derived spinal organoids has questioned the morphogenetic potential of BMP (Andrews et al., 2017; Gupta et al., 2018; Meinhardt et al., 2014; Ogura et al., 2018). Exposure to BMP triggered PNP or NP dorsalization in the organoids. However, regardless of the concentration of BMP used,

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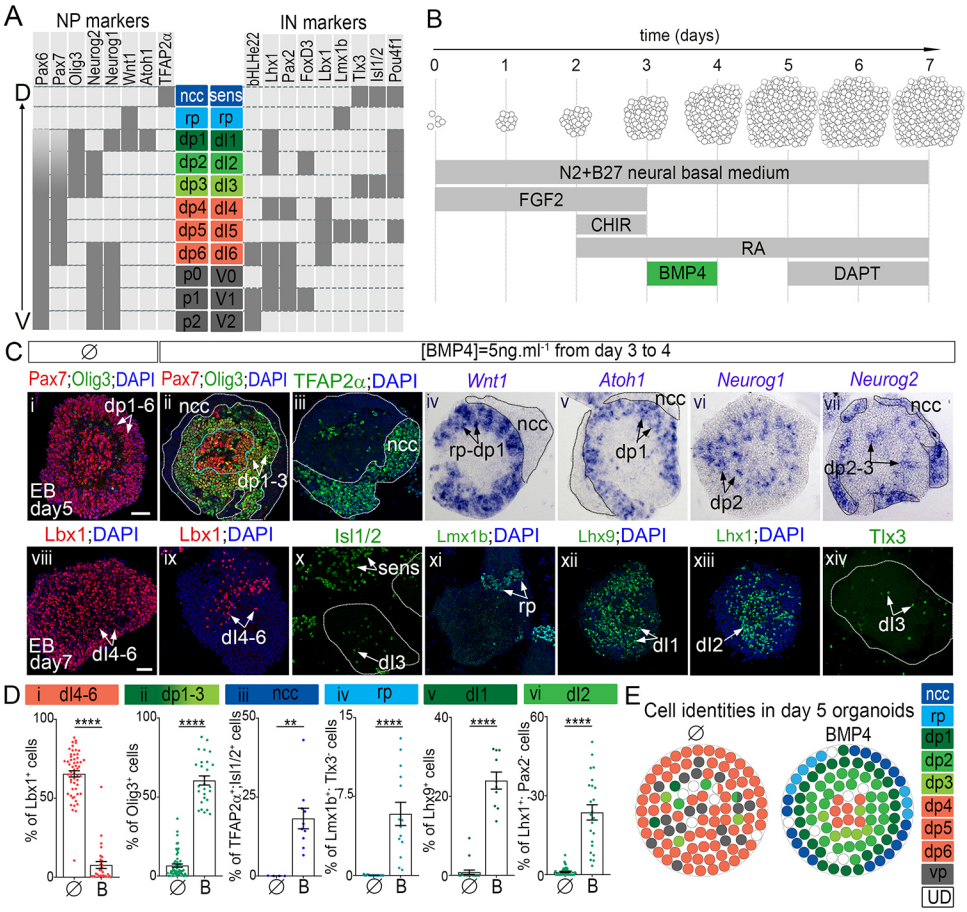


Fig. 1. BMP4 patterns mouse spinal organoids. (A) *In vivo* D-V expression patterns of neural progenitor (NP) and interneuron (IN) TF markers. (B) Schematics of differentiation conditions that generate dorsal spinal cellular subtypes in EBs. (C) Immunodetection (i-iii,viii-xiv) and *in situ* hybridization (iv-vii) for the indicated cell types. White and black outlines in ii-v,vii surround the NCC territory; blue outline in ii indicates cells less responsive to BMP4; white outlines in x and xiv indicate the EB contour. (D) Percentage of cell types harbouring the indicated TF code per image field (individual values; data are mean ±s.e.m.). $^{**}P\leq0.01$ and $^{****}P\leq0.0001$. (E) Graphs displaying the distribution and percentage of cell subtypes in day 5 organoids; white circles indicate cells with undetermined fate (UD). sens, peripheral sensory neurons. Scale bars: 60 µm.

several dorsal neural subtypes were generated in a non-organized fashion. This raised the possibility that BMP acts as a permissive, rather than an instructive, signal for the acquisition of dorsal cell fates (Andrews et al., 2017) and that additional signals or mechanisms are responsible for the *in vivo* spatial organization.

To tackle this issue, we have established robust embryoid body (EB)-based protocols to drive mouse or human PSCs into PNP cells that acquire a dp4 to dp6 state. In these EBs, BMP4 induces concentric patterns of several dorsal neural tube cell types that are reminiscent of those found along the D-V axis of the embryo. These patterns depend on the activity of the position-specific temporal profiles of the BMP transcriptional effectors Smad1, Smad5 and Smad9 within the emerging organoid epithelia. Varying the concentration, duration and timing of exposure to BMP4 modifies the cell types generated within these patterns. Furthermore, our data on human organoids revealed that, across evolution, the length of time windows for which PNP and NP cells are competent to generate discrete cell fates in response to BMP4 have been adapted to match species-specific temporal sequences of neural differentiation.

RESULTS AND DISCUSSION

Efficient generation of associating INs within spinal organoids

In order to generate dorsal spinal neurons in organoids derived from mouse embryonic stem cells (ESCs), we have adopted 3D EB-based differentiation and adapted culture conditions used for ventral spinal cells generation (Gouti et al., 2014; Maury et al., 2015; Wichterle et al., 2002). EBs were produced in neural basal medium supplemented with FGF2 for the first 3 days of culture and CHIR (CHIR99021), a GSK3 inhibitor, was used to activate Wnt signalling between day 2 and 3 (Fig. 1B). Many day 3 EB cells treated with these two compounds adopted a mesodermal fate, which could be prevented by adding the neuralizing cue RA on day 2 (Fig. 1A,B,D; data not shown) (Beccari et al., 2018; Henrique et al., 2015). CHIR drove the NPs towards caudal $Cdx2^+$ fate (Henrique et al., 2015), while FGF2 further caudalized the state of cells, as shown by the induction of brachial markers such as *Hoxc6* (Fig. S1C,D,E) (Liu et al., 2001). In line with *in vivo* studies (Alvarez-Medina et al., 2008; Lee and Deneen, 2012; Valenta et al., 2011; Zechner et al., 2003), CHIR-mediated Wnt signalling activation favoured dorsal fate acquisition, with 80% of cells exhibiting a dp4 to dp6 molecular identity (Fig. 1Ci,E, Fig. S2A,Bi-iii,C). Accordingly, at day 7, following a 48 h-long treatment with Notch signalling inhibitor DAPT (Fig. S3A), EBs contained mainly neurons expressing typical markers of the dI4 to dI6 associating INs (Fig. 1Cviii,Di, Fig. S3B,C). Thus, the identified combination of FGF2, Wnt agonist, RA and DAPT rapidly and efficiently produces mouse ESC-derived organoids containing brachial spinal associating progenitors (day 4, 5) or neurons (day 7) (Andrews et al., 2017; Meinhardt et al., 2014).

Concentric patterns of dorsal neuronal cell types formed in response to BMP4 exposure

We next examined the effects of BMP4, a BMP ligand implicated in neural patterning (Le Dréau and Martí, 2013), by exposing day 3 NPs to 5 ng ml^{-1} BMP4 for 24 h (Fig. 1B). Consistent with BMP4 being a 'dorsalizing' cue, it induced markers of NCCs, RPs, relay dp1/dI1 and dp2/dI2 cells (Fig. 1Cii-vii,ix-xiv,Dii-vi, Figs S2Biv-viii‴,C,Di, S3B). In day 4 and 5 EBs, these markers displayed

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stereotyped concentric patterns of expression organized along the outer-inner axis of the organoid, with their relative position matching the one found along the D-V axis of the neural tube (Fig. 1Cii-vii,E, Fig. S2Bviii). At day 7, the sensory neurons produced from NCCs in the outer ring detached in part from the EB to colonize the dish plate (Fig. 1Cx, not shown). RP cells clustered at the EB periphery and were surrounded by dI1 INs (Fig. 1Cxi, Fig. S3B). Inside the EB, dI2 and a few dI4-6 associating INs were intermingled (Fig. 1Cix, xiii). Altogether, these data demonstrate that, in addition to creating cellular diversity, BMP4 directs spatial arrangement so that the most dorsal cell types are found at a more peripheral position than the ventral ones (Fig. 1E). As shown in the developing spinal cord (Lai et al., 2016), this organization attenuates over time owing to NCC and post-mitotic IN delamination. This may explain why the patterning activity of BMP has not been noticed in previous studies as organoids were analysed solely for terminally differentiated neurons (Andrews et al., 2017; Gupta et al., 2018; Ogura et al., 2018).

Spatial and temporal dynamics in Smad1, Smad5 and Smad9 activity triggered by BMP4 exposure

We next sought to investigate whether BMP4-mediated EB patterning could stem from differential spatial and temporal activation of its intracellular downstream effectors. For this, we monitored the levels of the phosphorylated forms of the TFs Smad1, Smad5 and Smad9 (PSmads) from day 3 to 4 in presence or absence of BMP4 (Fig. 2A-C, Fig. S4).

At all time-points, PSmads^{+} cells formed a spatially restricted, three to five cell wide ring, at the periphery of EBs. In contrast, cells deeper in the organoid were devoid of PSmads signal (Fig. 2Avi-x, B, Fig. S4A). This organization could be recapitulated by exposing EB to BMP2 (Fig. S6), but such a spatial restriction in the cell response to signalling cues was not observed for other compounds (not shown) (Maury et al., 2015). This response to BMP was not due to a difference in competence between the outer and inner cells, as single cells obtained from dissociated day 3 EBs homogeneously activated Smads in response to BMP and all cells acquired a NCC state later on (Fig. 2E). This sharp spatial organization could thus emerge from the limited rate of BMP diffusion (Kicheva et al., 2007; Pomreinke et al., 2017; Zinski et al., 2017) or stem from the emergence of a specific tissue organization within EBs. Previous work has demonstrated that the extracellular matrix separating an outer epithelium from inner epithelia can act as a barrier that hampers BMP diffusion (Hu et al., 2004; Ma et al., 2017; Perrimon et al., 2012; Plouhinec et al., 2013; Ramirez and Rifkin, 2009; Wang et al., 2008) (Fig. 2D,F). The emergence of epithelia was revealed by analysing the distribution profiles of Par3, a member of apical protein complexes, and of laminin, a component of the basal lamina (Fig. 2D, Fig. S5i-iv). PSmads^{+} cells delineated exactly the outer epithelium whose apical side faced the culture medium (Fig. 2D,F).

The analysis also revealed that P-Smads levels within the outer epithelium varied in space and time. Smads activation displayed a decreasing inward gradient across this epithelium (Fig. 2B, Fig. S4B). The gradient amplitude decreased progressively over time (Fig. 2C), except in the outer-most layer of NCC where, after the initial decline, Smads activity rose again (Fig. 2Ax,C,F, data not shown). The temporal adaptation of BMP signalling was confirmed by the progressive decrease in the levels of BMP4 target genes (Fig. 2Axv, Fig. S2Dii). Negative feedback mediated by the BMP antagonist noggin could be at stake, as its gene was transcriptionally induced 3 h after BMP4 addition, when PSmads levels had declined (Fig. 2Axi-xv) (Brazil et al., 2015). Progressive depletion of

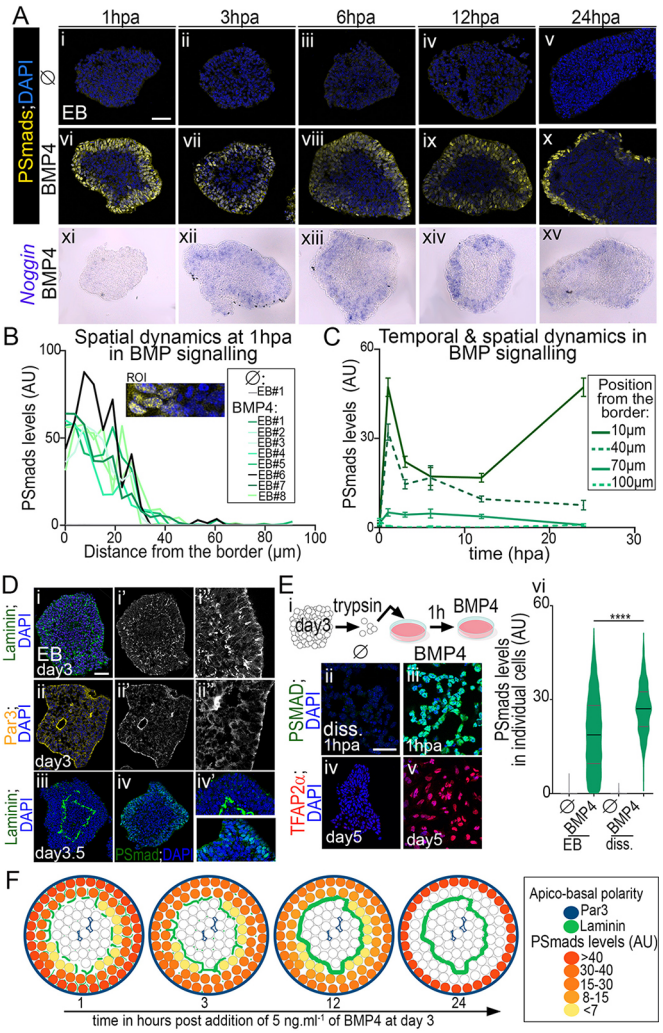


Fig. 2. Spatial and temporal dynamics of PSmads in response to BMP4. (A) Immunodetection of phospho-Smad1, Smad5 and Smad9 (PSmads), and DAPI labelling (i-x) and *in situ* hybridization against Noggin (xi-xv) in EBs cultured without or with 5 ng ml^{-1} BMP4 (added from day 3) and harvested several hours post-BMP4 addition (hpa). (B) Gradients of PSmads are measured in eight independent regions of interest (ROI; inset) 1 hpa. (C) Temporal and spatial dynamics in PSmad levels in cells at discrete distances from EB borders (data are mean±s.e.m.). (D) Laminin, Par3 and PSmad immunodetection, and DAPI labelling in day 3 and 3.5 EBs. (i′,ii′) Laminin and Par3 staining; (i″,ii″) higher magnification of i′,ii′. (E) (i) Schematics of experimental procedure used to assess the fate of dissociated cells from day 3 EBs exposed to 5 ng ml^{-1} BMP4 1 h post-dissociation. (ii-v) Immunostaining of the indicated markers in dissociated cells 1 hpa (ii,iii) or at day 5 (iv,v). (vi) PSmad levels in EBs or dissociated cells 1 hpa (violin plot; data are mean±quartiles). ****P≤0.0001. (F) Graphs representing temporal and spatial dynamics in PSmad levels and polarity markers. Scale bars: 60 µm.

receptors from the cell surface represents an alternative explanation for temporal adaptation (Miller et al., 2019). Together, these data indicate that stereotyped temporal profiles of intracellular signalling are generated in response to BMP4, according to the location of the cell within the organoid (Fig. 2C,F). This spatially graded Smad activity provides an explanation for the inability of BMP4 to drive cells in organoids towards a unique cell type (Andrews et al., 2017; Gupta et al., 2018), which creates an organized cellular diversity. Furthermore, the match between this position-specific signalling dynamics and the emergence of distinct cell types supports a model where ‘positional information’ results from

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a combination of the levels and the duration of PSmads (Tozer et al., 2013).

Three tuneable morphogenetic parameters of BMP4 exposure discriminate between relay IN subtypes

To assess directly the morphogenetic potential of BMP4 in organoids, we sought to modulate the three main parameters known to influence cell response to morphogens (Sagner and Briscoe, 2017): the ligand concentration, the exposure duration and the time point at which the ligand is added (Figs S7 and S8).

Increasing BMP4 concentration increased the number of PSmads^{+} cells (Fig. S7Avi), the mean levels in PSmads (Fig. S7Avii) as well as the duration for which Smads were active (see insets in Fig. S7Aii-v). Similarly, the duration of BMP4 exposure altered the temporal dynamics of signalling. Nuclear PSmads levels fell 6 h after BMP4 removal (Fig. S7B), arguing against a long-term memory of intracellular signalling by cells exposed to BMP (Tozer et al., 2013). Importantly, the modulation of PSmad dynamics by increasing BMP4 concentration or duration extended the total amount of BMP dependent cell-types generated and promoted more dorsal cell types at the expense of more ventral ones (Fig. 3A,C,D, Fig. S8A,B).

Changing the time at which EBs were exposed to BMP4 also had strong effects on cell identities (Fig. 3B, Fig. S8C and compare Fig. 1C,D to Fig. 3D). Cells displayed a 12 h competence time window for generating specific BMP4-dependent cell types, the most dorsal cell types requiring an earliest time of exposure than more ventral ones (Fig. 3Bii-vii). This is in agreement with the limitation in time for chick spinal NP to become NCCs in response to BMP4 (Sasai et al., 2014). Importantly, the switches in cell competence were not due to alteration in the ability of a cell to transduce BMP4 information intracellularly. The amplitude (Fig. S7Cvi), gradient (insets in Fig. S7Cii-iv) and temporal adaptation (not shown) of PSmad profiles remained invariant upon shifts in the timing of BMP4 exposure. Thus, the competence could stem from changes in the molecular state of NPs during the course of their differentiation (Fig. S2Bi-vi) (Sasai et al., 2014).

Finally, in agreement with concentration, duration and timing of exposure being morphogenetic parameters (Sasai et al., 2014; Tozer et al., 2013), we determined conditions needed to generate organoids containing a dominant relay IN subpopulation at their periphery (Fig. 3C,D), including dI2 and dI3, which were poorly generated in previous protocols (Andrews et al., 2017).

Species-specific changes in NP competence time windows are related to the evolution of neuronal differentiation temporality

Dynamics of neural differentiation and tissue sizes are highly variable between species (Ebisuya and Briscoe, 2018), raising the issue of whether morphogen interpretation is modulated to scale

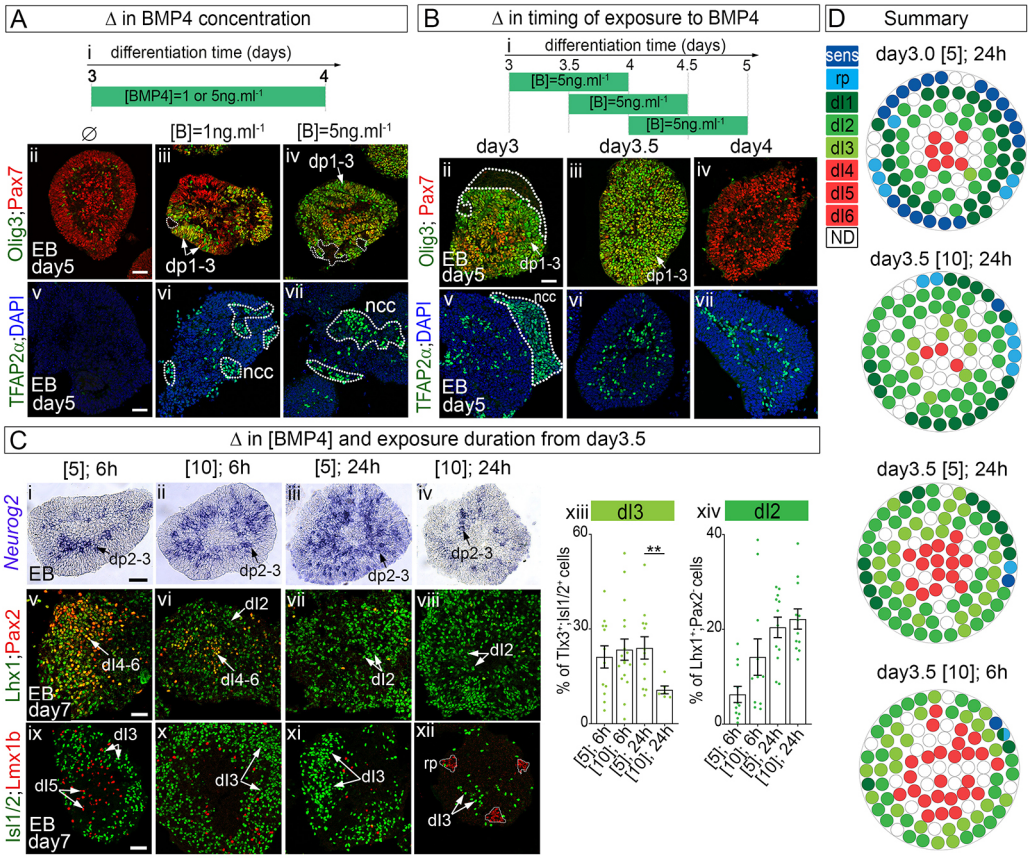


Fig. 3. Morphogenetic parameters of BMP4 exposure generate specific relay IN subtypes. Phenotypic characterization of EBs upon the modulation of BMP4 concentration (A), exposure timing (B) and both BMP4 concentration and exposure duration when adding BMP4 at day 3.5 (C). (Ai,Bi) Schematics indicating BMP4 exposure conditions. (Aii-vii,Bii-vii,Cv-xii) Immunostaining for the indicated markers in EBs grown under the indicated conditions at day 7. (Ci-iv) *In situ* hybridization for Neurog2 in day 5 EBs cultured under the indicated conditions. (Cxiii,xiv) Percentage of dI2 and dI3 INs harbouring the indicated TF code per image field (individual values; data are mean±s.e.m.). $^{**}P \leq 0.01$. (D) Graphs displaying the distribution and percentage of cell subtypes in day 7 organoids cultured under the indicated conditions. Scale bars: 60 µm.

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with these dynamics. To address this, we modified our protocol to generate ventral spinal neurons from human iPSCs (Maury et al., 2015) (Fig. 4Ai). As for mouse ESCs, removing the Shh agonist SAG was sufficient to generate spinal organoids containing mostly dp4-dp6 NPs at day 9 of differentiation and associating neurons at day 14 (Fig. 4Aii,iii). Importantly, although both human and mouse dorsal spinal organoids contained similar number of cells (not shown), the time taken to generate these cells in human was around ∼2.5 times longer. Hence, as for motor neuron generation, the speed of differentiation during dorsal differentiation varies greatly between vertebrate species (Maury et al., 2015; Wichterle et al., 2002).

Exposure of hiPSC-derived organoids to BMP4 revealed that the response of human cells is similar to that of mouse cells. Smad activation was restricted to the outer layer of cells, which was separated from more inner cells by a basal lamina (Fig. 4B, Fig. S5v-viii). PSmad levels were higher in the most peripheral cells and were decreased within the width of the epithelium (Fig. 4Bi,i′). Accordingly, different cell types were produced in a concentric manner, with the most-dorsal subtypes located at a more peripheral position than the ventral subtypes (Fig. 4D). Finally, both BMP4 concentration and exposure duration influenced the proportion of the BMP-dependent cell types produced (Fig. 4D,E). As in mouse, human NPs displayed competence time windows for the generation of NCCs versus dp1-dp3 cells (Fig. 4C). Yet these windows lasted about twice as long as they did in mouse (compare Fig. 4C with 3B) and paralleled the species-specific time frames of neuronal differentiation. This supports the idea that competence is a consequence of sequential transitions in the transcriptional states of differentiating NPs that occur at slower pace in humans (Sasai et al., 2014).

Together, using efficient and rapid protocols for the differentiation of human and mouse PSC in organoids containing specific subsets of associating and relay spinal neurons, we revealed key parameters for BMP4-mediated tissue patterning. First, 3D polarized epithelia emerging within the organoids participate in the generation of stereotyped profiles of Smad activity and of discrete subsets of cellular subtypes. Their emergence constrains the spatial distribution of not only proteins controlling ligand diffusion, but also of the basolaterally located BMP receptors, which together are likely to generate spatial and temporal dynamics in BMP intracellular signalling (Etoc et al., 2016; Miller et al., 2019; Pomreinke et al., 2017; Wang et al., 2008; Zhang et al., 2018preprint). Second, as shown for a great number of morphogens (Sagner and Briscoe, 2017), the timing of differentiation, i.e. the molecular state of the receiving cells, appeared to be the most constraining parameter for the response to BMP4, with species specificities. In terms of bioengineering of BMP-dependent cell

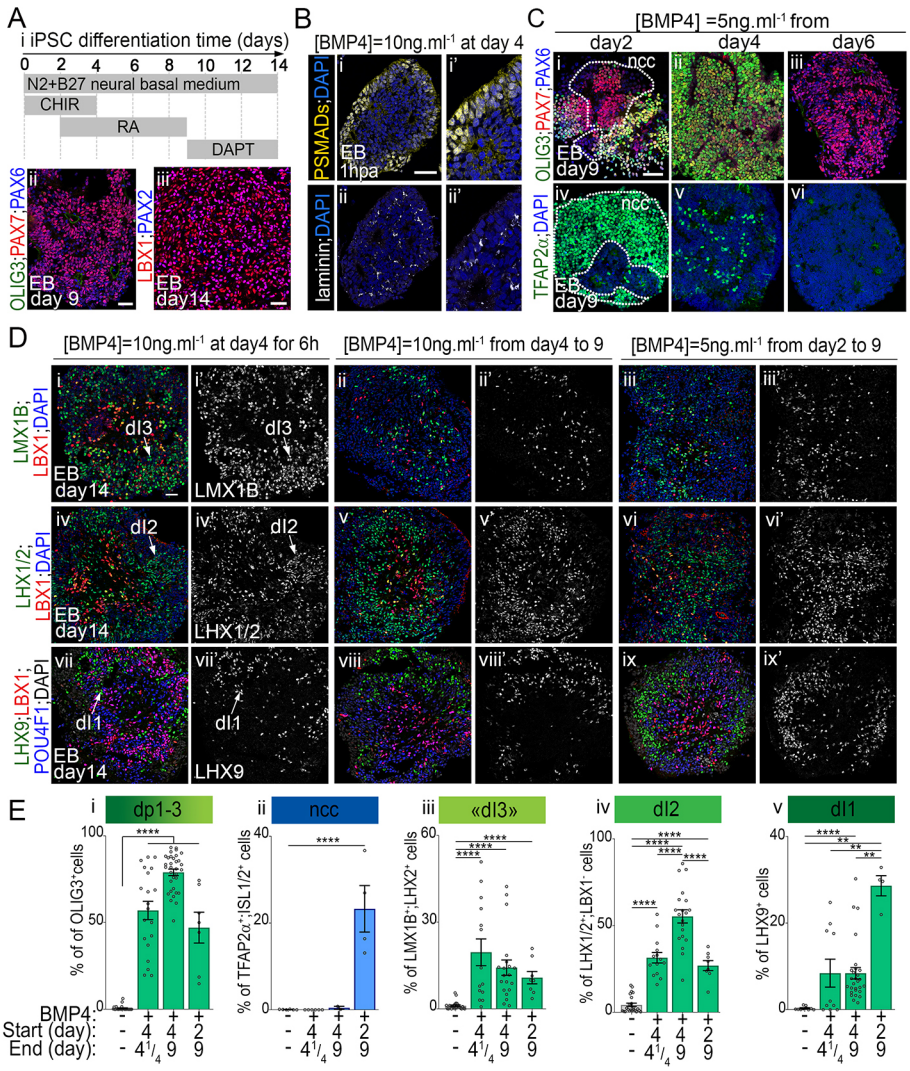


Fig. 4. Morphogenetic effects of BMP4 exposure on human iPSC-derived spinal organoids. (A) Generation of associating progenitors and IN from human iPSCs. (Ai) Schematics indicating drug treatments to convert human iPSCs into dorsal spinal cells. (Aii,iii) Immunostaining for the indicated NP (ii) and IN (iii) markers in day 9 and 14 organoids. (B) Immunodetection of pSmads or laminin and DAPI labelling in day 4 organoids treated for 1 h with BMP4. (C) Immunostaining for the indicated NP markers in day 9 EBs grown in presence of BMP4 added at the indicated time points and maintained up to day 9. (D) Immunostaining for the indicated post-mitotic markers in day 14 EBs grown under the indicated conditions. (E) Percentage of IN subtypes harbouring the indicated identities (individual values; data are mean±s.e.m.). $^{**}P\leq0.01$ and $^{****}P\leq0.0001$. Scale bars: 60 µm.

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types for basic and translational perspectives, our results call for the elaboration of new diffusible BMP pathway activators to generate homogenous populations of cells and for a precise temporal control of morphogen signalling with drug treatments.

MATERIALS AND METHODS

Cell line maintenance and differentiation

Mouse ESC line HM1 (Selfridge et al., 1992) at passages ranging from 15 to 19 were maintained on mitotically inactive primary mouse embryo fibroblasts in EmbryoMax D-MEM supplemented with 10% ESC-qualified foetal bovine serum (Millipore), L-glutamine, non-essential amino acid, nucleosides, 0.1 mM β-mercaptoethanol (Life Technologies) and 1000 U ml^{-1} leukaemia inhibitory factor (Millipore). Human iPSC WTSIi008-A cell and WTSIi002-A line (EBISC, European Bank for Pluripotent Stem Cells) were cultured in E8 medium on vitronectin (Life Technologies) as previously described (Maury et al., 2015).

To initiate mouse ESC embryoid bodies differentiation, cells were trypsinized and placed twice onto gelatinized tissue culture plates to remove feeders. Cells (5×10^4 cells ml^{-1}) were placed in ultra-low attachment petri dishes (Corning) and in Advanced Dulbecco's Modified Eagle/F12 and Neurobasal media (1:1, Life Technologies) supplemented with 1× B27 devoid of vitamin A and 1×N2 (Life Technologies), 2 mM L-glutamine (Life Technologies), 0.1 mM β-mercaptoethanol, penicillin and streptomycin (Life Technologies). At this concentration of cells, small EBs were formed from day 1 of differentiation, and grown as such up to day 7 of differentiation; medium was changed every day from day 2 onwards. Human PSC EB differentiation was performed as described by Maury et al. (2015), except the medium did not contain LDN193189 or SB431542. Cells (1.5×10^5 cells ml^{-1}) were seeded in ultra-low attachment six-well plates (Corning). Medium was changed on days 2, 4, 7, 9 and 11 of differentiation. Chemical drugs to inhibit or activate key developmental signalling pathways were used at the following concentrations: 10 ng ml^{-1} bFGF (FGF2, R&D), 3 µM CHIR99021 (Tocris or Axon Medchem), 10 nM retinoic acid (Sigma), 1 ng ml^{-1} to 15 ng ml^{-1} BMP4 (R&D), 10 µM DAPT (Stemgent). Figs 1B and 4A depict the time frames over which these drugs were applied to mouse and human EBs, respectively. Human iPSC experiments were declared (CD-2015-2559) and approved by local ethical Committee (CPP A95).

Dissociation of mouse ESC-derived neuroprogenitors for monolayer culture

EB at day 3 of differentiation were incubated with 0.05% trypsin-EDTA for 3 min. After fetal bovine serum (FBS) trypsin inactivation they were dissociated by pipetting and filtered (40 µm pore size). Single cell suspensions were plated at 10^5 cells $well^{-1}$ in a 24-well plate onto a glass coverslip coated with 20 µg ml^{-1} poly-ornithine (Sigma) and 5 µg ml^{-1} laminin (Life Technologies). Cells were allowed to adhere 1 h before the addition of fresh differentiation medium containing BMP4.

Expression analyses

RT-qPCR

Total RNA was extracted from 50 to 250 EBs collected at different time points using the NucleoSpin RNA kit (Macherey-Nagel) following manufacturer's instructions. cDNAs were synthetized using SuperScript IV (Thermo Fisher Scientific), random primers and oligo dT. For real-time quantitative PCR (RT-qPCR), SYBR Green I Master (Roche) and the LightCycler 480 II (Roche) were used. PCR primers were designed using Primer3 software (Table S1). Levels of expression per gene for a given time point was measured in biological duplicates or triplicates. Mouse gene expression levels were expressed relatively to TATA-box binding protein (TBP) mRNA levels and normalized to the expression in either GD11.5 dissected spinal cord or mouse ESCs.

Immunofluorescence and *in situ* hybridization

EB fixation, embedding and cryosectioning have been described previously (Maury et al., 2015), so have the immunolabelling and *in situ* hybridization protocols (Briscoe et al., 2000; Yamada et al., 1993). Details of the antibodies are provided in Table S2. Analyses were carried out using a Leica TCS SP5 confocal microscope or a Zeiss Axioplan 2, and images processed with Photoshop 7.0 software (Adobe Systems) or ImageJ v.1.43g image analysis software (NIH).

Quantification

The number of cells immunolabelled was calculated using Cell Profiler (Broad institute) after nuclei segmentation based on DAPI fluorescence signal with a signal intensity threshold and was expressed as the percentage of all detected nuclei. The EB area labelled by *in situ* hybridization probes was estimated using ImageJ v.1.43g image analysis software (NIH) and was expressed as the percentage of the whole EB surface. For each condition, these quantifications were performed on a minimum of five images per experiment and on a minimum of two independent experiments. Percentage of all cellular subtypes analysed were represented using a dot plot shaped as a circle; the position of the cellular subtypes reflected our observations. Levels of PSmad fluorescent signal intensity per cells were evaluated using Cell Profiler by subtracting background values from EBs that were not treated with BMP4. Violin plots were used to show the spread of these levels in five images per experiments and on a minimum of three independent experiments. The fluorescence intensity of PSmad expression along the EB outer-inner axis was measured in rectangles 16 µm wide and 100 µm long positioned perpendicular to an EB tangent line using ImageJ v.1.43g. Background measurements were obtained from EBs not treated with BMP4 and these were subtracted from each assayed profile. Statistical analysis was carried out and graphs were created using Prism Graphpad software. Non-parametric *t*-tests were used to evaluate pair-wise comparisons between conditions. *P*-values are: $*P\leq0.05$, $**P\leq0.01$, $***P\leq0.001$ and $****P\leq0.0001$.

Whole-mount immunolabelling, 3D scans and image processing

EBs fixed in 4% paraformaldehyde and rinsed with PBS were incubated in a blocking solution of 0.5% Triton X-100 and 1% BSA for 10 h. They were then incubated for 48 h with anti-PSmad antibodies (Table S2). After overnight washes in PBS and 0.5% Triton X-100, they were incubated overnight with a secondary antibody coupled to Alexa A488 (Table S2). After 1 day of washes in PBS and 0.5% Triton X-100, they were mounted in Vectashield mounting medium (Vector) between coverslips and a glass slide. They were imaged using a Leica TCS SP5 confocal microscope and the image segmentation and signal quantification were processed using Imaris (Bitplane). Notably, surfaces delimitating PSmad-positive nuclei in the EBs were defined using the surface segmentation tool (Fig. S4Ai-i″). Segmentation of PSmad-positive cells was established and the mean intensity values colour coded using the spot detection tool (Fig. S4B). All observations were carried out on at least seven EBs.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.D., S.N., V.C.R.; Methodology: N.D., C.V., T.C.B., Y.F., X.B., S.N., V.C.R.; Software: V.C.; Validation: N.D., C.V., T.C.B., V.C.R.; Formal analysis: N.D., C.V., T.C.B., Y.F., V.C., V.C.R.; Investigation: N.D., C.V., T.C.B., V.C.R.; Writing - original draft: V.C.R.; Writing - review & editing: N.D., C.V., S.N., V.C.R.; Visualization: V.C.; Supervision: S.N., V.C.R.; Funding acquisition: S.N., V.C.R.

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Supplementary information

Supplementary information available online at
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