FGF/MAPK signaling is required in the gastrula epiblast for avian neural crest induction

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SUMMARY
Neural crest induction involves the combinatorial inputs of the FGF, BMP and Wnt signaling pathways. Recently, a two-step model has emerged where BMP attenuation and Wnt activation induces the neural crest during gastrulation, whereas activation of both pathways maintains the population during neurulation. FGF is proposed to act indirectly during the inductive phase by activating Wnt ligand expression in the mesoderm. Here, we use the chick model to investigate the role of FGF signaling in the amniote neural crest for the first time and uncover a novel requirement for FGF/MAPK signaling. Contrary to current models, we demonstrate that FGF is required within the prospective neural crest epiblast during gastrulation and is unlikely to operate through mesodermal tissues. Additionally, we show that FGF/MAPK activity in the prospective neural plate prevents the ectopic expression of lateral ectoderm markers, independently of its role in neural specification. We then investigate the temporal participation of BMP/Smad signaling and suggest a later involvement in neural plate border development, likely due to widespread FGF/MAPK activity in the gastrula epiblast. Our results identify an early requirement for FGF/MAPK signaling in amniote neural crest induction and suggest an intriguing role for FGF-mediated Smad inhibition in ectodermal development.

KEY WORDS: Neural crest induction, FGF, MAPK, Smad, Neural plate border, Gastrulation, Pax7, Chick

INTRODUCTION
The neural crest (NC) is a population of multipotent embryonic cells that migrates from the dorsal neural tube to give rise to a diverse array of derivatives, including melanocytes, sensory neurons of the peripheral nervous system, and most of the bone and cartilage of the face and skull. NC progenitors are first identifiable by the expression of several transcription factors immediately following gastrulation at the neural plate border (NPB), a collection of ectodermal cells flanked medially by the neural plate (NP) and laterally by the non-neural ectoderm (NNE), with a layer of mesoderm found underneath. The NC is thought to be formed through an inductive mechanism, whereby interactions between ectodermal tissues and the mesoderm bring about the formation of the NPB (Liem et al., 1995; Mancilla and Mayor, 1996; Moury and Jacobson, 1990; Raven and Kloos, 1945; Selleck and Bronner-Fraser, 1995). The precise participation of the different tissues, however, seems to be species specific. The mesoderm, for example, is crucial to Xenopus NC induction (Bonstein et al., 1998; Hong et al., 2008; Marchant et al., 1998; Monsoro-Burq et al., 2003; Steventon et al., 2009), yet it appears to be dispensable in zebrafish (Ragland and Raible, 2004).

Several extracellular signaling pathways have been implicated in NC induction, with most studies focusing on bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wnt signaling (Chang and Hemmati-Brivanlou, 1998; Garcia-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Lewis et al., 2004; Liem et al., 1995; Mayor et al., 1997; Mayor et al., 1995; Nguyen et al., 1998; Saint-Jeannet et al., 1997; Selleck et al., 1998) [for further references, see Jones and Trainor (Jones and Trainor, 2005)]. Recent evidence from Xenopus and chick embryos supports a two-step model of NC induction, with an early phase requiring Wnt activation and BMP inhibition during gastrulation, followed by a later phase of both Wnt and BMP activation during neurulation (Patthey et al., 2009; Patthey et al., 2008; Steventon et al., 2009). Although Xenopus studies have identified the likely inductive molecules, their sources and the time at which they act, recent work in chick has only explored the timing. Furthermore, these experiments in chick used embryonic explants and lacked in vivo information on the presence or absence of Wnt and BMP activation throughout early development.

In contrast to BMP and Wnt signaling, the role of FGF signaling in NC induction has only been investigated in Xenopus (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). It is currently proposed that FGFs act on the paraxial mesoderm during gastrulation to bring about the expression of Wnt8, which then signals to the overlying ectoderm to induce the NC (Hong et al., 2008). Thus, FGF is thought to induce the NC indirectly through the mesoderm. Although the participation of FGF in NC induction has not been addressed in the chick, studies have identified an early requirement for FGF signaling in mesoderm induction (Bertocchini et al., 2004; Chuai et al., 2006; Storey et al., 1998), placode development (Adamska et al., 2001; Ladher et al., 2005; Litsiou et al., 2005), neural induction (Linker and Stern, 2004; Stavridis et al., 2007; Streit et al., 2000; Wilson et al., 2001) and later development in the caudal neural plate/stem zone (Akai et al., 2005; Delfino-Machin et al., 2005; Diez del Corral et al., 2003; Olivera-Martinez and Storey, 2007). The induction of neural tissue, which occurs in close spatial and temporal proximity to the formation of the NPB, requires FGF/MAPK signaling up until the gastrula stage, both to antagonize BMP signaling and to act directly on the prospective neural ectoderm (Linker and Stern, 2004; Sheng et al., 2003; Stavridis et al., 2007; Streit et al., 2000; Wilson et al., 2000; Wilson et al., 2001).
Here, using the chick model, we provide the first in vivo evidence that FGF/MAPK signaling is required for NC induction in amniotes and provide a different perspective to indirect mesoderm-based induction. Using a truncated, dominant-negative form of Fgfr1, the general FGF receptor inhibitor SU5402 and a cell-autonomous MAPK inhibitor, we identify a novel requirement for FGF/MAPK signaling during gastrulation within the prospective NP epiblast. In agreement, FGF receptors are expressed in the ectoderm but are not found in the mesoderm at the time of NC induction. We additionally find a different role for FGF/MAPK signaling in the prospective NP, where it prevents the ectopic expression of lateral ectoderm markers. Interestingly, this activity is independent of neural specification, suggesting that FGF/MAPK signaling has multiple separable roles in the development of ectodermal tissues. Given the established role of BMP signaling in NC induction, we analyze the temporal participation of Smad1/5/8 signaling, and provide in vivo evidence for its requirement after gastrulation. Last, we show that increased Smad1/5/8 signaling is likely to be responsible for the ectopic expression of lateral markers in NP tissues with attenuated FGF signaling. Our results identify a novel mechanism of FGF/MAPK action in NC induction, and suggest an important role for FGF/MAPK-mediated Smad inhibition in the early development of ectodermal tissues in amniotes.

MATERIALS AND METHODS

Expression constructs
The chick dominant-negative Fgfr1 sequence (a gift from S. Fraser, Caltech, Pasadena, CA, USA) was subcloned into pCIG after adding exogenous 5′ Xhol and 3′ ClaI sites. Full-length chick Mkp3 (also known as Dusp6) (bp 147-1295, NM_204254) was cloned into pCIG after adding exogenous 5′ Xhol and 3′ Xmal sites. Both pCαβ and cSmad6 pCαβ were gifts from C. Stern (UCL, London, UK).

In situ probes were generated as follows: cFgfr mRNA sequences were cloned into pBlu2SK+ after addition of exogenous 5′ Xhol and 3′ ClaI sites. Sequences used: cFgfr1, bp 606-975 (NM_205510); cFgfr2, bp 606-975 (NM_205519); cFgfr3, bp 485-861 (NM_205509). cBrachyury bp 707-1139 (NM_204940) was cloned into pBlu2SK+ after addition of exogenous 5′ Xhol and 3′ Xhol sites.

Other probes were gifts: cBmp4 (O. Pourquié, IGMCB, Strasbourg, France), cGata2 (D. Engel, U-M, Ann Arbor, MI, USA), cSox2 (A. Groves, BCM, Houston, TX, USA), cWnt8c (J. Dodd, Columbia University, NY, USA) and cThod6 (S. Mackern, NCI, NIH, Frederick, MD, USA). Fgfr4 was detected with a quail FREG probe (C. Marcell, Monash University, Clayton, Victoria, Australia).

Embryos and electroporation
Fertile hen eggs were obtained from Hardy’s Hatchery (Massachusetts, USA). Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951), with embryos having morphology between stages 3 and 4 and given the stage designation 3+/4− (supplementary material Fig. S1).

DNA for electroporation was used at 1 mg/ml in 0.1% Fast Green in Milli-Q water. For focused electroporation, sucrose was added to a final concentration of 6% (w/v). Electroporation was carried out in Howard Ringers (HR) in chambers containing a platinum plate below the embryo while holding a platinum electrode above, with five pulses of 6V (50 ms on, 100 ms off). Embryos were cultured at 38°C for 16-20 hours in EC culture (Chapman et al., 2001).

Explant preparation and culture
The hypoblast of stage 3 embryos was mechanically removed using glass needles, and a horizontal strip of epiblast tissue was cut from the center of the embryo. This strip was trimmed to include only the area pellucida and dissected into 10 equivalent-sized squares (each approximately 100 μm2) after discarding the primitive streak. These squares were cut in half diagonally and immobilized in separate collagen gels in four-well plates. Collagen gels were prepared by combining 90 μl 3.68 mg/ml collagen (BD Biosciences), 10 μl of 10% DMEM (Gibco) and 3.7 μl of 7.5% sodium hydrogen carbonate. Collagen gels were immersed in DMEM/F12 containing N2 supplement (Gibco) and either SU5402 (5 or 10 μM) or DMSO for 16 hours at 37°C. Explants were fixed in 4% paraformaldehyde for 15 minutes before immunostaining.

Bead grafting experiments
Agarose beads were soaked in PBS+0.1%BSA containing SU5402 (Calbiochem) or an equivalent volume of DMSO for at least 1 hour at room temperature, then washed three times in PBS+0.1%BSA immediately before grafting. SU5402 concentrations of 100, 50 and 25 μM were used, and all yielded similar results, so the results were combined. Beads were grafted between the upper (epiblast or ectoderm) and lower (hypoblast or mesoderm) layers. Embryos were cultured for 16 hours when beads were grafted at gastrulation stages, and cultured for 2-7 hours if grafted at later stages. Pax7 mRNA and protein were assayed, and both gave similar results, so the data were combined.

Protein and mRNA detection
For immunofluorescence, embryos were fixed in freshly-thawed 4% paraformaldehyde (w/v) for 30-45 minutes at room temperature (except for phospho-specific antibody staining, see below), then rinsed in PBS+0.1% Tween (PT). For in situ hybridization, embryos were first imaged in HR using a SPOT SE camera and software using a Nikon SMZ 1500 microscope, and then fixed for 2 hours at room temperature or overnight at 4°C.

Whole-mount in situ hybridization and in situ hybridization were performed as previously described (Basch et al., 2006). Primary antibodies were diluted as follows: 1:50 Pax7 (mlgG1, Developmental Studies Hybridomra Bank), 1:1500 Snail2 (rbIgG, Cell Signaling #9585), 1:60 Sox2 (gtlgG, R&D Systems #AF2018) and 1:40 GFP (rbIgG, Millipore, #AB3080). Secondary antibodies (Alexa 488, 568, or 633, from Invitrogen) were used at 1:3000. Staining for dual-phosphorylated Erk1/2 [rbIgG Phospho-p44/42 MAPK (Thr202/Tyr204), Cell Signaling #9101] was carried out as follows: embryos were fixed in ice-cold paraformaldehyde at 4°C for 1.5 hours or overnight, rinsed in ice-cold PT, then dehydrated to methanol and placed at –20°C overnight. Embryos were rehydrated to PT and kept at 4°C for the remainder of the protocol. Embryos were blocked with PBS (PBS+2% BSA+0.1% Tween+10% horse serum) overnight, incubated with dpERK antibody (1:50) in PBTS for 3-5 days, then washed with PT several times. Embryos were re-blocked for 30 minutes in PTBS, incubated with Biotinylated anti-rbIgG (1:200, Vector Labs #BA-1000) in PBTS overnight, then washed with PT several times. Embryos were re-blocked for 30 minutes in PBTS, incubated with Streptavidin-Alexa 568 conjugate (1:1500, Invitrogen –S-11226 in PBTS overnight, then washed in PT several times before imaging. Staining for phospho-Smad1/5/8 [rbIgG Phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad8 (Ser426/428), Cell Signaling #9511] was carried out similar to that for dpErk, but with only one overnight incubation in primary antibody followed by an Alexa-based secondary antibody.

Embryos were mounted in gelatin and sectioned at 12 μm using a Leica CM1900 Cryostat. Sections were mounted with Permafluor (Thermo Scientific) with or without DAPI (10 μg/ml). Images after immunostaining or in situ hybridization were acquired using a SPOT SE camera and software using a Nikon Eclipse 80i microscope, and processed in Adobe Photoshop. DAPI color in Figs 4 and 5 is selectively lightened to increase visibility. Student’s t-tests of statistical significance, assuming unequal variance, were performed, comparing treated and control embryos. P values are provided in supplementary material Table S1.

RESULTS

Inhibition of FGF signaling during gastrulation causes a medial expansion of lateral ectoderm markers and a loss of primitive streak markers
To evaluate the role of FGF signaling during gastrulation, we electroporated chick embryos at stage 3+/4− on the entire left side of the epiblast with a dominant-negative Fgfr1 (dnFgfr1) construct
cloned into a vector expressing nuclear-localized GFP (pCIG). Embryos were cultured for 16 hours, then assayed for the expression of lateral ectoderm markers Msx1, Bmp4 and Gata2. Msx1 and Bmp4, primarily expressed in the neural plate border (NPB) and in some non-neuronal ectoderm (NNE) tissue, are strongly expanded medially into the caudal neural plate (NP) and primitive streak upon FGF inhibition (Msx1, n=8/12, Fig. 1A; Bmp4, n=7/8, Fig. 1B). In addition, some of these embryos show a slight increase in the NPB domain (Msx1, n=6/8; Bmp4, n=2/7; not shown), whereas others are inhibited in the NPB (Msx1, n=2/8; Bmp4, n=5/7, Fig. 1B). Gata2, which marks NNE, is slightly expanded medially into the caudal NP after dnFgfr1 electroporation (n=8/11, Fig. 1C), but not to the extent that Msx1 or Bmp4 are. Control embryos electroporated with the empty vector pCIG display normal expression of Msx1 (n=11/12, Fig. 1A'), Bmp4 (n=8/9, Fig. 1B'), and Gata2 (n=12/12, Fig. 1C'). These results suggest FGF signaling is required in the medial regions of the embryo to prevent the ectopic expansion of lateral markers, and might have a role in NPB development.

Next, we analyzed Sox2, a specifier of neural tissue. Inhibition of FGF/MAPK signaling before or during stage 3 is known to cause a loss of Sox2 expression, whereas inhibition at stage 4 does not (Delfino-Machin et al., 2005; Stavridis et al., 2007; Wilson et al., 2000). Interestingly, Sox2 expression is unaffected by electroporation of dnFgfr1 pCIG at stage 3+/4+ when we see ectopic expression of lateral markers in the NP (n=7/8 normal, Fig. 1D). Earlier electroporation at stage 3 causes an inhibition of Sox2 expression, as previously reported (n=2/2, not shown). Embryos electroporated with pCIG appear normal (n=10/10, Fig. 1D'). Known targets of FGF signaling, Brachyury (Bra) (n=18/20, Fig. 1E) and Wnt8c (n=5/6, Fig. 1F) are inhibited by dnFgfr1 electroporation, whereas the empty vector has no effect (Bra, n=15/15 normal, Fig. 1E'; Wnt8c, n=3/3 normal, Fig. 1F'). Tbx6L, a lateral mesoderm marker, is also inhibited by dnFgfr1 (n=10/10) whereas control embryos appear normal (n=9/9) (not shown).

Statistical significance of P<0.05 was found in these experiments, and similar analysis was performed throughout our study and is provided as supplementary material Table S1. Taken together, these results demonstrate that FGF inhibition at gastrulation stages causes a medial shift in the expression of lateral ectoderm markers, and a loss of primitive streak and mesoderm markers.

**FGF signaling is required for neural plate border development during gastrulation**

Our analysis of Msx1 and Bmp4 suggests FGF signaling may have a role in NPB development, but we observe some inconsistencies in their responses to FGF inhibition, perhaps owing to slight variations in the stage of electroporation. Next, we performed a fine temporal analysis of FGF inhibition on Pax7, as it is expressed exclusively in the NPB at early stages and required for later NC development (Basch et al., 2006; Otto et al., 2006). The majority of embryos electroporated with dnFgfr1 at stage 3/3+ show reduced Pax7 expression (n=13/23, Fig. 2A). Interestingly, a smaller number of embryos electroporated at this stage (n=6/23) display a different phenotype altogether, presenting an ectopic expansion of Pax7 expression into the caudal NP accompanied by an increase in its endogenous neural fold domain anteriorly. If embryos are electroporated slightly later during gastrulation at stage 4+, most instead exhibit the expanded/increased phenotype (n=9/11, Fig. 2C,D). Some embryos electroporated at these stages

**Fig. 1. FGF signaling is required to prevent ectopic expression of lateral ectoderm markers in medial tissues. (A-F)** Inhibition of FGF signaling at gastrulation using a truncated Fgfr1 construct causes a strong medial expansion of NPB markers Msx1 (A) and Bmp4 (B), and a slight medial expansion of the NNE marker Gata2 (C). The neural marker Sox2 (D) is unaffected by electroporation at late gastrulation stages. FGF inhibition causes a complete or near-complete loss of the primitive streak markers Bra (E) and Wnt8c (F). (**A'-F'**) Embryos electroporated with the empty vector control appear normal. Insets show electroporated tissues (green) after culture, before in situ hybridization. Arrows indicate ectopic expression. Arrowheads indicate inhibition.
show both an inhibition in the NPB/neural folds and expansion into the caudal NP \( (n=2/23\) electroporated at stage 3/3+; \( n=1/11 \) at stage 4–). Electroporation of \( \text{dnFgfr1} \) at the end of gastrulation (stage 4) results in mostly normal embryos \( (n=6/10) \), with a minority having abnormal phenotypes \( (n=3/10 \text{ expanded/increased}, \ n=1/10 \text{ inhibited}) \). Embryos electroporated after gastrulation all display normal Pax7 expression \( (n=8/8 \text{ electroporated at stage 4+}, \ n=9/9 \text{ at stage 4}, \ n=12/12 \text{ if electroporated at stage 4+ or later}, \text{ not shown}) \).

To confirm that FGF signaling is required for Pax7 expression at the NPB, we grafted beads coated with SU5402, a chemical inhibitor of Fgfr transphosphorylation, or DMSO as a control during or after gastrulation (Fig. 2J). Beads were grafted between the prospective or proper NPB and the lower layers. We again saw Pax7 expression at the NPB was only sensitive to FGF inhibition during early stages of gastrulation \( (n=7/11 \text{ inhibited at stage 3/3+}, \ n=3/3 \text{ at stage 4–, } n=0/18 \text{ at stage 4+ to 7}) \). Taken together, these results suggest a delicate timing for FGF signaling in the early avian gastrula: the NPB appears to require FGF signaling until early gastrulation stages (3/3+), whereas the NP and primitive streak remain sensitive to FGF inhibition until late gastrulation stages (4–/4). In the stages following gastrulation, FGF signaling no longer contributes to Pax7 expression.

To determine whether FGF activity during gastrulation is necessary for later NC markers, we analyzed Snail2 expression. Most embryos electroporated with \( \text{dnFgfr1} \) at stage 3/3+ present an inhibition of Snail2 expression \( (n=5/9, \text{ Fig. 2B}) \), whereas fewer embryos show increased expression \( (n=2/9) \). Electroporation at stage 4–/4 causes a lower frequency of these effects \( (n=2/8 \text{ inhibited}; \ n=1/8 \text{ increased}) \), and any electroporation from stage 4+ until stage 8 has no effect \( (n=0/13, \text{ not shown}) \). All control embryos appear normal \( (n=8/8 \text{ electroporated at stage 3/3+}, \ n=13/13 \text{ at stage 4–/4}, \ n=3/3 \text{ at stage 4+}, \ n=7, \text{ or } 8) \). These results demonstrate that FGF signaling is essential up until gastrulation for NC induction in amniotes.

**Early distributions of FGF receptor expression support a role for FGF signaling during gastrulation**

Next, we examined the mRNA expression of the four FGF receptors at gastrula stages. \( \text{Fgfr1} \) is broadly expressed in much of the epiblast immediately preceding gastrulation at stage 2 (not shown), and by stage 3+ still encompasses the prospective NP (pNP) and prospective NPB (pNPB), but is excluded from the most lateral regions of the embryo that give rise to NNE (Fig. 3A,D). At later stages of gastrulation (stage 4–), \( \text{Fgfr1} \) is expressed most highly in the pNP, with lower levels found in the surrounding ectoderm (Fig. 3B), and
by stage 4/4+ continues to be most highly enriched in the NP (Fig. 3C). Throughout all early stages of development, Fgfr1 is expressed at a low level in the primitive streak. Fgfr2 and Fgfr3 are not expressed strongly until stage 4, when they are found primarily in neural ectoderm and caudal NNE, respectively (supplementary material Fig. S2). Fgfr4 is expressed strongly in much of the epiblast, as well as the node and the caudal primitive streak at gastrulation stages (Fig. 3E,F). By the end of gastrulation at stage 4/4+, the expression of Fgfr4 has decreased and become more restricted (Fig. 3G). Combined, the spatiotemporal expression profiles of Fgfr1 and Fgfr4 demonstrate FGF signaling is capable of acting on the pNP during gastrulation. Importantly, there is no FGF receptor expression in the mesoderm during gastrulation when FGF signaling is required for NC induction.

**Erk activity in the early embryo depends on FGF signaling and is required for proper neural plate border development**

As many recent studies demonstrate that FGFs act through the mitogen-activated protein kinase (MAPK) signaling pathway during embryogenesis, we examined the activation state of Erk1/2 proteins, the downstream effectors of MAPK signaling, using an antibody specific to the activated, dual-phosphorylated forms (abbreviated, dpErk). At stage 3+, dpErk is found in most nuclei throughout the gastrula, with the strongest levels found in the primitive streak and pNP (Fig. 3I). Sections demonstrate that epiblast cells within a given region display a wide variation in dpErk levels, but, overall, the medial epiblast contains a greater number of nuclei with high levels of dpErk when compared with the lateral epiblast (supplementary material Fig. S3A,D,E). Embryos stained in parallel without the primary antibody show no nuclear signal (supplementary material Fig. S3B,C). This demonstrates the presence of activated Erk1/2 proteins in the pNP, and suggests a subtle gradient of MAPK activity in the epiblast during gastrulation.

Next, we electroporated stage 3+/4– embryos with dnFgfr1, and then assayed dpErk levels. Embryos treated with dnFgfr1 show a strong inactivation of Erk1/2 proteins in all three tissue layers and the primitive streak (n=9/9, Fig. 4A,B), whereas embryos electroporated with the control vector display normal Erk activation (n=7/7 normal, Fig. 4C). We conclude that FGF signaling is responsible for the majority of Erk signaling in the early avian embryo.

To determine whether the MAPK signaling pathway is required for neural crest development, we overexpressed MAP kinase phosphatase 3 (Mkp3, also known as Dusp6), which dephosphorylates and inactivates Erk1/2, serving as a cell-autonomous inhibitor of Erk signaling. Embryos electroporated with Mkp3 pCIG display a strong inhibition of Bra, a direct target of Erk1/2 signaling (n=9/9, Fig. 4D), whereas embryos electroporated with pCIG appear normal (n=8/8, Fig. 4G). We then immunostained these embryos for Pax7 and GFP. In agreement with our dnFgfr1 electroporation results, Mkp3 overexpression also creates two different effects – inhibition of Pax7 in the domain of the neural folds and pNP, while also causing an ectopic medial expansion of Pax7 into the caudal NP and primitive streak (n=5/5, Fig. 4D’-F; control n=0/5, Fig. 4G’).

NPB cells expressing high levels of GFP (high levels of Mkp3) express very little or no Pax7 compared with their neighboring cells (Fig. 4D’,D’, arrowhead). In the caudal NP, we observed GFP-positive cells ectopically expressing Pax7, but also found that cells expressing the highest levels of GFP did not display ectopic Pax7 expression. This implies that some level of Erk activity is necessary for cells to express Pax7. We additionally found cells that expressed little to no GFP that still displayed ectopic Pax7 expression, suggesting that there might be some non-cell-autonomous contributions to this phenotype.

**FGF/MAPK signaling acts directly on the gastrula epiblast to specify the neural plate border and to prevent ectopic expression of lateral markers in the neural plate**

Embryos electroporated broadly with FGF/Erk-inhibiting constructs during gastrulation stages exhibit an underdeveloped mesoderm (Fig. 4F, arrowhead), do not express the mesoderm
markers *Bra* and *Tbx6L*, and display a curved primitive streak (see Fig. 1). As FGFs are involved in mesoderm development and mediating cell ingress through the primitive streak (Amaya et al., 1991; Amaya et al., 1993; Ciruna and Rossant, 2001; Ciruna et al., 1997; Hardy et al., 2011; Sun et al., 1999), some of the ectodermal changes might be secondary to the perturbation of mesodermal signals. To address this issue, we first tested the effects of FGF inhibition using an explant assay. Previous work from our lab has demonstrated that pNPB epiblast explants from stage 3 embryos cultured in isolation will go on to express Pax7 and form migratory NC cells and derivatives independently of mesoderm markers *Bra* and *Tbx6L* (Basch et al., 2006). Here, we dissected a strip of epiblast from the equator of the embryo (hypoblast removed) and cut it into ten equivalent-sized explants, excluding the primitive streak. Each of these pieces was then cut in half and cultured for 16 hours, with one half in media containing 10 μM SU5402, and the other half in media containing DMSO. Explants cultured in DMSO exhibited a normal distribution of Pax7 expression, with pNPB explants (2, 3, 8 and 9) having Pax7-positive nuclei, while more lateral (NNE, 1 and 10) and more medial explants (pNP, 4, 5, 6 and 7) displayed minimal or no Pax7 expression at all. By contrast, pNPB explants cultured in 10 μM SU5402 do not express Pax7, whereas pNP explants 4, 5, 6 and 7 now ectopically express Pax7 (*n*=8; Fig. 5A). This supports the requirement of FGF signals in the pNPB epiblast to specify Pax7 expression, and in the pNP epiblast to prevent ectopic Pax7 expression.

To confirm whether FGF/MAPK signaling acts directly on the ectoderm, we restricted electroporation of Mkp3 pCIG to the pNPB at early stages of gastrulation (3/3+ to the pNP at late gastrulation (4–)). This results in expression of our construct exclusively in the ectoderm of the embryo, without adverse effects on mesoderm ingress. Mkp3 overexpressed in prospective NC cells caused a cell-autonomous loss of Pax7 expression in its endogenous domain (*n*=8/8, Fig. 5B–E; control *n*=0/6, not shown), whereas electroporation in the pNP generates ectopic Pax7 expression in the NP (*n*=6/7; control *n*=0/4; supplementary material Fig. S4). Similarly, NP-restricted electroporation of dnFgfr1 generates ectopic expression of Pax7 (*n*=8/8, Fig. 5F,G), Msx-1 (*n*=6/9, not shown) and Bmp4 (*n*=4/7, not shown), while control electroporation has no effect (Pax7 *n*=0/7, Msx-1 *n*=0/9, Bmp4 *n*=1/9, not shown). Some of these embryos display restricted electroporation to the anterior NP (and not to the posterior NP), and present cell-autonomous ectopic expression of lateral markers, demonstrating that FGF/MAPK inhibitory effects are not limited to the caudal NP. Henceforth, we refer to NP effects in general, without axial quality. These results, together with explant experiments (Fig. 5A), demonstrate that FGF/MAPK signaling acts directly on the gastrula epiblast to both specify the NPB and prevent the ectopic expression of NPB markers in the NP.

FGF/MAPK signaling is known to be necessary for the expression of the neural specifiers *Sox2* and *Sox3* up until gastrulation (Delfino-Machin et al., 2005; Stavridis et al., 2007; Wilson et al., 2000). Our results complement these studies, but suggest that *Sox2* is insensitive to FGF inhibition at late gastrulation stages (Fig. 1D). In order to determine whether the ectopic expression of NPB markers in the NP is accompanied by the loss of the neural tissue, we electroporated embryos with dnFgfr1 pCIG in the pNP at stage 3+/4–, then co-stained for Pax7 and an antibody that recognizes both Sox2 and Sox3. Surprisingly, we found that neural specification is unaffected by inhibition of FGF signaling at late gastrulation stages as indicated by normal Sox2/3 expression, though cells fated to become NP do indeed express Pax7 (*n*=5/5; control *n*=0/6; supplementary material Fig. S5). These findings demonstrate a narrow window during gastrulation when the NP has been specified, but still remains sensitive to inhibition of FGF signaling.

**Smad1/5/8 signaling becomes active after gastrulation and is required for proper neural plate border development**

Several reports have implicated BMP signaling in avian NPB formation and NC development (Basch et al., 2006; Liem et al., 1997; Liem et al., 1995; Linker et al., 2009; Patthey et al., 2009; Patthey et al., 2008; Selleck et al., 1998; Streit and Stern, 1999), and one study monitored BMP/Smad signaling (Faure et al., 2002), but Smad activation has not been directly related to early neural crest development. We demonstrated that Pax7 and *Bmp4* have responded similarly to FGF inhibition, and wondered to what...
extent BMP/Smad signaling might regulate Pax7 expression at early developmental stages. As a first step, we analyzed the distribution of phosphorylated Smad1/5/8 (pSmad1) and Pax7 expression from gastrulation to neurulation (Fig. 6A-I). Interestingly, pSmad1 is barely detectable at early gastrulation stages (Fig. 6A, n=6) and first becomes apparent in the posterior territory of the embryo at late gastrulation stages (Fig. 6B, n=5), suggesting there is very little or no BMP activity during gastrulation (also see supplementary material Fig. S6). By stage 4/4+, the caudal primitive streak and caudal ectoderm begin to display high levels of pSmad1 (n=8). By early stage 5, pSmad1 is found in much of the NNE and primitive streak, as well as in NPB cells that also express Pax7, but is noticeably absent from the NP (Fig. 6D, n=11). Although many Pax7-expressing cells have a strong nuclear enrichment of pSmad1 (Fig. 6E, closed arrowheads), some cells with robust Pax7 expression have low levels of pSmad1 (Fig. 6E, open arrowheads). Still, all cells that express Pax7 have detectable levels of pSmad1, greater than that observed in the NP.

At stage 7, the NNE, primitive streak and NPB continue to express high levels of pSmad1, as do the anterior neural folds (Fig. 6F-I). Additionally, the NP has a low pSmad1 level at this stage, with slightly more activity in the caudal NP (Fig. 6F). Sections suggest that the neural fold/NPB, marked by Pax7 (Fig. 6H, arrowheads), has an overall intermediate level of Smad1/5/8 activity when compared with that seen in the NNE and the NP. However, cells that express Pax7 appear in a territory of higher Smad1/5/8 activity (Fig. 6I, bounded by dotted lines) when compared with cells immediately adjacent that do not express Pax7. Embryos processed in parallel without incubation in primary antibody show no nuclear signal (supplementary material Fig. S6).

This analysis suggests that Smad signaling plays a role in NPB formation and maintenance, although it is likely to be required after gastrulation.

To directly assay whether signaling through Smad1/5/8 proteins is required for NPB formation, we overexpressed Smad6, an intracellular inhibitor of Smad1/5/8 signaling, using a vector (pCAβ) that also expresses non-localized GFP. Electroporation of Smad6 pCAβ at stage 3+/4+ causes a strong decrease in pSmad1 levels (n=4/4; control, n=0/3; supplementary material Fig. S7A-D) and a complete loss of Gata2 (n=3/3; control, n=0/3; supplementary material Fig. S7B,E), a direct target of BMP/Smad signaling.

We then analyzed the NPB markers Msx1 and Pax7 in response to Smad6 overexpression. Widespread electroporation of Smad6 pCAβ causes a reduction and overall disorganization of Msx1 (n=3/3; supplementary material Fig. S7C) and Pax7 (n=7/7, Fig. 7A,A′), with Pax7 being expanded laterally into the NNE along the entire rostrocaudal axis. These embryos also display a similar lateral expansion of Sox2/3, though expression in the NP appears unaltered (n=7/7, Fig. 7A,A′). Embryos electroporated with the control vector appear normal (Msx1, n=3/3; supplementary material Fig. S7C) and Pax7 (n=7/7, Fig. 7B,B′), with Pax7 being expanded laterally into the NNE along the entire rostrocaudal axis. These embryos also display a similar lateral expansion of Sox2/3, though expression in the NP appears unaltered (n=7/7, Fig. 7A,A′).
Ectopic Pax7 expression in the neural plate depends on attenuated FGF signaling and active Smad1/5/8 signaling

As Smad1/5/8 signaling is required for the proper expression of Pax7, and because loss of FGF signaling in the NP causes an ectopic expression of both Pax7 and Bmp4 in that domain, we argued that ectopic Pax7 expression in the NP might be a consequence of medially expanded Smad1/5/8 signaling. To determine whether ectopic Pax7 expression was dependent on Smad signaling, we inhibited both FGF and Smad signaling in the pNP by co-electroporating dnFgfr1 pCIG and Smad6 pCAβ at late gastrulation stages 3+/4+. Electroporation of both empty vectors into the pNP caused no effect on Pax7 expression (n=9/9 normal, Fig. 8A), as did electroporation of Smad6 pCAβ + pCIG (n=4/4 normal, Fig. 8B). As expected, electroporation of dnFgfr1 pCIG + pCAβ generated ectopic Pax7 expression in the NP (n=6/8, Fig. 8C). Significantly, co-electroporation of both inhibitory vectors dnFgfr1 pCIG + Smad6 pCAβ did not yield ectopic Pax7 expression (n=6/8 normal, Fig. 8D), demonstrating that Smad1/5/8 signaling is required for Pax7 to be ectopically induced in the NP. These results demonstrate Smad1/5/8 signaling is necessary for ectopic Pax7 expression when FGF signaling is inhibited in the prospective NP.

DISCUSSION

FGF/MAPK signaling and the tissues involved in avian neural crest induction

We have characterized the participation of the FGF/MAPK signaling pathway in early NC induction during gastrulation and unveiled multiple roles in the development of ectodermal tissues. Using the chick model, our work constitutes the first major effort to analyze the participation of FGF signaling in NC induction in amniotes. Recent evidence from Xenopus suggests that FGF induces the NC indirectly, by activating Wnt8 expression in the lateral mesoderm (Hong et al., 2008). By contrast, our results demonstrate a novel requirement for FGF signaling within the pNPB epiblast of the gastrula, independent of mesodermal signals (Fig. 5). Multiple studies from Xenopus have demonstrated the capacity of the ectoderm to respond to exogenous FGF signals in the absence of mesodermal tissue, producing strong induction of NC markers (Bang et al., 1997; Hong et al., 2008; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1995; Monsoro-Burq et al., 2003; Villanueva et al., 2002). Two of these studies also show that FGF signaling is necessary for NC markers in BMP-compromised ectoderm when conjugated with mesoderm (Monsoro-Burq et al., 2003; Villanueva et al., 2002). However, FGF has not yet been shown to act on the prospective NC in intact Xenopus embryos.

Yet our results do not directly refute any additional input from mesodermal signaling in the amniote gastrula. The mesoderm underlying the pNPB lacks FGF receptor expression during gastrulation (Fig. 3D,H), suggesting it does not receive FGF signals during the first step of NC induction in the chick. FGFs are crucial
for mesoderm formation (Hardy et al., 2011), however, and thus could be considered to be responsible for mesodermal signaling. Although the mesoderm has not yet been implicated in amniote NPB formation, at later stages of development, both mesoderm and ectoderm are capable of inducing the NC in chicks (Selleck and Bronner-Fraser, 1995).

In *Xenopus*, the current model of early NC development proposes an initial inductive step during gastrulation that is dependent on Wnt activation and BMP inhibition, followed by a maintenance step during neurulation where both BMP and Wnt signaling are active (Steventon et al., 2009). This two-phase model is supported by chick explant studies (Patthey et al., 2009), but in vivo confirmation is lacking. Our analysis of the spatiotemporal distribution of Smad1/5/8 activation fits with the model; we find minimal Smad1/5/8 activity during gastrulation when FGF/MAPK signaling acts on much of the epiblast, and then a robust Smad1/5/8 activation colocalizing with Pax7 during neurulation (Fig. 6) when most of the FGF/MAPK activity is restricted to the neural plate (Fig. 3) (Lunn et al., 2007). We note that FGF may participate in BMP-antagonism in the pNPB during gastrulation, allowing the NPB to proceed (Delaune et al., 2005). At gastrula stages, we see a modest difference in levels of activated Erk1/2 proteins in nuclei of lateral epiblast until gastrulation (Stavridis et al., 2007). Together, these studies suggest that FGF, via the Erk signaling cascade, acts on the medial epiblast from the blastula through to the gastrula stage to specify neural tissue. Our results suggest a similar requirement for FGF/MAPK signaling within the pNPB epiblast, with a crucial point during gastrulation when FGF activities cease to affect the endogenous domain of Pax7. This lends support to a potential shared mechanism of neural and NC specification, orchestrated by FGF/MAPK signaling. However, in sharp contrast to promoting the expression of NPB makers in the pNPB epiblast, we find FGF/MAPK signaling is required in the pNP instead to prevent the expression of these same markers, independently of its role in neural specification. We note that some effects of FGF/MAPK inhibition in the NP are found anterior to the node, whereas other electroportations also encompass the caudal NP, a region capable of yielding mesodermal and neural derivatives termed the ‘stem zone’ (Delfino-Machin et al., 2005). As lateral ectoderm markers can be induced in the NP for a short period after FGF signaling is necessary for neural and NPB specification, these activities may be separable. Thus, FGF/MAPK signaling may act similarly on both the pNP and pNPB early on, but inhibition of FGF signals has different outcomes in the two regions at the gastrula stage.

It has been proposed that cells respond to varying levels of MAPK signaling, such that very high levels specify mesoderm tissue, and lower levels are needed to allow the neural program to proceed (Delaune et al., 2005). At gastrula stages, we see a modest difference in levels of activated Erk1/2 proteins in nuclei of lateral versus medial epiblast (Fig. 3; supplementary material Fig. S3), suggesting the low level in the lateral epiblast might specify the NPB, while progressively higher levels promote the generation of neural and mesodermal tissue. As Mkp3 overexpression causes a cell-autonomous loss of Pax7, we reason that some level of Erk1/2 signaling in the pNPB is required for Pax7 expression. In support, Pax7 is not ectopically induced in NP cells expressing extremely high levels of Mkp3 (cells that have the highest level of Erk inhibition). Still, the difference in Erk activation between nuclei is subtle, and may not be significant enough to elicit differential gene expression.

**Implications for neural induction and early signaling in neural crest induction**

In the chick, studies on neural induction point to a direct requirement for FGF signaling within the epiblast from pre-gastrula stages (Streit et al., 2000; Wilson et al., 2000; Wilson et al., 2001). More recently, Erk1/2 signaling was shown to be necessary for neural induction up until gastrulation (Stavridis et al., 2007). To this, together, these studies suggest that FGF, via the Erk signaling cascade, acts on the medial epiblast from the blastula through to the gastrula stage to specify neural tissue. Our results suggest a similar requirement for FGF/MAPK signaling within the pNPB epiblast, with a crucial point during gastrulation when FGF activities cease to affect the endogenous domain of Pax7. This lends support to a potential shared mechanism of neural and NC specification, orchestrated by FGF/MAPK signaling. However, in sharp contrast to promoting the expression of NPB makers in the pNPB epiblast, we find FGF/MAPK signaling is required in the pNP instead to prevent the expression of these same markers, independently of its role in neural specification. We note that some effects of FGF/MAPK inhibition in the NP are found anterior to the node, whereas other electroportations also encompass the caudal NP, a region capable of yielding mesodermal and neural derivatives termed the ‘stem zone’ (Delfino-Machin et al., 2005). As lateral ectoderm markers can be induced in the NP for a short period after FGF signaling is necessary for neural and NPB specification, these activities may be separable. Thus, FGF/MAPK signaling may act similarly on both the pNP and pNPB early on, but inhibition of FGF signals has different outcomes in the two regions at the gastrula stage.

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**Fig. 8. Ectopic Pax7 expression in the NP depends on attenuated FGF signaling and active Smad1/5/8 signaling.**

(A-D) Electroporation of the pNP with empty control vectors pCIG and pCAG (A) or inhibition of Smad1/5/8 signaling using Smad6 pCAG+pCIG (B) causes no change in Pax7 expression. Inhibition of FGF signaling using dnFgfr1 pCIG+pCA (C) causes an ectopic increase in Pax7 expression. When FGF signaling and active Smad1/5/8 signaling is inhibited, ectopic expression is prevented (D).
Although NP and NPB markers require FGF/MAPK signaling up until gastrula stages, the progenitors of these populations appear to be specified earlier. Explant studies that isolate the pNP and pNPB epiblast demonstrate that neural tissue is specified during or before the blastula stage in the chick (Wilson et al., 2000; Wilson et al., 2001), whereas the NC seems to be specified just before gastrulation (Patthey et al., 2009). These findings suggest that cells of the pNP and pNPB are inherently different by the gastrula stage, and probably respond distinctly to FGF inhibition due to their different intracellular characteristics. What caused these differences? Our study, together with other recent findings, suggests prospective neural and NC populations both require BMP inhibition and active FGF/MAPK signaling, but they differ in the status of canonical Wnt signaling (Patthey et al., 2009; Wilson et al., 2000; Wilson et al., 2001). Interestingly, nuclear-localized β-catenin and Wnt ligands are found in the lateral epiblast, but are absent from the medial, neural-specified epiblast at blastula stages (Roeser et al., 1999; Skromne and Stern, 2001), prompting an analysis of the intrinsic differences in the epiblast at this stage.

**The participation of FGF/MAPK signaling machinery**

What molecules in the FGF signaling machinery are likely to participate in NC induction? Our expression data demonstrate that both Fgfr1 and Fgfr4 could potentially serve as receptors for prospective neural or NC tissue during gastrulation (Fig. 3). Several canonical FGF ligands are expressed during chick gastrula stages, including Fgf3, Fgf4, Fgf8 and Fgf18 (Karabagli et al., 2002; Wilson et al., 2000; Wilson et al., 2001). Of these, Fgf3 and Fgf8 have been implicated in neural induction and could also mediate FGF effects on the pNP. Fgf3 is expressed in the epiblast from pre-gastrula stages (Wilson et al., 2000; Wilson et al., 2001), whereas Fgf8 is expressed in the hypoblast as well as the primitive streak and newly ingressing mesendoderm (Karabagli et al., 2002; Streit et al., 2000). In addition to its regulation of Wnt8 expression in Xenopus NC induction (Hong et al., 2008; Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005), Fgf8 has been shown to induce Mesp1 (Streit and Stern, 1999) and many neural markers (Albazerchi and Stern, 2007) in the chick.

MAPK signaling is known to regulate relevant patterning genes such as Wnt ligands and BMP antagonists (Branney et al., 2009; Hardy et al., 2011), but the mechanism of MAPK action has yet to be determined in this context. Interestingly, Erk1/2 can phosphorylate the linker domain of Smad1/5/8 proteins, promoting polyubiquitylation by Smurf1 and subsequent degradation of the Smad, thereby inhibiting the pathway cell-autonomously (Fuentealba et al., 2007; Kretzschmar et al., 1997; Sapkota et al., 2007). This pathway is crucial for Xenopus neural development (Fuentealba et al., 2007; Pera et al., 2003), and may also operate in the lateral avian epiblast fated to become NC cells. We have shown that inhibition of FGF/MAPK signaling in the pNP causes ectopic upregulation of Pax7, cell-autonomously (Fig. 5), and that this upregulation requires Smad signaling (Fig. 8). Potentially, the inhibition of FGF/MAPK signaling alleviates the phosphorylation of the Smad linker domain, resulting in a higher level of Smad signaling in the forming NP and invoking the expression of Smad1/5/8 targets without modifying C-terminal phosphorylation. This mechanism could account for the inability of Noggin (a secreted BMP antagonist) to eliminate lateral markers from medial epiblast explants treated with an FGF inhibitor (Wilson et al., 2001), and argues that FGF-mediated MAPK phosphorylation of the Smad1/5/8 linker domain is a fundamental mechanism to promote neural development. An attractive hypothesis is that MAPK-dependent linker phosphorylation could also underlie the absence of Smad1/5/8 activity in the epiblast during gastrulation. Thus, the widespread distribution of Erk1/2 activity during gastrulation would explain the lack of observed Smad1/5/8 activation until neurulation, when ectodermal Erk1/2 activity is then restricted to the NP.

FGF signaling in Xenopus and zebrafish is also known to contribute to the expression of Chordin and Noggin, secreted BMP inhibitors (Branney et al., 2009; Fletcher and Harland, 2008; Kudoh et al., 2004). If we suppose FGF in the chick is also responsible for the activation of Wnt signaling during the gastrula stage of NC induction, it is possible that the proper level of FGF/MAPK activity could be sufficient to induce the initial NC program (Fig. 9). However, GSK3 was also shown to phosphorylate the Smad1 linker and contribute to the degradation pathway, though it first requires MAPK phosphorylation to ‘prime’ the linker domain. The same study also demonstrated that canonical Wnt signaling can prolong the period of Smad1 activity by inhibiting GSK3 and preventing or slowing degradation of Smad1 (Fuentealba et al., 2007). Thus, if FGF activates Wnt signaling, it would counterbalance MAPK inhibition of Smad signaling. The real challenge in the years to come is to determine which mechanisms of crossregulation are relevant to NC induction and how they integrate to activate the key regulatory transcription factors. In conclusion, our results illustrate the complex time-dependent roles of FGF/MAPK signaling in the early ectodermal development of amniotes and provide a new perspective into its role in NC induction.

![Fig. 9. Proposed mechanism of FGF participation in early neural crest development in avians. (A) FGF and Wnt activity both contribute to neural crest induction at gastrula stages in the pNPB epiblast. It is not yet clear whether FGF activates the Wnt signal responsible for induction, but FGF probably contributes to BMP inhibition on multiple levels. The source of the Wnt signal has not yet been identified. (B) During neurulation, FGF is no longer required in NC cells, but is active in further neural development. Wnt and BMP signaling cooperate to maintain the NC in the NPB. Wnt6 in the NNE and Bmp4/7 in the NPB are probably involved, although other molecules may also participate.](image-url)
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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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