

Expression of hindbrain boundary markers is regulated by FGF3

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Biology Open 1, 67–74
doi: 10.1242/bio.2011032

Summary

Compartment boundaries act as organizing centers that segregate adjacent areas into domains of gene expression and regulation, and control their distinct fates via the secretion of signalling factors. During hindbrain development, a specialized cell-population forms boundaries between rhombomeres. These boundary cells demonstrate unique morphological properties and express multiple genes that differs them from intra-rhombomeric cells. Yet, little is known regarding the mechanisms that controls the expression or function of these boundary markers.

Multiple components of the FGF signaling system, including ligands, receptors, downstream effectors as well as proteoglycans are shown to localize to boundary cells in the chick hindbrain. These patterns raise the possibility that FGF signaling plays a role in regulating boundary properties. We

provide evidence to the role of FGF signaling, particularly the boundary-derived FGF3, in regulating the expression of multiple markers at hindbrain boundaries. These findings enable further characterization of the unique boundary-cell population, and expose a new function for FGFs as regulators of boundary-gene expression in the chick hindbrain.

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Key words: Hindbrain, boundary cells, rhombomere, FGF, signaling pathway, chick embryo

Introduction

The embryonic hindbrain is divided along its antero-posterior (AP) axis to several compartments, named rhombomeres (r), each displays a unique molecular signature that underlies the formation of several neuronal and cranial tissues (Lumsden and Keynes, 1989; Lumsden and Krumlauf, 1996). The rhombomeres are separated one from the other by a specialized sub-set of cells, termed hindbrain boundary cells (Cooke and Moens, 2002; Fraser et al., 1990; Jimenez-Guri et al., 2010; Kiecker and Lumsden, 2005), which exhibit different characteristics from those of the intra-rhombomeric cells. Boundary cells are larger, they proliferate at a slower pace, they have enlarged intercellular spaces and they display reduced junctional permeability. Moreover, boundary cells express various types of genes including extracellular matrix (ECM) proteins, structural genes and transcription regulators (Guthrie et al., 1991; Heyman et al., 1995; Heyman et al., 1993; Martinez et al., 1992). Yet, the role of boundary cells during hindbrain development is not clear.

Compartment boundaries act as organizing centers that segregate adjacent areas into domains of gene expression and regulation, and control their distinct fates via the secretion of signaling factors (Figdor and Stern, 1993; Larsen et al., 2001; Lawrence and Struhl, 1996; Liu and Joyner, 2001; Mann and Morata, 2000; Raible and Brand, 2004; Rubenstein et al., 1994; Wurst and Bally-Cuif, 2001). Some examples in the CNS include the zona limitans intrathalamica (ZLI), which serves as an interface between the thalamic and prethalamic primordia, and expressed *sonic hedgehog* (*SHH*), *Wnts* and *fibroblast growth factors* (*FGFs*) (Guinazu et al., 2007; Lim and Golden, 2007;

Scholpp et al., 2006; Shimamura et al., 1995), and the midbrain-hindbrain boundary (MHB), which expresses *FGF8* and regulates the fates of the mesencephalon and anterior hindbrain (Lekven et al., 2003; Mason et al., 2000; Wassef and Joyner, 1997; Zervas et al., 2005). In hindbrain boundaries of multiple species, various signaling factors have also been shown to be expressed, such as *Wnts* in zebrafish (Amoyel et al., 2005; Riley et al., 2004), and *FGFs*, as well as the *TGF β* inhibitor *follistatin*, in chick and mice (Connolly et al., 1995; Kurose et al., 2004; Mahmood et al., 1995; Nittenberg et al., 1997; Sela-Donenfeld et al., 2009; Weisinger et al., 2008). However, what role these factors may play in boundaries remains vague. Our previous studies have suggested that, in the chick, boundary cells control the downregulation of rhombomeric genes by, as of yet, an unknown secreted factor (Sela-Donenfeld et al., 2009).

This study focuses on the FGF signaling pathway in chick hindbrain boundaries. The expression of FGF ligands, receptors, downstream effectors as well as proteoglycans is shown at inter-rhombomeric boundaries, raising the hypothesis that this signaling system mediates the expression of multiple boundary markers. Loss-and-gain-of-function experiments demonstrate for the first time that FGF signaling, particularly the specific ligand FGF3, plays a role in regulating the expression of several subtypes of boundary markers, which are responsible for the unique properties of hindbrain boundaries as regions of enriched ECM, axonal accumulation and neural differentiation. Our data brings us a step forward in potentiating the suggestion for an organizing role for hindbrain boundaries, mediated by FGF3 signaling.

Results

During hindbrain development, several members of the FGF superfamily and their receptors are expressed and act in pattern formation (Aragon and Pujades, 2009; Hatch et al., 2007; Labalette et al., 2011; Lunn et al., 2007; Mahmood et al., 1995; Marin and Charnay, 2000a; Walshe and Mason, 2000; Weisinger et al., 2010; Weisinger et al., 2008). We wished to establish whether FGF signaling also functions at later hindbrain stages by regulating expression of markers unique to boundary cells. As a first step, the expression of multiple components of FGF signaling pathway was studied in the chick hindbrain at stage 19 HH (35 somites), which represents well-established boundary structures (Guthrie and Lumsden, 1991; Heyman et al., 1995; Heyman et al., 1993).

FGF signaling components at hindbrain boundaries

FGF3, a member of the FGF superfamily of secreted proteins, is dynamically expressed in the chick hindbrain; early at rhombomeres, where it governs regional identities (Aragon et al., 2005; Mahmood et al., 1995; Weisinger et al., 2010), and later exclusively in boundary cells (Mahmood et al., 1995; Weisinger et al., 2008), where, as confirmed here, it is confined to the ventral half of the r2/3–r5/6 boundaries (Fig. 1A). Notably, an additional member of the FGF superfamily of soluble signals, FGF19, was also shown at hindbrain boundaries (data not shown and see also (Gimeno and Martinez, 2007; Kurose et al., 2004; Weisinger et al., 2010). Yet, in contrast to FGF3, its expression is punctuated and less intense than FGF3, and it is also not exclusive to boundaries.

Four types of FGF receptors (FGFRs) mediate the FGF signal (Itoh and Ornitz, 2004). The boundary expression of FGFRs 1–3 has been demonstrated (Nishita et al., 2011; Walshe and Mason, 2000; Weisinger et al., 2010) and is also shown here, together with the expression of FGFR4, which is demonstrated at

hindbrain boundaries for the first time. While *FGFRs 1, 3, 4* (Fig. 1C,E,F, respectively) are expressed throughout the hindbrain, but are much more prominent along the dorsal-to-ventral (DV) axis of all rhombomere boundaries, *FGFR2* (Fig. 1D) is more restricted and is expressed only in the dorsal half of the boundaries.

Dual phosphorylated Erk1/2 (dpErk1/2) is a key player in the MAPK signaling cascade, which transduces the FGF signal from its transmembrane receptors into gene regulatory events (Eswarakumar et al., 2005), and was previously shown in hindbrain rhombomeres at early stages of development (Lunn et al., 2007; Weisinger et al., 2010). Here we show that, in accordance with the FGFRs, dpErk1/2 is predominantly apparent throughout the DV area of all hindbrain boundaries (Fig. 1B). Notably, addition of only the secondary antibody used for dpERK1/2 detection (anti-rabbit HRP antibody), did not reveal HRP staining at hindbrain boundaries (data not showing). Taken together, the co-localization of FGFs with their receptors and dpERK suggests active FGF signaling at hindbrain boundaries.

Proteoglycans in the ECM are necessary co-factors for FGF signaling (Ornitz and Marie, 2002). One such protein, Heparan Sulfate Proteoglycan (HSPG), which is required for optimal FGF signaling by forming an active complex with FGFRs (Pellegrini et al., 2000; Rapraeger et al., 1991), is abundant in the ECM. HSPG is demonstrated here to be apparent throughout the DV axis of the boundaries (Fig. 1I). Chondroitin Sulfate Proteoglycan (CSPG), another type of proteoglycan, has previously been shown at hindbrain boundaries (Heyman et al., 1995), and is re-presented here to be apparent throughout the DV aspect of the boundaries (Fig. 1J), similar to HSPG. Further support for the enriched proteoglycan distribution at rhombomere borders came from our staining with the cationic dyes Alcian blue (AB) and Safranin-O (SO), which specifically stain acid glycosaminoglycans in the ECM, such as chondroitin and

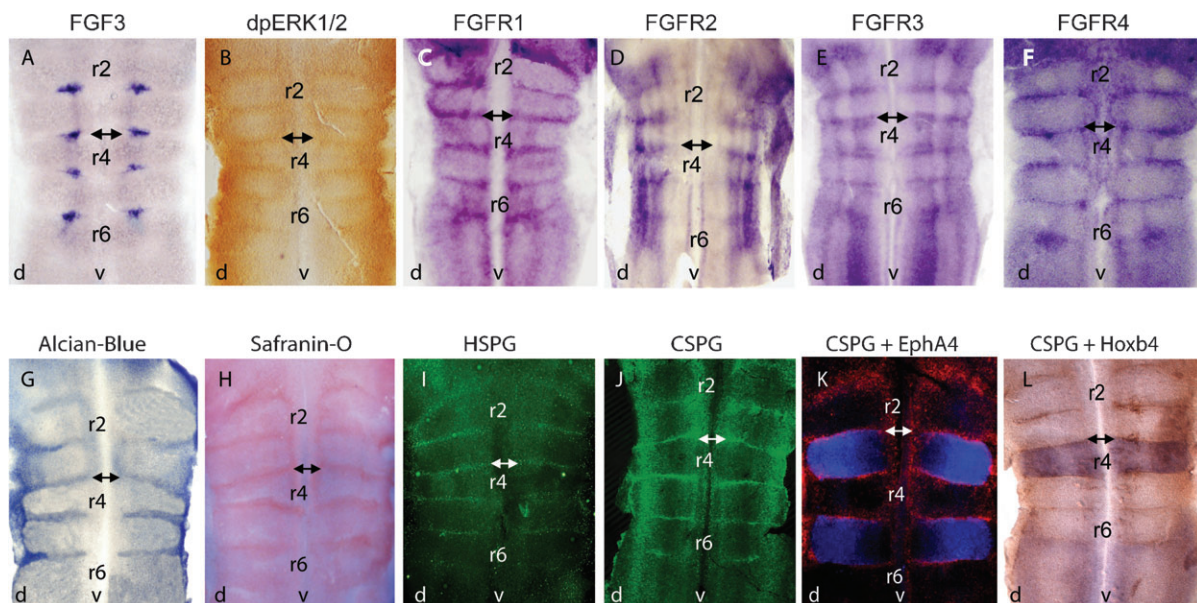


Fig. 1. The expression of FGF signaling pathway components in hindbrain boundary cells. (A, C–F, L) In situ hybridization was carried out to detect the expression of (A) *FGF3*, (C–F) *FGFRs 1–4* and (L) *Hoxb1*. (G,H) Staining of (G) Alcian blue and (H) Safranin-O. (B,I–K) immunohistochemistry was carried out to detect the expression of (B) DpERK1/2, (I) HSPG, (J–L) CSPG and (K) EphA4. All images are of 35 somite-old embryos and are shown in flat-mount preparations of the hindbrain. Rhombomeres are numbered, probes and antibodies are indicated; double-headed arrow indicated r3/r4 boundary and anterior is at the top. Rhombomere (r), dorsal (d), ventral (v).

heparin sulphate proteoglycans (Prent, 2009; Reich et al., 2010; Rosenberg, 1971). Both AB (Fig. 1G) and SO (Fig. 1H) are clearly evident throughout the entire DV aspect of the boundaries, with the exception of AB in the r6/7 boundary. Notably, staining with another ECM dye, Trichrome, which marks collagen fibers, did not reveal any boundary-specific stain (data not shown), confirming the specificity of the above dyes. Moreover, to further validate the specificity of our in situ hybridization or immunostaining at the boundaries, embryos underwent either in-situ hybridization with *Hoxb1* probe or immunostaining with EphA4 (both known to be expressed within specific rhombomeres opposed to boundaries) and co-stained with CSPG. In both cases, the rhombomeric markers EphA4, expressed in r3,r5, (Fig. 1K, blue) and *Hoxb1*, expressed in r4, (Fig. 1L, purple), are adjacent to the CSPG-stained boundaries (red or brown, respectively). Together, the accumulation of HSPG and CSPG as well as the staining of AB and SO at boundary regions further highlights the unique ECM characteristics of these hindbrain sites, as well as supports a possible function for FGF signaling there.

FGF signaling is required for the expression of boundary-specific markers via FGF3.

Boundary cells express various genes that comprise diverse functions such as ECM molecules, structural proteins and transcriptional regulators (Guthrie et al., 1991; Heyman et al., 1995; Lumsden and Keynes, 1989). Based on the presence of FGF signaling components at boundaries (Fig. 1), we set out to determine whether FGF signaling is involved in the regulation of boundary marker expression. The following representative markers were tested (supplementary material Fig. S1); two ECM proteins (i) CSPG (Fig. 1J) and (ii) laminin, a major component of basal lamina which has previously been shown at hindbrain boundaries (Lumsden and Keynes, 1989), (iii)

Neuronal Stem Cell Leukemia 1 (NSCL1), a bHLH transcription factor which play roles in survival/maintenance of post-mitotic neurons (Theodorakis et al., 2002), (iv) The transcription factor *Brn3a*, which is expressed in sensory neurons (Dykes et al., 2010; Eng et al., 2007; Gruber et al., 1997; Helms and Johnson, 2003; Huang et al., 1999; Wang et al., 2002), and here we show its presence in hindbrain boundary cells), and (v) the neurofilament-associated antigen 3A10, which labels axonal neurofilaments, and although is seen in many hindbrain axons, it accumulates in the boundaries more extensively (Lumsden and Keynes, 1989).

First, FGFR activity was blocked in hindbrains of 14–15 HH embryos, stages in which FGFs and FGFRs are becoming confined to boundary cells (Weisinger et al., 2008), such that their activity is predominantly inhibited at these sites. Beads soaked in SU5402 (a pharmacological inhibitor of FGF receptor) or in DMSO were implanted into the hindbrain lumen for 18 hrs. SU5402-treated embryos presented a clear decrease in the levels of each of the markers (Fig. 2B,D,F,H,J respectively; CSPG n=7/8, Laminin n=8/8, 3a10 n=16/16, *Brn3a* n=11/12 and *NSCL1* n=15/18), as compared to the control (Fig. 2A,C,E,G,I respectively; CSPG n= 0/11, Laminin n=0/9, 3a10 n=0/14, *Brn3a* n=0/11 and *NSCL1* n=0/13). Notably, in both control and SU5402-treated embryos, some NSCL1 and 3A10-expressing cells can still be evident in rhombomeres, pointing against the possibility of a general abolishment of gene expression in the hindbrain by SU5402. Yet, the finding of much less expression of 3A10 and NSCL1 also in the rhombomeres upon su5402 treatment may indicate that boundary-derived FGF acts on adjacent hindbrain regions. As another control, we analyzed the expression of *Follistatin*, which is also expressed in hindbrain boundaries but is not regulated by FGFs (Weisinger et al., 2008). Both control (Fig. 2K n=15/15) and SU5402 (Fig. 2L n=15/15) treated embryos showed similar *Follistatin* expression,

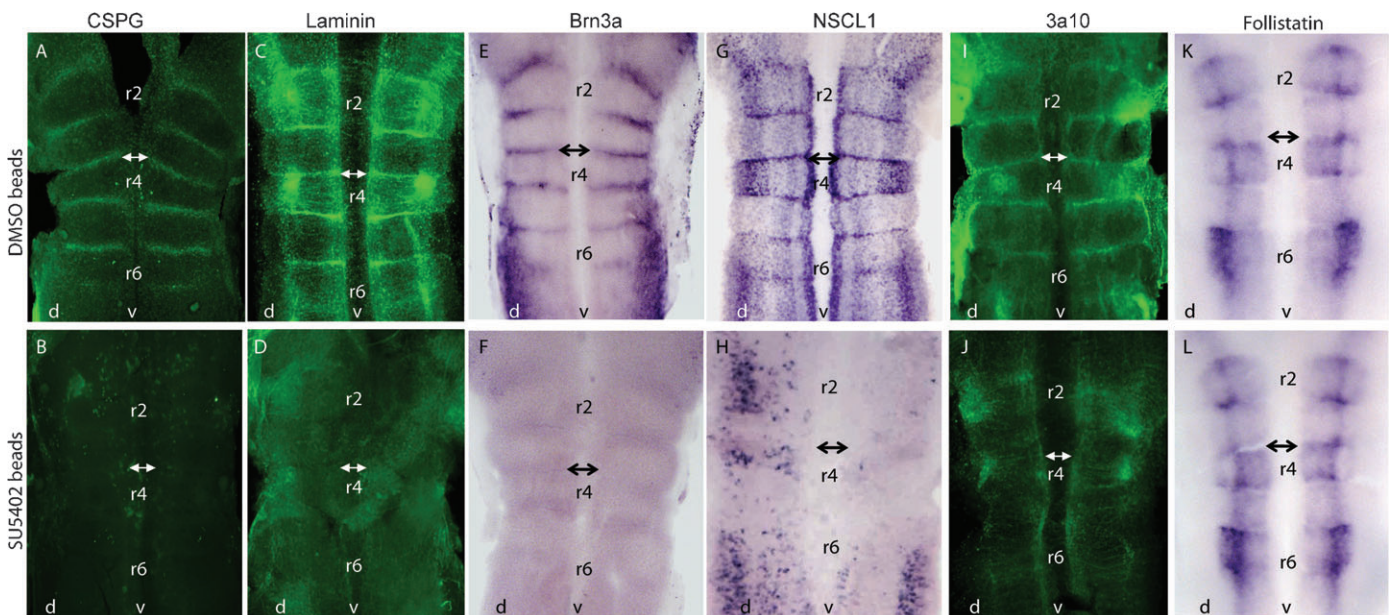


Fig. 2. FGF signaling regulates the expression of boundary markers. Embryos (14–15 HH) were implanted with beads soaked in DMSO (A,C,E,G,I,K) or SU5402 (B,D,F,H,J,L), and stained 18 hrs later for (A,B) CSPG, (C,D) Laminin, (E,F) *Brn3a*, (G,H) *NSCL1*, (I,J) 3a10 and (K,L) *Follistatin*. Rhombomeres are numbered, probes and antibodies are indicated, double-headed arrow indicated r3/r4 boundary, and anterior is at the top. Rhombomere (r), dorsal (d), ventral (v).

indicating that SU5402 effects do not cause general decrease on any boundary marker or delay in boundary marker appearance due to a hindrance in hindbrain development. Furthermore, no excessive cell death was apparent in the treated embryos, as tested by TUNEL assay (data not shown, see also Weisinger 2010). All together, these results indicate that FGF activity in the hindbrain is required for the expression of several boundary-specific genes.

The ligand FGF3 is restricted to boundary cells (Fig. 1A). We next assessed its direct contribution to the expression of the boundary markers by examining whether manipulation of FGF3 will affect their expression. FITC-conjugated morpholino antisense oligonucleotides (MO) directed against the FGF3 sequence, or a control MO, were electroporated into the hindbrain of stage 14–15 HH, stages in which FGF3 is already confined to boundaries (Weisinger et al., 2010). The expression of CSPG, Laminin, 3a10, *Brn3a* and *NSCL1* was analyzed 18–20 hr later. Importantly, this strategy was successfully used in a recent study to knock-down rhombomeric FGF3, which led to perturbation of hindbrain patterning at earlier stages (Weisinger et al., 2010). These MOs were shown to specifically act by decreasing FGF signaling activity, as demonstrated by the downregulation of the MAPK-downstream gene *Pea3*, without affecting genes which are not-related to the FGF pathway or cell death in the hindbrain (Weisinger et al., 2010). Here we show that all the boundary markers were normal in embryos electroporated with control MO (Fig. 3A,D,G,J,M respectively; CSPG n=11/13, Laminin n=18/19, 3a10 n=8/8, *Brn3a* n=14/16 and *NSCL1* n=16/17), but were greatly reduced in embryos electroporated with FGF3 MO (Fig. 3B,E,H,K,N, respectively; CSPG n=13/14, Laminin n=12/15, 3a10 n=9/12, *Brn3a* n=16/18 and *NSCL1* n=18/20). Green or red staining (Fig. 3A'–I' and J–O, respectively) indicate cells expressing the MO-FITC as detected by fluorescence (green) or by immunostaining (red). These findings suggest that FGF3 regulates the expression of ECM proteins (laminin, CSPG) and transcription factors (*Brn3A*, *NSCL1*), in boundary-cells as well as the accumulation of axons (shown by 3A10) at boundary cells. Notably, in agreement with the su5402 experiment, we show here that FGF3 MO also affects expression of 3A10 and *NSCL1* at non-boundary-regions, suggestive for an organizing role of boundary-derived FGF3 in the hindbrain. Another observation is that the FGF3 MO seems to cause non-cell autonomous effects, since the level of expression of the boundary markers were sometimes lowered even in domains which did not seem to contain FGF3-MO. This effect fits well with the suggested activity of FGF3 as a soluble ligand.

In order to confirm the requirement of FGF3 for these markers, as well as to further ascertain the specificity of the knock-down technique, we performed rescue experiments. The MO experiment was repeated and thereafter we added FGF3-soaked beads to examine whether exogenous FGF3 reverses the FGF3 MO effect on the expression of the selected boundary markers. The exogenous addition of FGF3 to the treated embryos rescued the FGF3 MO phenotype and yielded levels of expression which were close to controls (Fig. 3C,F,I,L,O, respectively; CSPG n=6/8, Laminin n=5/7, 3a10 n=10/15, *Brn3a* n=8/9 and *NSCL1* n=4/4), demonstrating the specific effect of the MO and of exogenous FGF3 on these particular markers. Furthermore, we ruled out the possibility that the MOs induce excessive cell death by performing TUNEL assay on the electroporated embryos, which revealed similar levels of TUNEL staining in both the

electroporated and control hindbrains (Fig. 3P,Q, see also (Weisinger et al., 2010)).

In our previous work, we showed that FGF3 has an early role in regulating *Krox20* expression (Weisinger et al., 2010). We, therefore, wanted to ensure that the results observed here are not due to defects caused by any disruption of initial hindbrain segmentation, but rather due to a later role for FGF3 in the boundaries. Embryos were electroporated as above and analyzed 20 hr later for both, gross hindbrain morphology as well for expression of *Krox20*. The segmentation of the hindbrain and the expression of *Krox20* seemed similar and intact in both, the FGF3 MO (Fig. 3S n=15/17, and 3U n=4/4) and control MO (Fig. 3R n=15/15, and 3T n=3/3). These experiments demonstrate that the segmental patterning of the rhombomeres is not altered and is not likely to be the cause for the reduction in the boundary markers. Moreover, this assay further shows that FGF3 MO does not yield unspecific effects on other genes or general developmental delay, as also previously shown in (Weisinger et al., 2010).

Altogether, these data show for the first time that boundary-derived FGF signaling, in particular FGF3, regulates the expression of molecules responsible for ECM integrity as well as genes supportive of neuronal differentiation and organization of boundary cells.

Discussion

The localization of FGFs, FGFRs and dpERK at hindbrain boundaries is suggestive of an active FGF signalling pathway at these sites. Similar expression of some FGF signaling components, such as FGF3, is also apparent at mice hindbrain boundaries (Powles et al., 2004), but not in other species such as zebrafish. Thus, although FGF signalling is well-known to be active at the developing hindbrain, its distribution and possible actions varies between different species. Moreover, our data shows that the distribution of the different FGF-signaling molecules is not identical at the boundaries (see also (Walshe and Mason, 2000)). For instance, FGFRs 1,3,4 spans the entire DV axis of boundaries (excluding the floor plate), whereas FGFR2 is more dorsally restricted. This suggests that some receptors are more prominent than others at different parts of the boundaries. Unravelling which of the FGFRs (and their precise spliced isoforms; Nishita et al., 2011) are indeed active at hindbrain boundaries will be necessary.

The accumulation of HSPG and CSPG, as well as the enhanced staining of proteoglycans, supports the possibility of the ECM involvement in promoting FGF signaling at the boundaries. A central role for FGF signalling is well-documented at the adjacent MHB, where FGF8 is expressed and regulates patterning, survival, proliferation and neurogenesis in the posterior midbrain, cerebellum and anterior hindbrain (Alexandre et al., 2006; Basson et al., 2008; Brand et al., 1996; Canning et al., 2007; Crossley et al., 1996; Jukkola et al., 2006; Liu and Joyner, 2001; Mason et al., 2000; Raible and Brand, 2004; Wassef and Joyner, 1997; Zervas et al., 2005). In addition, FGF signaling was shown to regulate the properties of the MHB itself, such as the morphological constriction and cell cycle rate of the boundary cells (Trokovic et al., 2005). Furthermore, proteoglycans were shown to be expressed at elevated levels at the MHB (Teel and Yost, 1996). Here we show that FGF signaling, and in particular FGF3, regulates the expression of multiple hindbrain boundary markers, indicating FGFs as regulators of hindbrain boundary properties. For instance, the finding that boundary-derived FGF3

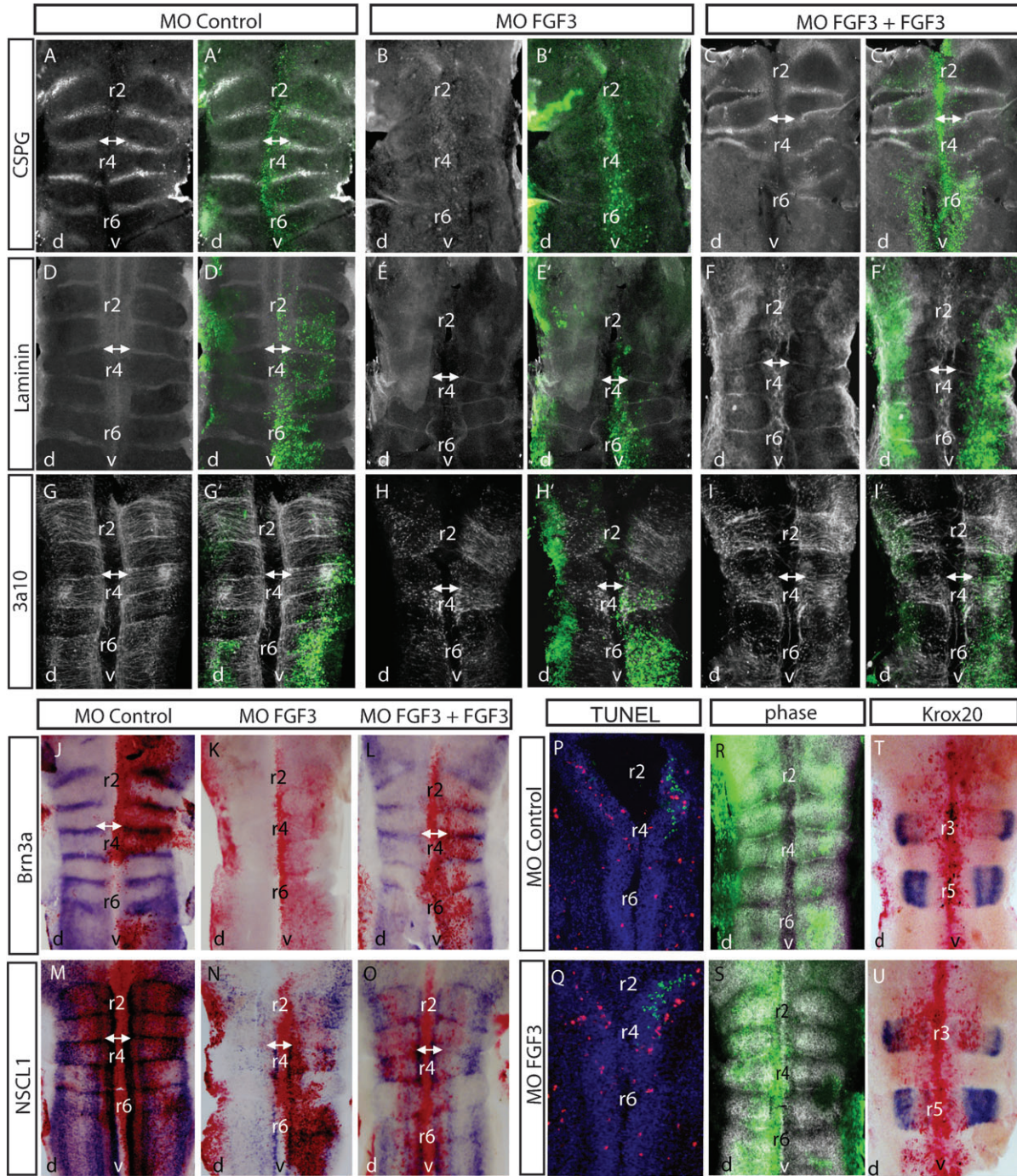


Fig. 3. FGF3 is required for expression of boundary markers. Embryos (14–15 HH) were electroporated with (A,D,G,J,M,P,R,T) control-MO or (B,E,H,K,N,Q,S,U) FGF3-MO, incubated for 18–20 hrs, and stained for (A–C) CSPG, (D–F) Laminin, (G–I) 3a10, (J–L) *Brn3a*, (M–O) *NSCL1*, (T,U) *Krox20* and (P,Q) TUNEL assay. Hindbrains are shown in flat mount preparations or in coronal section for the TUNEL images. (C,F,I,L,O) Rescue of embryos electroporated with FGF3 MO and thereafter FGF3-soaked beads were added. (R,S) Phase images of flat-mounted hindbrains after electroporation with (S) FGF3 MO or (R) control MO. All tagged images show the staining of their respective images merged with the area of electroporation. Red staining in (J–O, T, U) corresponds to MO-expressing cells detected with anti-fluorescein antibody. Green staining in (A'–I', P–S) corresponds to MO-expressing cells detected by fluorescence. Rhombomeres are numbered, probes and antibodies are indicated, double-headed arrow indicated r3/r4 boundary, and anterior is at the top. Rhombomere (r), dorsal (d), ventral (v).

positively regulates CSPG and laminin expression indicates its involvement in establishing the unique boundary ECM-properties. Yet, the significance of the enriched ECM at the rhombomere borders is not clear. It is possible that, in addition to optimize FGFR activity, the boundary-enriched proteoglycans

and basal lamina may be involved in axonal growth, since axonal guidance is influenced by the ECM in many instances (reviewed in (Kiryushko et al., 2004; Włodarczyk et al., 2011)). The well-known accumulation of axons at hindbrain boundaries (Lumsden and Keynes, 1989; Trevarrow et al., 1990), as well as the

perturbation of, both ECM accumulation and axonal localization that we show upon inhibition of FGF signalling, is supportive for such an option also in the hindbrain. Indeed, the MHB, as well as the DV boundaries of the neural tube (roof-plate and floor-plate), control axonal guidance, either as attractants or repellents (Augsburger et al., 1999; Bourikas et al., 2005; Butler and Dodd, 2003; Colamarino and Tessier-Lavigne, 1995; Hernandez-Montiel et al., 2003; Irving et al., 2002; Kennedy et al., 2006; Tessier-Lavigne et al., 1988). In addition, FGF signals were shown to act as direct chemoattractants in spinal-cord axons, or to control trochlear axonal growth in the MHB (Irving et al., 2002; Shirasaki et al., 2006).

Our data shows that the boundary-expression of the neuronal markers *Brn3a* and *NSCL1* is also controlled by FGF3. The significance of these markers at boundary cells remains to be explored. This is especially intriguing since the roles of *NSCL1* in other regions of the CNS are only partially understood (Chuan-Ming et al., 1999; Theodorakis et al., 2002) and since *Brn3a* is mostly considered as a marker for sensory neurons (Dykes et al., 2010; Lanier et al., 2007), and therefore its expression at boundary cells is surprising. Notably, MHB-derived FGF8 is known to either promote or inhibit differentiation of different types of neural progenitors at different DV parts of the midbrain and r1 (Alexandre et al., 2006; Basson et al., 2008; Brand et al., 1996; Canning et al., 2007; Crossley et al., 1996; Jukkola et al., 2006; Liu and Joyner, 2001; Mason et al., 2000; Raible and Brand, 2004; Saarimäki-Vire et al., 2007; Wassef and Joyner, 1997; Zervas et al., 2005). Moreover, in the zebrafish, FGF20, which is expressed at rhombomere centres, prevents neuronal differentiation at these sites, allowing neurogenesis to occur only at neighbouring regions (Gonzalez-Quevedo et al., 2010). The activity of FGF3 to induce boundary *Brn3a* and *NSCL1* gene expression is suggestive of neuronal regulative roles of FGF signaling at hindbrain boundaries.

Our data shows that the knockdown of FGF3 results in the downregulation of the multiple boundary markers non-cell autonomously. Such a result can be expected from a soluble signal that acts in some distance from its secretion source to positively regulate the transcription of these genes (Chen et al., 2009; Lahti et al., 2011; Tabata and Takei, 2004; Yu et al., 2009). Moreover, previous data from other's and our's works (Aragon and Pujades, 2009; Aragon et al., 2005; Marin and Charnay, 2000a, b; Maves et al., 2002; Walshe et al., 2002; Weisinger et al., 2010; Weisinger et al., 2008), have indicated non-cell autonomous actions of FGFs at earlier hindbrain stages in regional specification of rhombomeres.

In summary, this work provides the first evidence that boundary-derived FGF signaling, in particular FGF3, regulates the expression of multiple molecules responsible for ECM integrity, as well as genes involved in neuronal differentiation and axonal organization at boundary cells, and thus contribute to the understanding of how boundary-unique properties arise. Further evaluations are required to understand the precise mechanism(s) by which FGF3 controls the expression of these multiple markers, and the role of these genes at the hindbrain.

Materials and Methods

Embryos:

Fertile Loman chicken eggs were incubated at 38°C until embryos reached required somite stage. Following the incubation embryos were excised, fixed in 4% paraformaldehyde, dehydrated in 100% methanol, and stored at -20°C.

In ovo electroporation:

FITC-conjugated FGF3 or control antisense morpholino (MO) oligonucleotides (GeneTools, OR USA) were diluted in PBS to a working concentration of 2 mM. The sequences used are as follows: FGF3 MO: 5'-GCAGCAGGAGCCAGATCACGAGCAT-3' Control MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3'. MO oligonucleotides were injected into the hindbrain lumen of 22–25 somite-old embryos (14–15 Hamburger Hamilton stage) by using a pulled glass capillary. Following injection, electroporation was performed using a BTX 3000 electroporator with four 45 millisecond pulses of 16–18 volts and pulse intervals of 300 milliseconds (Itasaki et al., 1999). Embryos were incubated for a further 18–20 hrs.

Bead implantation:

AGX-100 beads were soaked at room temperature for 2 hr in SU5402 (200 µM, Calbiochem, CA USA) or DMSO. Heparin acrylic beads were soaked at 4°C for 2 hr in FGF3 (R&D systems, MN USA) diluted to 1 mg/ml in PBS-0.3%BSA. Beads were implanted in the hindbrain lumen of 20–25 ss (14–15 HH) embryos, which were then incubated for a further 18–20 hr. For rescue experiments, beads were implanted 2 hrs after electroporating the embryos with MO's. Beads were removed prior to in situ hybridization or immunostaining procedures.

Whole-mount in situ hybridization, immunohistochemistry and proteoglycan staining:

Whole-mount in situ hybridization was performed as described (Sela-Donenfeld and Kalchauer, 1999), using probes for chick *FGF3* (EST clone 812g6, MRC Geneservice), *FGF19* (a gift from H. Ohuchi), *NSCL1* (chick EST clone 474F24, BBSRC, UK), FGF receptors (*FGFRs*) 1,2,3 (a gift from E. Pasquale), *FGFR4* (a gift from C. Kalchauer), *Hoxb1* (a gift from R. Krumlauf) and *Brn3a* (a gift from A. Graham). The DIG labelled probes were detected using NBT/BCIP as substrate (Roche, Basel Switzerland), as described previously (Weisinger et al., 2008).

Alcian Blue staining was performed on hindbrains fixed overnight in 4% PFA in PBS at 4°C and thereafter washes in PBS. Safranin-O staining was performed on fresh hindbrains stored in PBS only. Both staining were carried out by incubating the hindbrains with 0.6% Alcian blue 8 GX or Safranin-O, respectively, for 5–10 minutes. Both dyes were washed with 70% ethanol in PBS (Dan et al., 2009; Reich et al., 2010).

Whole-mount immunohistochemical localisation of 3A10, Laminin and CSPG, was carried out by incubating embryos in PBS with 0.1% Tween20 (for CSPG and 3A10) or 0.1% Triton (for Laminin), and 5% goat serum for 2 hours, followed by addition of the following antibodies for overnight: mouse anti-3A10 (1:50, DSHB, USA), mouse anti-Laminin (1:50, DSHB, USA), mouse anti CSPG (1:50, Sigma USA), and rabbit anti EphA4 (1:250) ((Sela-Donenfeld et al., 2009). Following PBS + 0.1% triton washes, either anti-mouse Alexa 488, anti-mouse Alexa 594 or rabbit Alexa 613 (all 1:400, Molecular Probes) antibodies were added. Whole-mount immunohistochemical localisation of dual phosphorylated (dp) Erk1/2 proteins was performed as described previously (Corson et al., 2003; Weisinger et al., 2010), using rabbit anti-Erk1/2 antibody (1:350, Cell signaling technology, DV, USA), secondary anti-rabbit HRP-conjugated antibody (1:500, EnVision, Dako, Denmark) and AEC substrate system (Lab Vision, Thermo Fisher Scientific, CA, USA), to visualize HRP staining. Detection of FITC-conjugated MO oligonucleotides following in situ hybridization was performed using sheep anti-fluorescein (1:2000, Roche, Basel Switzerland) and Fast Red staining, as described elsewhere (Weisinger et al., 2008).

TUNEL assay:

Cell death detected in cryo-sectioned electroporated embryos by terminal transferase UTP nicked end labelling (TUNEL) using In Situ Cell Death Detection Kit, TMR red (Roche, Basel Switzerland), according to manufacturer's protocol.

Acknowledgments

This study was supported by the Israel Science Foundation and by the Niedersachsen-Israel Research Cooperation Program.

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