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FGF Signaling Transforms Non-neural Ectoderm into Neural Crest

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Abstract

The neural crest arises at the border between the neural plate and the adjacent non-neural ectoderm. It has been suggested that both neural and non-neural ectoderm can contribute to the neural crest. Several studies have examined the molecular mechanisms that regulate neural crest induction in neuralized tissues or the neural plate border. Here, using the chick as a model system, we address the molecular mechanisms by which non-neural ectoderm generates neural crest. We report that in response to FGF the non-neural ectoderm can ectopically express several early neural crest markers (*Pax7*, *Msx1*, *Dlx5*, *Sox9*, *FoxD3*, *Snail2*, and *Sox10*). Importantly this response to FGF signaling can occur without inducing ectopic mesodermal tissues. Furthermore, the non-neural ectoderm responds to FGF by expressing the prospective neural marker *Sox3*, but it does not express definitive markers of neural or anterior neural (*Sox2* and *Otx2*) tissues. These results suggest that the non-neural ectoderm can launch the neural crest program in the absence of mesoderm, without acquiring definitive neural character. Finally, we report that prior to the upregulation of these neural crest markers, the non-neural ectoderm upregulates both BMP and Wnt molecules in response to FGF. Our results provide the first effort to understand the molecular events leading to neural crest development via the non-neural ectoderm in amniotes and present a distinct response to FGF signaling.

Keywords

Neural crest; FGF; non-neural ectoderm; induction; Pax7

Introduction

The neural crest (NC) is a multipotent cell population that arises at the border of the neural plate (NP) and non-neural ectoderm (NNE) during early vertebrate development. NC cells delaminate from the NP border (NPB) after undergoing an epithelial-to-mesenchymal transition and migrate along stereotypic pathways throughout the whole vertebrate body, contributing to a varied array of derivatives. NC cells produce the majority of the neurons and glia of the peripheral nervous system and melanocytes of the skin. Additionally, the cranial NC contributes to craniofacial bone and cartilage, smooth muscle, adipose tissue, and tooth-forming odontoblasts (as well as other derivatives) (Le Douarin and Kalcheim, 1999). NC

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occupy a prominent place in human biology because defects in their development lead to a wide range of health conditions and diseases such as cleft palate, Waardenburg's syndrome, and neuroblastoma, amongst many others (Bolande, 1997; Etchevers et al., 2006; Farlie et al., 2004)

In the last 20 years considerable progress has been gained regarding the molecular mechanisms underlying NC development. Interactions among signaling pathways, are thought to launch NC development via the induction of specific transcription factors at the border of the NP, referred to as border specifiers (Msx1/2, Dlx3/5, Pax3, and Pax7). The border specifiers, along with additional signaling, trigger the expression of later transcription factors known as NC specifiers (FoxD3, Snail2, Sox9, and Sox10) which further control the expression of NC-effector genes responsible for the migration and differentiation of NC cells (reviewed by Betancur et al., 2010; and Meulemans and Bronner-Fraser, 2005; and Stuhlmiller and García-Castro, 2012a).

In terms of inductive signaling, Bone Morphogenetic Proteins (BMPs), Fibroblast Growth Factors (FGFs) and Wnt molecules have dominated the focus of NC induction studies. A requirement for BMP molecules in this process has been supported through expression and functional studies in avian embryos (Liem et al., 1995; Selleck et al., 1998; Streit and Stern, 1999). Instead in *Xenopus*, assays of NC induction required BMP inhibition, and a threshold of BMP signaling was suggested to control NC development (Marchant et al., 1998; Tribulo et al., 2003). In zebrafish, however, loss of three BMP inhibitors does not prevent NC formation (Ragland and Raible, 2004). Wnt molecules are also thought to be critical for NC formation. In frogs, neuralized animal caps will express NC markers when treated with Wnts, and ectopic application of Wnts enhances NC marker expression in the NPB (Chang and Hemmati-Brivanlou, 1998; Hong et al., 2008; Hong and Saint-Jeannet, 2007; LaBonne and Bronner-Fraser, 1998; Monsoro-Burq et al., 2005; Saint-Jeannet et al., 1997; Sasai et al., 2001). Conversely, inactivation of Wnt signaling prevents NC formation at the NPB in *Xenopus* (Abu-Elmagd et al., 2006; Carmona-Fontaine et al., 2007; Deardorff et al., 2001; Heeg-Truesdell and LaBonne, 2006; Hong et al., 2008; LaBonne and Bronner-Fraser, 1998; Monsoro-Burq et al., 2005; Steventon et al., 2009). In chick, Wnts are sufficient to induce NC formation (García-Castro et al., 2002; Patthey et al., 2009), and they have been proposed to be necessary, as Wnt antagonists applied to the prospective NPB (*in vivo* and *in vitro*) prevent expression of NC markers such as Snail2 (Basch et al., 2006; García-Castro et al., 2002; Litsiou et al., 2005; Patthey et al., 2009; Patthey et al., 2008; Schmidt et al., 2007). While most evidence suggests an involvement of the Wnt/ β -catenin pathway on NC induction, reports from both *Xenopus* and chick embryos also support the participations of non-canonical Wnts (Ossipova and Sokol, 2011; Schmidt et al., 2007).

FGFs are also important during NC induction, although the exact mechanism appears to differ between model organisms. Several studies in *Xenopus* have shown that FGFs, specifically Fgf8, can trigger NC induction (Hong and Saint-Jeannet, 2007; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997; Mayor et al., 1995; Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005; Villanueva et al., 2002). It has been suggested that Fgf8 acts indirectly, by activating expression of Wnt8 in the mesoderm, which then induces NC in the overlying ectoderm (Hong et al., 2008). However, recent evidence from *Xenopus* suggests that FGFs can act directly on NC formation through activation of the Stat pathway (Nichane et al., 2010). In chick, recent evidence shows that FGF activity is required in the ectoderm during gastrulation, as a targeted blockade of FGF signaling at the NPB prevents expression of NC markers (Stuhlmiller and García-Castro, 2012b).

Our current understanding of the relationship between these three pathways in chick can be summarized in a two-step process characterized by an initial phase of FGF and Wnt

signaling and low or negative BMP signaling, followed by a second phase where Wnt and BMP activation are required to sustain NC development (Patthey et al., 2009; Stuhlmiller and García-Castro, 2012a ; Stuhlmiller and García-Castro, 2012b).

NC precursors appear in the region between the NP and the NNE, underlain by mesoderm, and these tissues have been proposed to be the source of inductive signals for NC formation. Studies in axolotl, *Xenopus*, and chick embryos have shown that juxtaposition of mesoderm and ectoderm induces NC (Mitani and Okamoto, 1991; Raven and Kloos, 1945; Selleck and Bronner-Fraser, 1995), yet evidence from zebrafish and *Xenopus* suggest mesoderm is dispensable for NC formation (Ragland and Raible, 2004; Wu et al., 2011). Experiments involving the juxtaposition of the NP and NNE have highlighted the relevance of interactions between these two during NC induction in amphibians (Mancilla and Mayor, 1996; Moury and Jacobson, 1990; Rollhäuser-ter Horst, 1979, 1980) and avians alike (Basch et al., 2000; Dickinson et al., 1995; Liem et al., 1995; Ruffins and Bronner-Fraser, 2000; Selleck and Bronner-Fraser, 1995; Streit and Stern, 1999). Collectively these studies support a model of classic induction in which the NNE signals to the NP which responds by generating NC.

Importantly, results from both *Xenopus* and chick suggest that the NNE is capable of producing NC. Experiments in *Xenopus* using animal caps show that NC are induced in response to BMP inhibition (Marchant et al., 1998; Tribulo et al., 2003), FGF activation (Monsoro-Burq et al., 2003), or the combination of BMP inhibition with Wnt or FGF activation (Chang and Hemmati-Brivanlou, 1998; Hong et al., 2008; Hong and Saint-Jeannet, 2007; LaBonne and Bronner-Fraser, 1998; Monsoro-Burq et al., 2005; Saint-Jeannet et al., 1997; Sasai et al., 2001). However some controversy exists over whether animal caps truly represent naïve ectoderm (Knecht et al., 1995; Lamb et al., 1993; Wills et al., 2010) or whether they actually represent a pro-neural state (Dale and Slack, 1987; Gamse and Sive, 2001; Linker et al., 2009; Sagerström et al., 2005). Experiments using fluorescently labeled grafts of NP into NNE of *Xenopus* embryos suggest that both NP and NNE can contribute to NC (Mancilla and Mayor, 1996). In the chick, NNE-NP juxtapositions have shown that NC markers and migratory NC can be found in cells derived from both NP and NNE tissue (Ruffins and Bronner-Fraser, 2000; Selleck and Bronner-Fraser, 1995; Streit and Stern, 1999). It was suggested that after contact with the NP, the NNE could directly form NC, or become neuralized first and then respond to adjacent NNE to make NC, or that perhaps the NP responded to NNE by generating cells of undefined identity which secondarily induced NNE to form NC (Selleck and Bronner-Fraser, 1995). Yet to date these possibilities have remained untested, and we do not know how the avian NNE generates NC.

To address the molecular mechanisms by which NNE generates NC in the chick we applied signaling molecules associated with NC development via bead implantation in the anterior NNE of early chick embryos and monitored NC induction. We report that FGF4 is sufficient to induce ectopic expression of early NPB markers (*Pax7*, *Msx1*, and *Dlx5*) as well as later NC markers (*Sox9*, *FoxD3*, *Snail2*, and *Sox10*), indicating a recapitulation of the NC developmental program in the NNE. While FGF4 is able to induce mesoderm, which could cause a secondary, indirect induction of NC in the NNE, we consistently observed ectopic NC independent of mesoderm formation. Furthermore, *Sox2* (a definitive neural marker in chick) was not induced; instead the prospective neural marker *Sox3* was often induced, suggesting that an incomplete neuralization of the ectopic tissue may accompany NC induction. Finally, we demonstrate that NNE tissue upregulates both *Bmp4* and *Wnt8c* expression in response to FGF4 application prior to launching the expression of NC markers. Our work demonstrates the capacity of NNE to generate NC in the absence of mesoderm induction and without acquiring a definitive neural character. Furthermore, the

FGF-induced upregulation of both BMP and Wnt ligands suggests a putative role for FGF in coordinating both pathways during NC formation.

Materials and Methods

Embryos and Bead Implantation

Fertile hen eggs were obtained from Hardy's Hatchery (Massachusetts, USA). Embryos were staged according to Hamburger and Hamilton (1951). Approximately 50 to 100 Affi-Gel Blue beads (Bio-Rad) were coated with a mixture of molecules suspended in 1x PBS and 0.1% BSA (w/v). Beads were incubated in the mixture for one hour at room temperature, and subsequently kept at 4°C or on ice. Prior to implantation, beads were rinsed briefly in 1x PBS and 0.1% BSA. Beads were then implanted under the anterior hypoblast of St. 3–4 chick embryos, at the border of the area pellucida (Figure 1A). Embryos were cultured at 38°C for 16–20 hours in EC culture (Chapman et al., 2001). Recombinant proteins were used to coat beads; concentrations and sources are as follows: mouse Chordin (5µg/ml, R&D); mouse Noggin (5µg/ml, R&D); human WIF (10µg/ml, R&D); human Frizzled-5/FC (10µg/ml, R&D); human FGF12 (25µg/ml, R&D); human FGF4 (0.125, 0.625, 1.25 or 2.5µg/ml, R&D); mouse Wnt3a (10µg/ml, R&D); and Wnt agonist (5µM, Calbiochem #681665). Bioactivity of mouse Wnt3a and Wnt agonist were demonstrated by their ability to promote survival and proliferation in human embryonic stem cells and their ability to regulate gene expression (data not shown). N2 supplement (Gibco) was also included within certain mixes (see below).

In Situ Hybridization and Immunofluorescence

Whole-mount *in situ* hybridization and immunofluorescence was performed as previously described (Basch et al., 2006). The *in situ* probe for *cBrachyury* (bp707–1139, NCBI Reference Sequence: NM_204940) was cloned into pBlu2SK+ through the addition of exogenous 5' *XhoI* and 3' *XbaI* sites. The *in situ* probe for *cMsx1* (bp639–1228, NCBI Reference Sequence: NM_205488.2) was cloned into pBlu2SK+ through the addition of exogenous 5' *BamHI* and 3' *PstI* sites. The *in situ* probes for *cDlx5* (bp582–1175, NCBI Reference Sequence: NM_204159.1) and *cFoxD3* (bp955–1406, NCBI Reference Sequence: NM_204951.1) were cloned into pBlu2SK+ through the addition of exogenous 5' *EcoRI* and 3' *BamHI* sites. The *cPax7* and *cSox9* probe have been previously described (Basch et al., 2006). The probe for *cOtx2* (chEST291J16) was obtained from ARK-Genomics. Other probes were gifts from the following sources: *cSox2* (A. Groves), *cBmp4* (O. Pourquie), *cWnt8c* (J. Dodd), and *cSox3* (H. Kondoh).

Primary antibodies were diluted as follows: 1:25 Pax7 (mIgG1, Developmental Studies Hybridoma Bank), 1:100 Bra (gtIgG, R&D #AF2085), 1:1500 Snail2 (rIgG, Cell Signaling #C19G7), 1:2000 Sox10 (rbIgG, a generous gift from the laboratory of Vivian Lee, Medical College of Wisconsin). All secondary antibodies (Alexa 488 or 568, Invitrogen) were used at 1:2000.

Images for *in situ* hybridization or immunofluorescence were acquired using a SPOT SE camera and SPOT software (version 4.5) and either a Nikon Eclipse 80i or Nikon SMZ1500 microscope; images were processed in Adobe Photoshop. Exposure time for Figure 6I and 6L were the same, no subsequent adjustments were made. Exposure time for Figure 7I and 7L were the same, levels in both images were adjusted exactly the same using Adobe Photoshop.

For sectioning, embryos were mounted in gelatin and sectioned at 12µm using a Leica CM1900 Cryostat. Sections were mounted with Permaflour (Thermo Scientific), with or without the addition of DAPI (10µg/ml).

Results

Combined BMP inhibition, Wnt inhibition and FGF signaling induce Pax7 in the NNE

To better understand what signals are sufficient to convert the NNE to NC, an *in vivo* bead implantation strategy was employed. Beads coated with a mixture of signaling molecules were implanted under the hypoblast at the anterior border of the area pellucida of gastrulating chick embryos (St. 3–4, (Hamburger and Hamilton, 1951)). Embryos were cultured overnight (16–20 hours) and subsequently analyzed for markers via *in situ* hybridization (ISH) (Figure 1A).

Previous experiments have shown that grafts of Hensen's node are capable of inducing a secondary axis and ectopic NP and NPB markers when placed in the area opaca which normally gives rise to extraembryonic tissue (Litsiou et al., 2005; Storey et al., 1992; Streit and Stern, 1999). Work in our laboratory has shown that Pax7 expression can be induced by grafts of quail node into chick area opaca (García-Castro, unpublished observations). Further investigations have shown that emulating the signals emanating from the node using a combination of BMP inhibition (Smad6), Wnt inhibitors (Dkk, NFz8, Crescent, and Cerberus), and FGF (FGF2, FGF3, FGF4 or FGF8) was not sufficient to induce neural tissue. These studies did not investigate the potential of such combinations to induce NC (Linker and Stern, 2004). To test this possibility, we implanted beads coated with a similar cocktail containing BMP inhibitors (Chordin and Noggin), Wnt inhibitors (WIF and Frizzled5/Fc), and FGFs (FGF12 and FGF4). Additionally, we included the cell-culture media supplement N2 – which contains insulin, progesterone, putrescine, transferrin and selenium – intended to promote neural differentiation; this supplement has been widely used in chick explant assays for neural and NC development (Basch et al., 2006; Bottenstein and Sato, 1979; García-Castro et al., 2002; Linker et al., 2009; Patthey et al., 2009; Selleck et al., 1998; Wilson et al., 2000; Wilson et al., 2001), and we reason that it could be involved in disparate results between reports of induction using coculture experiments *in vitro* and *in vivo* approaches.

We performed double ISH and analyzed the expression of the transcription factor *Pax7*, given its restricted expression and associated role with early NC development (Basch et al., 2006; Khudyakov and Bronner-Fraser, 2009; Otto et al., 2006), and the expression of the mesodermal marker *Brachyury* (*Bra*). Neither *Pax7* nor *Bra* was induced by control beads (0.1% BSA) (0/19, Figure 1B–C). The mix of BMP inhibitors, Wnt inhibitors, and FGFs induced *Bra* in 22/82 beads (27%) and *Pax7* in 20% of the beads implanted (16/82; Figure 1D–G). *Pax7* was accompanied by *Bra* in 7 out of 16 instances (44%), and the remaining 56% of *Pax7* expression occurred in the absence of *Bra*. While expression of *Pax7* and *Bra* tends to occur in distinct domains (see Figure 1F–G) instances of dual expression within the same cell do occur, which are reminiscent of the endogenous patterns of expression for both molecules at the border of the caudal most portion of the open neural plate (see Figure 1B, 2G, 2M). These results suggest that these node-like signals are sufficient to trigger *Pax7* expression in the NNE in the absence of mesoderm.

BMP Inhibition and FGF4 are sufficient to induce Pax7 in NNE

Given the complexity of the node-like cocktail, we aimed to determine the minimal signals that could still induce ectopic *Pax7* in the NNE. Wnts are required for NC formation at the NPB, however the anterior of the embryo endogenously expresses Wnt inhibitors such as Dkk1, Crescent, and Sizzled (Chapman et al., 2004; GEISHA database; Patthey et al., 2009). In an effort to enhance NC formation, we eliminated Wnt inhibitors from the cocktail. Additionally, as FGF is a potent inducer of mesoderm, we eliminated FGF12 and reduced the concentration of FGF4 used in our mix from 2.5 µg/ml to 1.25 µg/ml. This new mix,

containing Chordin, Noggin, FGF4 and N2, lead to an overall reduction in *Bra* expression (16% vs. 27% seen with the full mix) without affecting the previously observed ectopic *Pax7* (with or without *Bra*) expression in the NNE (25%, 17/67 beads; Figure 2A–C, P). Importantly, sectioned *Pax7*⁺ *Bra*-induced tissue displays no underlying mesenchymal cells, further supporting the absence of mesoderm (Figure 2B–C). While we had anticipated that removing Wnt inhibitors from the original cocktail would lead to an increase in the proportion of *Pax7* induction, these results suggest that the additional Wnt inhibitors had little (or no) effect on *Pax7* induction in the NNE (16/82 beads, 20% vs. 17/67 beads, 25%).

FGF4 is sufficient for ectopic *Pax7* induction in the NNE

To test if FGF was required to induce NC in NNE, we removed FGF4 and tested a mixture containing only Chordin and Noggin, however this mix did not produce any ectopic *Pax7* (0/19 beads; data not shown). These results suggested that BMP inhibitors alone were not sufficient to induce NC in the NNE. Because Wnt signaling is required for avian NC induction and can induce NC in naïve neural plate (García-Castro et al., 2002; Patthey et al., 2009), we tested the capacity of Wnt3a or a Wnt agonist (whose bioactivity was verified in parallel experiments; see materials and methods) to induce *Pax7* in the NNE. However, a mix containing Wnt3a combined with Chordin and Noggin was unable to trigger ectopic expression of *Pax7* or *Bra* (0/39 beads, Figure 2D–F, P). Similarly, no *Pax7* induction was observed when embryos were incubated with beads containing a Wnt agonist (0/12 beads, data not shown).

Next we tested if FGF4, without Wnt and BMP inhibitors, was sufficient for the ectopic expression of *Pax7* in the NNE. Mixes containing 0.125 or 0.625 µg/ml of FGF4 did not yield any ectopic *Pax7* (0/6 beads for each concentration, data not shown). However, implanted beads containing FGF4 at 1.25 µg/ml had a higher incidence of *Bra*-independent *Pax7* expression than other mixes tested (21%, 4/19 beads; Figure 2G–I, P). Overall *Bra* expression was reduced compared to previous mixes tested (1/19 beads; Figure 2P).

Importantly, all of the above mixes contained the N2 media supplement which could potentially influence the formation of ectopic tissue. To address this, we monitored expression of *Bra* and *Pax7* in response to beads coated in N2 alone. Neither marker was induced (0/22 beads; Figure 2M–O, P). While this strongly suggests that N2 is not sufficient to trigger ectopic induction of these markers, its ingredients may still modify the response of the NNE to other signaling molecules; therefore, we tested FGF4 in the absence of N2, addressing directly if FGF4 is sufficient to induce *Pax7* in NNE. Implanted beads containing FGF4 (1.25 µg/ml) without N2 lead to an increased ectopic induction of *Bra* (56%, 22/39 beads; Figure 2P), the majority of which co-express *Pax7* (20/22; Figure 2P). Most importantly however, *Bra*-independent expression of *Pax7* is still observed (10%, 4/39 beads; Figure 2J–L, P). The absence of mesenchymal tissue was again confirmed in sectioned tissues, where ectopic *Pax7*⁺ tissue exhibits a thickened ectoderm surrounding the bead (Figure 2L). Additional evidence of mesoderm independent induction of *Pax7* in the NNE by FGF was obtained by monitoring the expression of the paraxial mesoderm marker *Tbx6L*. Control beads soaked in BSA elicited no ectopic expression (0/8 beads, data not shown), instead FGF4 (without N2) beads induced *Tbx6L* in 31 out of 57 cases (54%). However, *Pax7* was induced in the absence of *Tbx6L* in 12% of cases (7/57 beads, Figure 3), which is comparable to the 10% (4/39 beads) of *Pax7*⁺/*Bra*[−] ectopic tissue. This data further supports the notion that *Pax7* expression can be induced in the absence of mesodermal markers. While FGF4 with N2 lead to less *Bra*, the overall *Pax7* induction observed in the absence of N2 was far greater, therefore all further experiments were performed using FGF4 (1.25 µg/ml) only.

FGF4 upregulates Neural Plate Border Specifiers and Neural Crest Specifiers

Our findings indicate that FGF4 is sufficient to trigger ectopic *Pax7* expression in the NNE. We have previously shown that *Pax7* function is critical for NC development and that its early expression marks the NPB (Basch et al., 2006). At early stages the NPB contains placodal and neural crest progenitors. Slightly later however, only NC precursors, but not placodal precursors, express the set of transcription factors known as NC specifiers and are found in the neural folds, separated from the placodal precursors (Bailey and Streit, 2005; Litsiou et al., 2005; Schlosser, 2008; Streit, 2007). To better establish the character of the ectopic tissue induced by FGF4 we analyzed by *in situ* hybridization the expression of NPB and NC specifiers. As expected, FGF4 coated beads induced ectopic expression of the NPB specifiers *Msx1* (4/7 beads) and *Dlx5* (6/11 beads) (Figure 4A–F). Of note, NC specifiers *FoxD3* (8/16 beads), and *Sox9* (7/13 beads) were also induced (Figure 4G–L). Additionally we analyzed by immunofluorescence the expression of other NC specifiers. In multiple examples we identified *Snail2* and *Pax7* coexpression (not shown). Importantly, analysis of ectopic tissue adjacent to an FGF bead in consecutive serial sections revealed expression of *Pax7*, *Snail2*, and *Sox10* (Figure 4M–T). Furthermore, the upregulation of these NC specifiers can occur in the absence of mesoderm as indicated by the lack of *Bra*, which is clearly identified in endogenous locations but was not found in the ectopic tissue (Figure 4M, S). Thus, FGF4 is capable of inducing the NC regulatory program upregulating NPB and NC specifiers in the NNE in the absence of mesodermal markers.

NNE exhibits a prospective, but not definitive, neural character in association with ectopic NC induction

Previous reports have suggested that upon NP-NNE juxtaposition, the NNE could generate NC cells through different mechanisms. NNE could respond directly to signals from the NP, or alternatively the NNE could be first neuralized, and then in a secondary inductive interaction the newly neuralized tissue could respond to signals from the NNE to form NC (Baker and Bronner-Fraser, 1997; Selleck and Bronner-Fraser, 1995). To address the possibility that the ectopic tissue had been neuralized, we monitored the expression of the prospective neural marker *Sox3*, the definitive neural marker *Sox2*, and the anterior neural marker *Otx2*.

FGFs induce early neural markers such as *Sox3* and *ERNI* (Albazerchi and Stern, 2007; Pinho et al., 2011; Streit et al., 2000), and in agreement, we observed robust expression of *Sox3* in the NNE after 16 hours of incubation with FGF4 coated beads (38%, 16/42 beads, Figure 5A, B). Consistent with previous reports, FGF4-coated beads implanted in the NNE did not induce ectopic *Sox2* expression (0/47 beads, Figure 5C, D). Ectopic tissue morphologies similar to those in which *Pax7* was induced are readily observed (Figure 5D). Furthermore, immunostaining of *Pax7* protein after sectioning these ectopic regions reveal the presence of *Pax7* positive cells in the *Sox2*-negative tissue (Figure 5E). We also tested the expression of *Otx2*, which marks anterior neural tissue at later stages, and in agreement with the lack of *Sox2*, no *Otx2* was observed after 16 hours of FGF4 treatment (0/17 beads, Figure 5F–G). Our results agree with other data showing that FGFs have the potential to initiate neural induction but are unable to lead to definitive neural character (Streit et al., 2000; Wilson et al., 2000). Thus we propose that after application of FGF4, appearance of ectopic NC coincides with a partial neural induction that does not lead to a full-fledged neural character.

BMP upregulation precedes NC induction by FGF in the NNE

In an effort to understand how FGF4 was affecting the NNE, we investigated the expression of other signaling molecules known to be involved in NC formation. During chick gastrulation, *Bmp4* is expressed in the prospective epidermis and becomes restricted to the

neural folds at later stages (Liem et al., 1995; Streit and Stern, 1999; Watanabe and Le Douarin, 1996); furthermore, *Bmp4* can trigger the formation of NC when added to NP explants, mimicking the results observed using ectoderm (Liem et al., 1995). To determine if FGF4 altered *Bmp4* expression in the NNE, we examined its temporal expression via ISH after bead implantation (Figure 6). FGFs have been reported to inhibit BMP expression in the prospective NP and NPB (Stuhlmiller and García-Castro, 2012b; Wilson et al., 2001), in contrast, our findings in the NNE revealed ectopic *Bmp4* expression 4 hours after bead implantation in over 50% of the beads (35/66 beads; Figure 6A, B). Ectopic *Bmp4* expression is maintained at 6 hours (7/20 beads) and at 16 hours (32/49 beads; Figure 6C–F). To assess the formation of NC in regions ectopically expressing *Bmp4*, we sectioned embryos and found that Pax7 is co-expressed with *Bmp4* (Figure 6H). This embryo simultaneously provides an example of *Bmp4* and Pax7 induction in the absence of Brachyury (Figure 6I). These results provide evidence for a novel role of FGF signaling in upregulation of BMP expression in the NNE.

NC induction in the NNE by FGF is accompanied by Wnt8c expression

Wnts are another crucial player in NC development. It has been suggested that Wnt6 could induce NC in chick embryos (García-Castro et al., 2002; Schmidt et al., 2007), however, its expression has only been reported after gastrulation (stage 6 onwards), and we have not been able to detect *Wnt6* expression at earlier stages. Instead, FGF signaling has been shown to regulate *Wnt8c* expression in the primitive streak and in the caudal NP (Olivera-Martinez and Storey, 2007; Stuhlmiller and García-Castro, 2012b), and therefore we sought to determine if FGF application modulated its expression in the NNE.

Similar to the BMP analysis, we examined *Wnt8c* expression via ISH after bead implantation (Figure 7). Unlike *Bmp4*, *Wnt8c* was infrequently induced at 4 hours (25%, 15/60 beads; Figure 7A, B) and absent at 6 hours (0/18 beads; Figure 7C, D) after bead implantation. However, a large proportion of implanted beads analyzed at 16 hours after treatment display ectopic *Wnt8c* expression (81% 25/31 beads, Figure 7E–F). Sections through ectopic tissue at 16 hours show that Pax7 is co-expressed with *Wnt8c*⁺ tissue (Figure 7H). In this example, the ectopic tissue also expresses Brachyury (Figure 7I). These results suggest that FGF4 initiates a complex pattern of Wnt expression in conjunction with ectopic NC formation in the NNE.

Discussion

FGF signaling can trigger the generation of NC from NNE

Previous work on amniote or chick NC induction has focused on signals required to produce NC from neural/neuralized tissue (Basch et al., 2006; Basler et al., 1993; García-Castro et al., 2002; Liem et al., 1995; Patthey et al., 2009; Patthey et al., 2008; Streit and Stern, 1999; Stuhlmiller and García-Castro, 2012b). Our study is the first dedicated to examining the inductive signals that enable the NNE to contribute to NC formation. *In vivo*, we show that addition of FGF4 to the NNE leads to distinct patterns of BMP and Wnt expression, and ultimately leads to the expression of NPB specifiers (*Pax7*, *Msx1*, *Dlx5*) and NC specifiers (*FoxD3*, *Sox9*, *Snail2*, *Sox10*). In *Xenopus*, FGFs, and particularly mesodermal Fgf8a, have been proposed to participate in NC induction (Hong et al., 2008; Hong and Saint-Jeannet, 2007; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997; Mayor et al., 1995; Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005; Villanueva et al., 2002). In chick, no mesodermal FGF has been identified with an expression profile suggestive of a role similar to that seen for Fgf8a in *Xenopus*. During chick gastrulation Fgf2, 3, 4, 8, 13, 18, and 19 are expressed in the primitive streak, while Fgf12 is distributed throughout the epiblast (Karabagly et al., 2002; Kurose et al., 2004). Fgf4 and Fgf8 have been previously associated

with avian neural induction (Huang et al., 2010; Streit et al., 2000; Streit and Stern, 1999); however, no evidence has been provided to identify the possible participation of these or any other FGF ligands in NC induction. Future work should aim to resolve this pending issue. Several FGF receptors (FGFRs) are expressed in the ectoderm and could mediate a response to FGF ligands. Both *Fgfr1* and *Fgfr4* are expressed broadly in the early ectoderm of St. 3 gastrula embryos encompassing the prospective NP and epidermis. Towards the end of gastrulation (St. 4+) their expression becomes progressively more restricted from the lateral edges of the embryo. By the end of gastrulation, *Fgfr2* and *Fgfr3*, which are not expressed initially, can be detected in the lateral edges of the embryo (Lunn et al., 2007; Stuhlmiller and García-Castro, 2012b).

NNE, prospective neural tissue, and the generation of NC

NC appear at the border between neural and NNE and tissue juxtaposition assays from different laboratories have suggested that in addition to the NP, NNE tissues also generate NC derivatives (Mancilla and Mayor, 1996; Mitani and Okamoto, 1991; Moury and Jacobson, 1990; Ruffins and Bronner-Fraser, 2000; Selleck and Bronner-Fraser, 1995; Streit and Stern, 1999). It has been argued, that data supporting the NC induction on the NNE were inconclusive based on lack of proper molecular markers for NC tissue, improper labeling of grafts, or failure to determine if NNE was neuralized prior to NC induction (Schlosser, 2006, 2008); yet a recent study from the same author demonstrates that *Xenopus* NNE can respond to inductive NPB signals by expressing NC markers (Pieper et al., 2012). Furthermore, in chick embryos it has been demonstrated that NNE transplanted to the lumen of the neural tube, is induced to express *Snail2* and generate migratory HNK1+ cells, strongly supporting the NC forming capacity of the NNE (Ruffins and Bronner-Fraser, 2000). Here we have provided further evidence by showing that NNE can be induced to form NC, demonstrating the expression of early NPB specifiers (*Pax7*, *Msx1*, and *Dlx5*), early NC specifiers (*Sox9*, *FoxD3*, *Snail2*), and a later NC specifier (*Sox10*) (Figures 1–4). In particular, the expression of the NC specifiers distinctly identifies NC and not placode precursors in endogenous tissue (Litsiou et al., 2005), and thus observation of these markers in FGF4-induced ectopic tissue in the NNE offers strong support for a NC character. However, to our knowledge no definitive evidence in model organisms has demonstrated absolute lineage-restricted compartments associated with these gene expression domains (Bailey and Streit, 2005). Yet, work focused on crest contributions made to the nasal epithelium using murine cre lines (*Pax7* and *Wnt1*) and quail chick grafts (Barraud et al., 2010; Forni et al., 2011; Murdoch et al., 2010), have clearly established NC contributions in territories previously thought to be made by placodes alone. These studies support our interpretation that the FGF4-induced ectopic tissue adopts a NC character.

It has been proposed that during NP-NNE juxtapositions, NNE could be neuralized prior to forming NC (Selleck and Bronner-Fraser, 1995). Signals from the surrounding NNE (such as BMPs or Wnts), could then launch a second inductive event upon the neuralized NNE triggering NC formation. Alternatively, NC induction may occur directly in the NNE as a result of the combination of signals emanating from the NP (e.g. FGF, BMP antagonists) and the NNE (e.g. BMP, Wnt). Here we show that NC and NPB markers are induced in the absence of the definitive neural marker *Sox2* (Figure 5); however, ectopic application of FGF4 does induce the prospective neural marker *Sox3*. *Sox3* is thought to mark ectoderm that is competent to form neural tissue after receiving additional signals from the node or mesoderm (Albazerchi and Stern, 2007; Rex et al., 1997). Thus it may be that priming the ectoderm towards a neural fate is a prerequisite to the normal formation of NC; this is supported by the fact that, in chick, the expression domain of *Sox3* extends more laterally than prospective NP, encompassing the adjacent NPB (Rex et al., 1997). Recent work in *Xenopus* further supports the contribution of NC from NNE and suggests that neural

competence precedes NC formation (Pieper et al., 2012). Pieper *et al.* also show that loss of the transcription factors *Dlx3* and *GATA2*, which confer non-neural competence to ectodermal cells, prevents proper NC formation in a non-cell autonomous manner. It will be interesting to determine the requirement of *Sox3*, and other early neural induction molecules that respond to FGF signaling like *ChCh* and *ERNI*, for NC formation in the NPB and from the NNE.

Mesoderm is dispensable during induction of NC from NNE

In the developing embryo, lateral mesoderm underlies the NPB, and studies in *Xenopus* suggest that signals from the lateral mesoderm play a crucial role in NC formation (Hong et al., 2008; Mitani and Okamoto, 1991; Steventon et al., 2009). In the chick, however, there is little evidence in support of a mesodermal role during NC induction (Basch et al., 2006; Patthey et al., 2009; Patthey et al., 2008; Selleck and Bronner-Fraser, 1995; Stuhlmiller and García-Castro, 2012b). FGF is known to be a potent inducer of mesoderm (Linker et al., 2009; Linker and Stern, 2004; Slack et al., 1987; Storey et al., 1998; Streit and Stern, 1999), however, our experiments used much lower concentrations than previous reports, and only induced *Bra* expression in half of our treatments (Figure 2P). Therefore we suggest that at the doses we used here, only a fraction of the mesoderm inducing capacity is elicited. Yet, this lower dose robustly induced *Pax7*, and in as much as 20% of the treated samples, *Pax7* expression appears independently of underlying mesoderm and *Bra* expression. Additionally, *Pax7* expression is induced in the absence of the paraxial mesodermal marker *Tbx6L* (12%). These results agree with others demonstrating that in avians, NC induction can proceed *in vitro* in the absence of mesoderm (Basch et al., 2006; Patthey et al., 2009; Stuhlmiller and García-Castro, 2012b).

Importantly, in *Xenopus* *Fgf8*, expressed by the mesoderm, has been identified as an indirect NC inducer, mediating its effect through the induction of Wnt ligands in the mesoderm. It has been postulated that the Wnt signal travels to the overlaying ectoderm, and is in effect, the NC inducer (Hong et al., 2008; Steventon et al., 2009). Instead, we previously reported that FGF/ERK signaling at the NPB is required during avian gastrulation to maintain the expression of *Pax7* and other NPB markers (Stuhlmiller and García-Castro, 2012b). Furthermore, in chick, it is the epiblast, and not the mesoderm at this stage, which expresses FGF receptors (Lunn et al., 2007; Stuhlmiller and García-Castro, 2012b), making a mesodermal FGF-mediated signal unlikely in avians.

Perhaps during normal development at the NPB both neural and mesodermal tissues provide a supportive role for endogenous NC induction through spatial and temporal control of requisite signaling molecules. For instance, FGF and BMP antagonists emanate from the node and act to reduce BMP signals (from the ectoderm) in the initial stages of NC development. And though the exact Wnt ligand acting during the early stages of NC has not yet been identified, several Wnts (*Wnt3a*, *Wnt5b*, and *Wnt8c*) are expressed in the mesoderm (especially the PS) (Chapman et al., 2004), and may serve to fulfill this role.

N2 and mesoderm induction in the NNE

In our assays, N2 supplement, which is commonly added to explant and neural cultures, appears to have an inhibitory effect on mesoderm formation. Mixes which contain N2 exhibit less *Bra* expression compared to a mix containing FGF4 without N2 (see Figure 2P). This effect could be due to insulin, an important component of N2 supplement, as insulin-like growth factors (IGFs) have been shown to be required for anterior neural formation and to inhibit expression of mesodermal markers in *Xenopus* explants (Pera et al., 2003; Pera et al., 2001). It seems relevant in this context to further examine the role of insulin and other N2 components (like progesterone) during NC induction.

Ectopic NC formation in the NNE occurs in the presence of BMP and Wnt

Interestingly, in response to FGF4, we observed early ectopic *Bmp4* and *Wnt8c* expression in the NNE, and robust signal for both ligands was identified concomitant with NC markers at 16 hrs (Figures 6 and 7). Interestingly, Litsiou *et al.* (2005), reported that after a common phase for border fates requiring FGF signaling, Wnt and BMP activation favors NC formation, while inhibition of these pathways leads to placodal derivatives. It is therefore possible that our FGF modulation in the NNE triggers both Wnt and BMP establishing the required conditions for NC development. The FGF, BMP and Wnt pathways have been shown to extensively cross-regulate one another. FGFs are known to inhibit BMPs (Branney *et al.*, 2009; Fletcher and Harland, 2008; Kretzschmar *et al.*, 1997; Pera *et al.*, 2003; Sapkota *et al.*, 2007; Wilson *et al.*, 2001), and have also been shown to upregulate *Wnt8c* in *Xenopus* and chick (Hong *et al.*, 2008; Olivera-Martinez and Storey, 2007). Wnts can inhibit FGF activity in the NP (Wilson *et al.*, 2001), and have also been shown to upregulate BMP expression in the NP (Patthey *et al.*, 2009).

During early chick development, FGF signaling in the prospective neural plate has been shown to inhibit BMP expression (Stuhlmiller and García-Castro, 2012b; Wilson *et al.*, 2000). Instead FGF activity in more lateral NNE seems necessary for *Bmp4* expression (Stuhlmiller and García-Castro, 2012b). In agreement with this, we report here that FGF beads induce *Bmp4* in the NNE, and together would suggest that FGF is necessary and sufficient to trigger *Bmp4* expression in NNE. Our results also indicate that BMP signaling is important for NC formation in the NNE. Ectopic *Pax7* expression by FGF4 is reduced by 60% in the presence of Chordin and Noggin, from a total of 24/39 beads (62%) for FGF4 alone to 17/67 beads (25%) using FGF4, Chordin, and Noggin (Figure 2P). Similarly, ectopic expression of NPB and NC specifiers is reduced between 17% and 46% when Chordin and Noggin are included in addition to FGF4 (data not shown). A lack of total abrogation of *Pax7* expression may be explained by insufficient BMP inhibition by Chordin and Noggin alone. It is possible that *Bmp4* expression is induced indirectly, downstream of FGF4-induced *Wnt* expression (this work, Olivera-Martinez and Storey, 2007). It has been reported that explants of prospective NP increase *Bmp4* expression in the presence of *Wnt3a*, while *Bmp4* expression is reduced in prospective NPB explants in the presence of a Wnt antagonist (Patthey *et al.*, 2009).

Given that the current model of NC induction proposes an early phase of FGF/ Wnt followed by a later phase of Wnt/BMP activation (Patthey *et al.*, 2009; Steventon *et al.*, 2009; Stuhlmiller and García-Castro, 2012b), it seems important to determine if the ectopic expression of these ligands is matched by activation of their pathways. It also remains to be established if the induction of BMP and Wnt expression by FGF in our experiments is direct or mediated indirectly.

Is FGF-mediated NC induction in the NNE equivalent to that occurring in the NPB?

Early in development the epiblast gives rise to the primitive ectoderm from which neural and NNE are derived. The border between them is composed of cells from both territories. NC cells are thought to arise at the border through a classic induction mechanism, and it is clear that both neural and NNE cells can contribute to NC. It has been proposed that the NNE must be neuralized to be able to respond to NC inductive signals and generate NC (Pieper *et al.*, 2012; Selleck and Bronner-Fraser, 1995). Here we have shown that the NC developmental program can be elicited by FGF signals delivered to the NNE, and that the expression of NC related markers arises in the absence of mesoderm and the definitive neural marker *Sox2*. Our results suggest two possible mechanisms by which FGF4 causes the NNE to adopt a NC fate. One possibility is that the NNE responded to FGF by launching a prospective neural state (marked by the expression of *Sox3*) which was then able to

respond to signals from adjacent non-transformed NNE to form NC in a similar fashion to what occurs to lateral prospective NP cells expressing Sox3 at the NPB. Alternatively, it is plausible that the NC program could be launched directly in the NNE in response to FGF signaling without further involvement from adjacent NNE. FGF signaling may have triggered expression of Wnt activating the NC program while simultaneously quenching BMP signaling through at least two known mechanisms, phosphorylating the linker region of R-SMADs (Kretschmar et al., 1997; Pera et al., 2003; Sapkota et al., 2007), and increasing the expression of TGF- β inhibitors (Branney et al., 2009; Fletcher and Harland, 2008; Kudoh et al., 2004). While our current results cannot definitively distinguish between these two possibilities, further examination of the requirement of Sox3 (and other prospective neural makers) as well as investigations on the activation status of the Wnt, BMP, and FGF pathways (and the cross-talk that exists between their downstream effectors), will clarify the mechanism by which NC arise in the NNE in response to FGF signaling. This system offers novel opportunities to explore the requirements and interactions at play during NC development.

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Highlights

- FGF signaling in the non-neural ectoderm induces neural crest cells
- FGF induces neural crest without mesodermal or definitive neural markers
- Non-neural ectoderm induced neural crest exhibit prospective neural markers
- FGF induced *Bmp4* and *Wnt8c* expression precedes neural crest formation.

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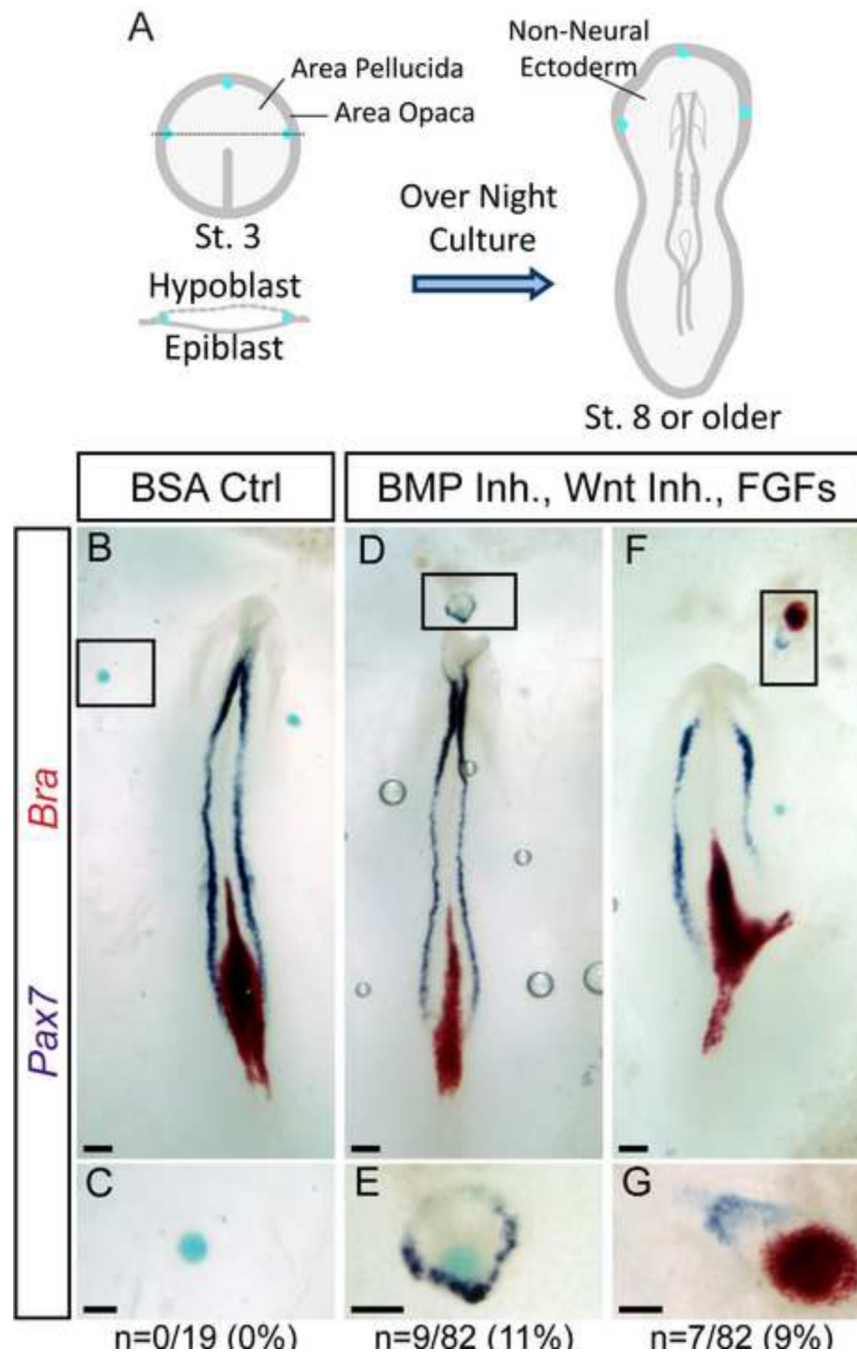


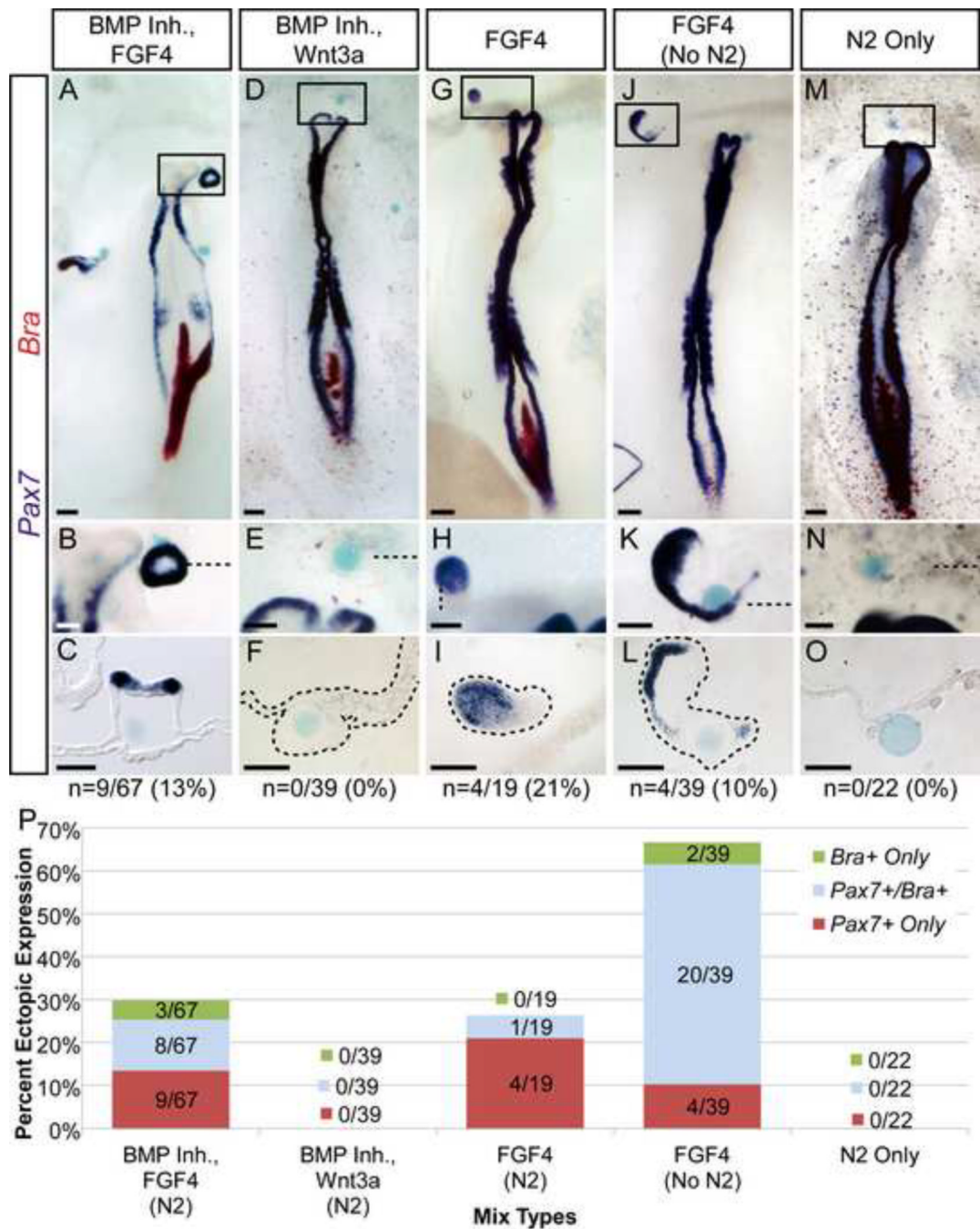
Figure 1. A mix of BMP inhibitors, Wnt inhibitors, and FGFs induce the expression of Pax7
A) Affigel blue beads coated with molecules are inserted under the hypoblast at the area pellucida/area opaca border of gastrula stage embryos. After overnight culture, embryos are assessed for marker expression. **B–C)** Control, BSA-coated beads do not induce expression of *Pax7* or Brachyury (*Bra*). **D–G)** Beads coated with Chordin, Noggin, WIF, Fz5-Fc, FGF12, FGF4 and N2 media supplement induce ectopic expression of *Pax7* independent of *Bra* (**D–E**) or in combination with *Bra* in the non-neural ectoderm (**F–G**). Boxed regions in B, D, and F indicate magnified regions shown in C, E, and G. Total number of samples

showing any ectopic expression of *Pax7* alone (C, E) or combined *Pax7* and *Bra* (G) are indicated (“n”). Scale bars are 200µm (B, D, F) and 100µm (C, E, G).

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inclusion of N2) greatly increased the frequency of total *Pax7* expression (red and blue bars combined), while *Pax7* alone (red bar only) is seen in 10% of beads. Scale bars are 200 μ m (A, D, G, J, M) and 100 μ m (B, C, E, F, H, I, K, L, N, O).

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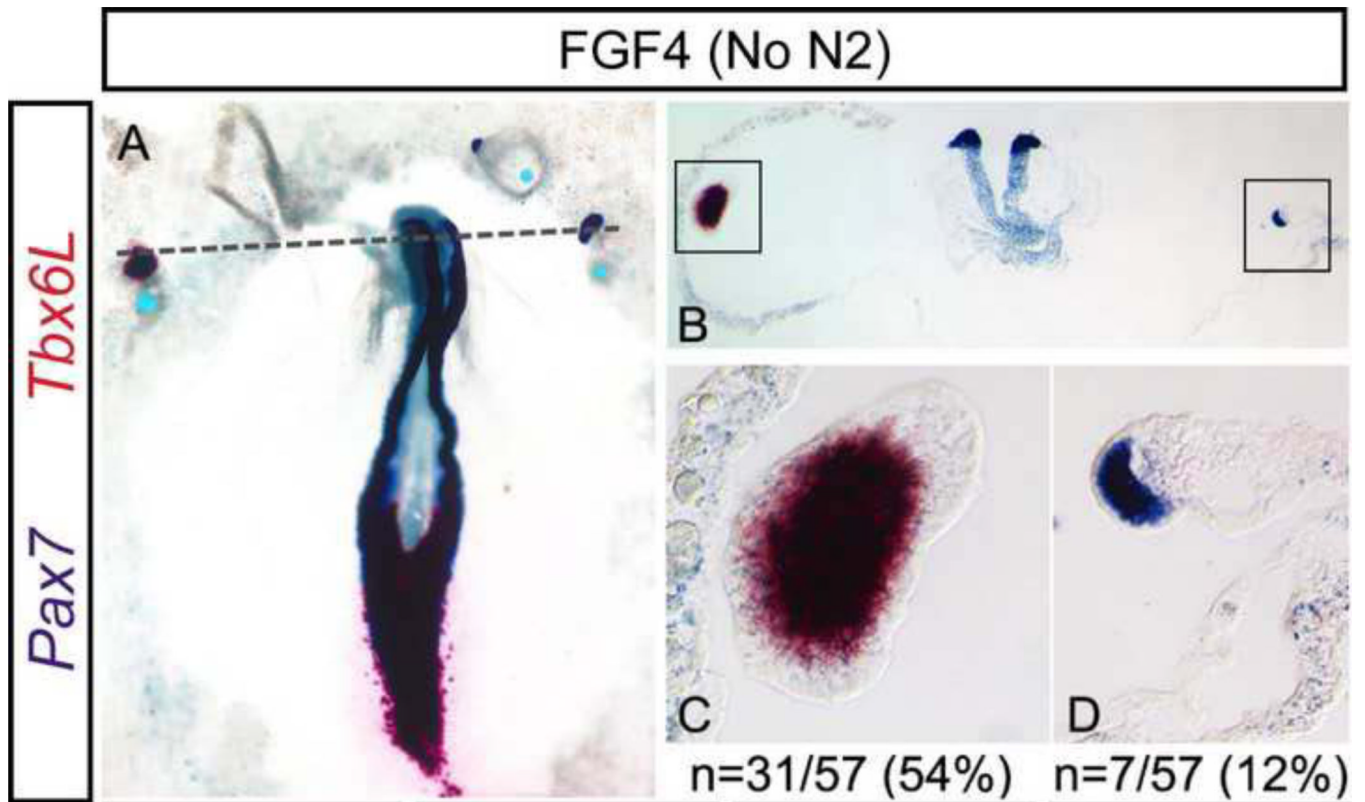


Figure 3. Pax7 is induced in the absence of Paraxial Mesoderm

Beads coated with FGF4 induce ectopic expression of *Pax7* and the paraxial mesoderm marker *Tbx6L* in the non-neural ectoderm **A–D**. Examples of independent expression of *Tbx6L* (**C**) or of *Pax7* (**D**) are shown. Dashed line in **A** indicates the plane of section shown in **B**. Boxed regions in **B** indicate magnified regions shown in **C** and **D**. Total incidence of ectopic *Tbx6L* expression (**C**) and of *Pax7*^{+/}*Tbx6L*⁻ tissue (**D**) are indicated (“n”). Scale bars are 200μm (**A**, **B**) and 50μm (**C**, **D**).

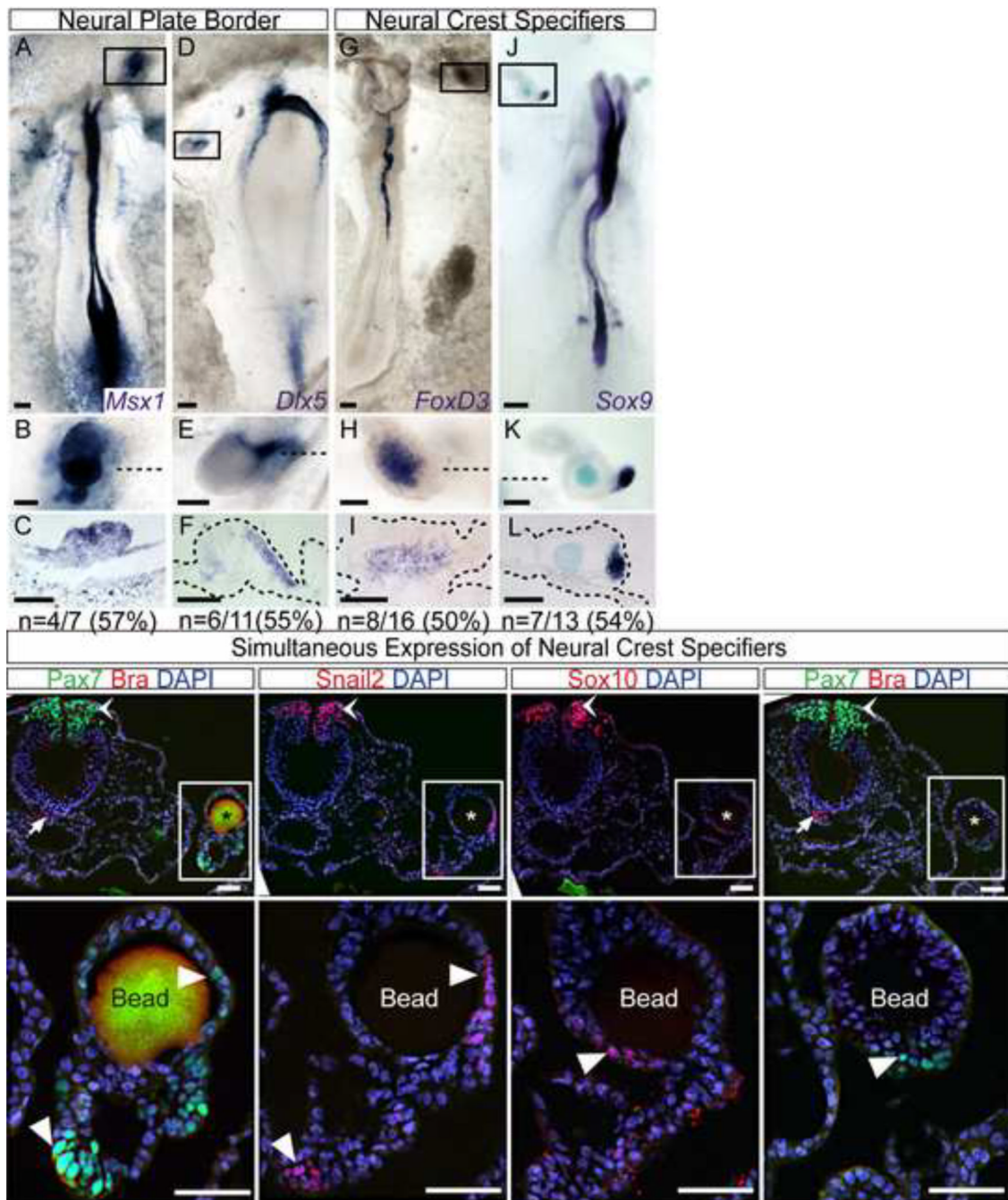


Figure 4. FGF4 induces Neural Plate Border and Neural Crest Markers

A–F) FGF4 is sufficient to induce ectopic expression of the neural plate border markers *Msx1* (A–C) and *Dlx5* (D–F). G–L) Neural crest specifiers *FoxD3* (G–I) and *Sox9* (J–L) are also ectopically induced. Boxed areas in whole mounts indicate magnified regions shown below each sample, and dashed lines indicate plane of corresponding sections. Incidence of ectopic gene expression is indicated (“n”). Immunofluorescence in consecutive sections of FGF4-induced ectopic tissue reveals additional crest markers in the absence of mesoderm. Pax7 (M, N, S, T), Snail2 (O, P) and Sox10 (Q, R) are detected in FGF4-induced ectopic tissue (white arrowheads) in addition to their endogenous NF location (chevron). Instead Bra

is only detected in the notochord, its endogenous location (**M, S**; white arrows). Boxed areas indicate magnified regions shown below each sample. Location of the bead is indicated by an asterisk in M, O, Q, and S. Scale bars are 200 μ m (A, D, G, J), 100 μ m (B, C, E, F, H, I, K, L), and 50 μ m (M–T).

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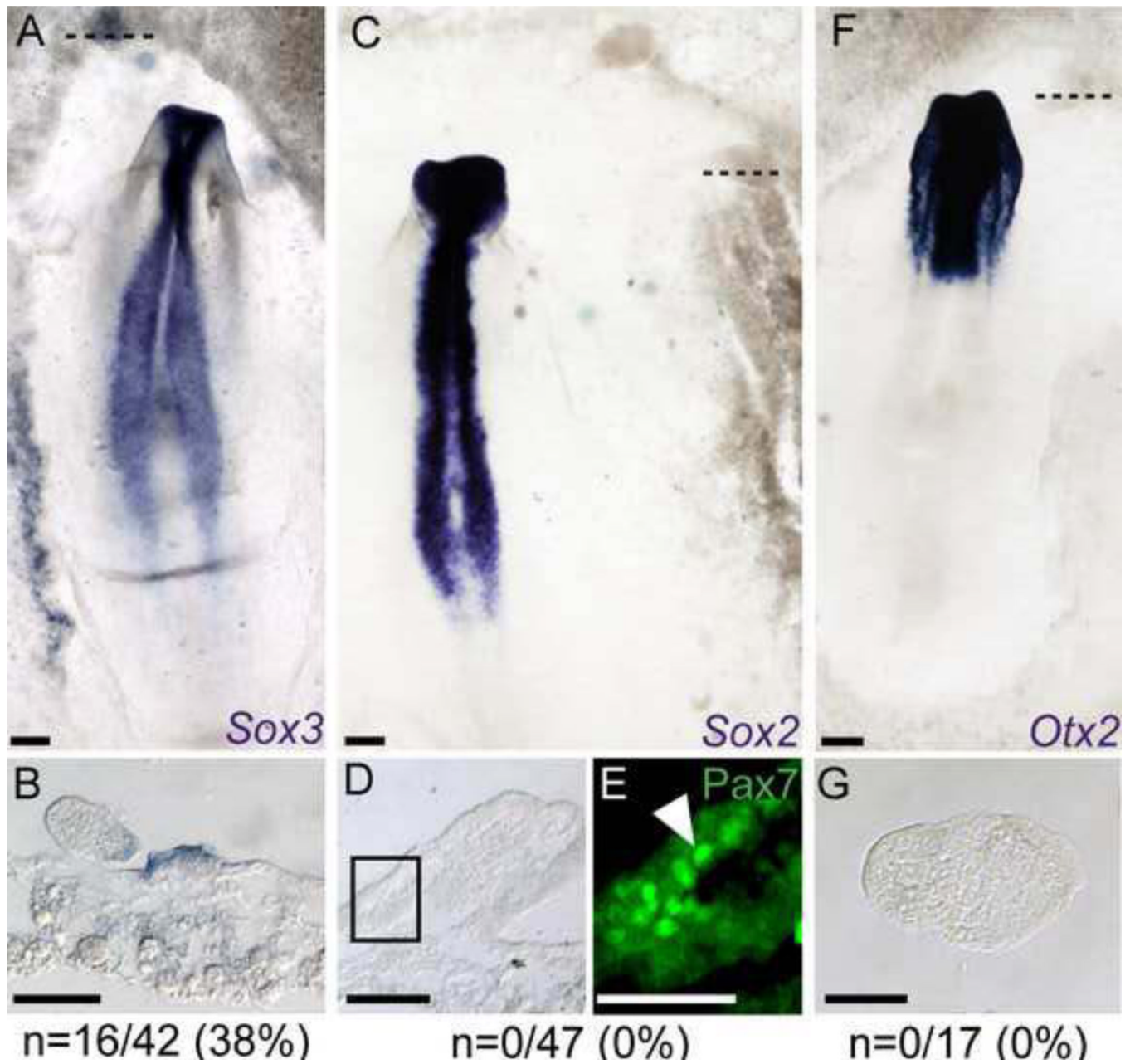


Figure 5. FGF4 induces prospective neural, but not definitive neural tissue

A–B) FGF4 coated beads induce the prospective neural marker *Sox3*. C–E) FGF4 does not induce definitive neural (*Sox2*, F–G) or anterior neural (*Otx2*) markers in the NNE. Dashed lines indicate the plane of section shown below. E) Magnified region of boxed area in D displays Pax7 immunofluorescence (arrowhead) in a *Sox2*-negative tissue. Scale bars are 200µm (A, C, F), 50µm (B, D, G), and 25µm (E).

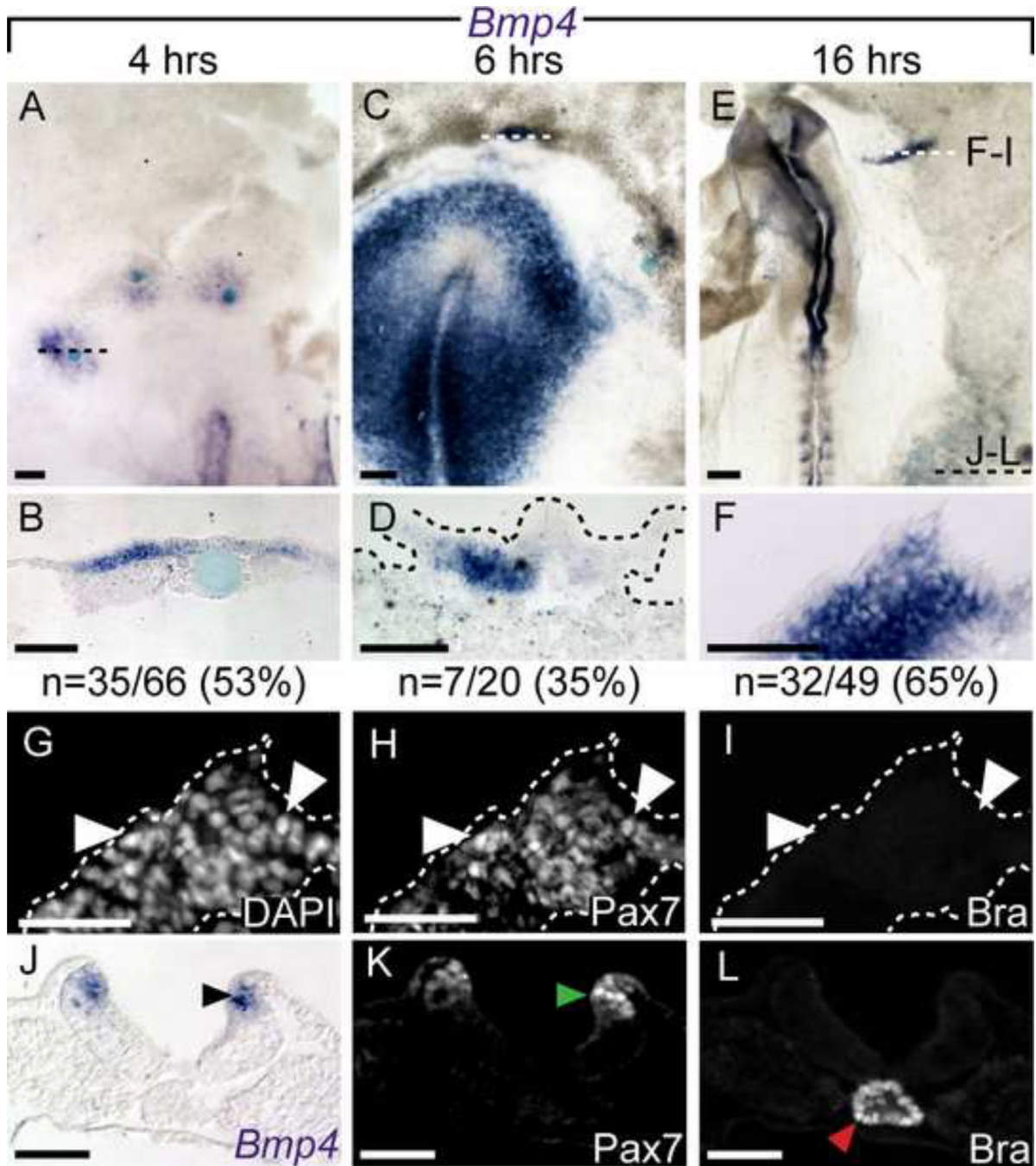


Figure 6. *Bmp4* expression is upregulated by FGF4

Using *in situ* hybridization, *Bmp4* can be detected as early as 4 hours (A, B), 6 hours (C, D) and 16 hours (E, F) after FGF4 application. Dashed lines in A, C, and D indicate the plane of sections shown below. Further analysis of panel F displaying DAPI nuclear staining (G), Pax7+ (H arrowheads) and Bra- (I) immunofluorescence. A section from caudal region of embryo E displays endogenous signal for *BMP4* (J, black arrowhead) and Pax7 (K, green arrowhead) in the neural folds, and Bra (L, red arrowhead) in the notochord. Scale bars are 200 μ m (A, C, E), 100 μ m (B, D, F) and 50 μ m (G-L).

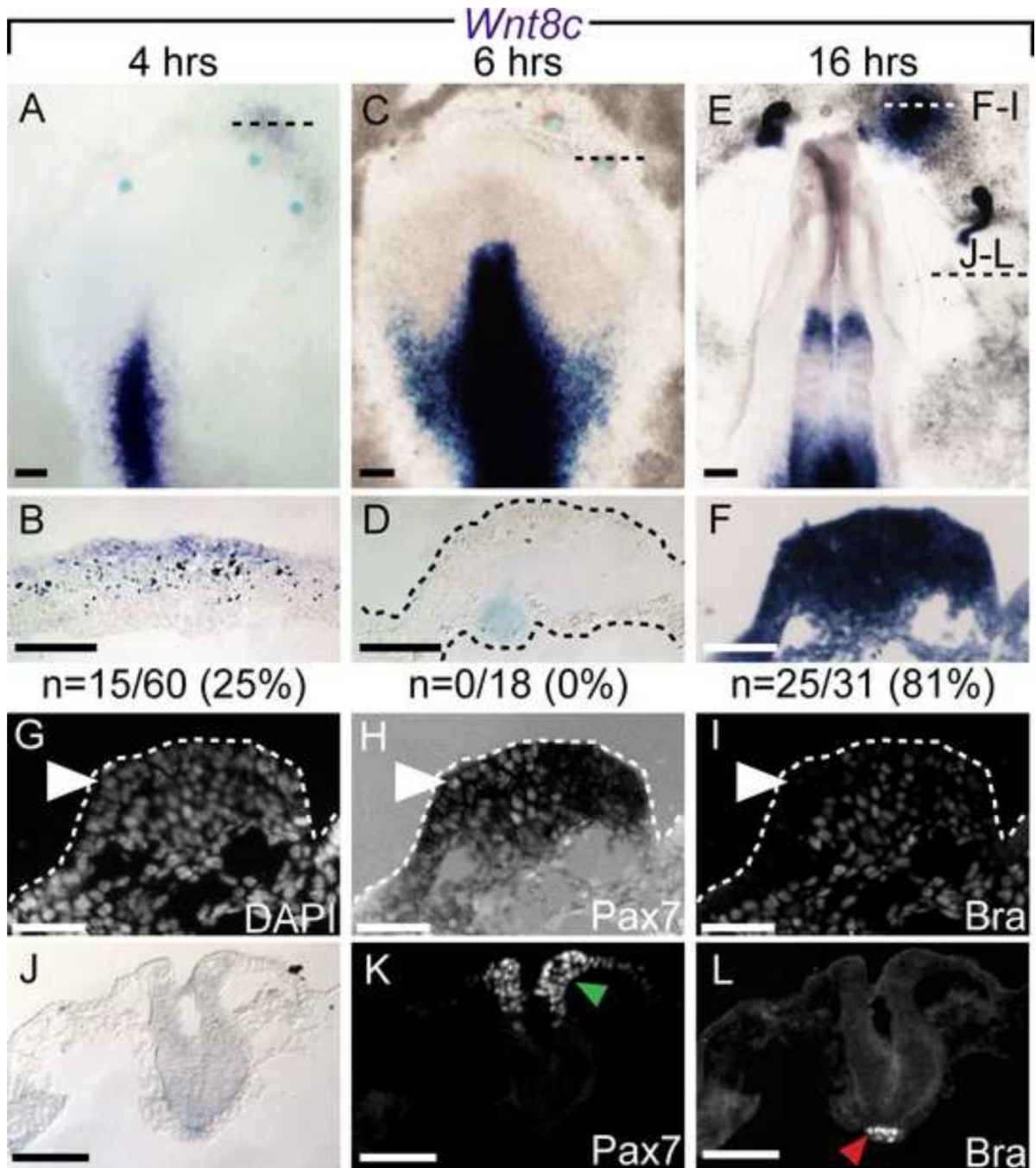


Figure 7. *Wnt8c* expression is upregulated by FGF4

Wnt8c mRNA dynamic expression after FGF4 induction was detected 4 hours (A–B), and 16 hours (E–F) after FGF4 application, but not after 6 hours of application (C–D). Dashed lines in A, C and E indicate the plane of section shown below. Further analysis of panel F displaying DAPI (G), Pax7 (H), and Bra (I) immunofluorescent signal. While both markers were induced, instances of Pax7+/Bra-negative cells are indicated (arrowhead). J–L) Midbrain level section of E, showing endogenous Pax7 (K green arrowhead) and Bra (L, red arrowhead) signal. Scale bars are 200µm (A, C, E), 100µm (B, D) and 50µm (F–L).