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Hindbrain patterning: FGFs regulate *Krox20* and *mafB/kr* expression in the otic/preotic region

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SUMMARY

Krox20 and _mafB/kr_ are regulatory genes involved in hindbrain segmentation and anteroposterior (AP) patterning. They are expressed in rhombomeres (r) r3/r5 and r5/r6 respectively, as well as in the r5/r6 neural crest. Since several members of the fibroblast growth factor (FGF) family are expressed in the otic/preotic region (r2-r6), we investigated their possible involvement in the regulation of _Krox20_ and _mafB/kr_. Application of exogenous FGFs to the neural tube of 4- to 7-somite chick embryos led to ectopic expression in the neural crest of the somitic hindbrain (r7 and r8) and to the extension of the _Krox20_- or _mafB/kr_-positive areas in the neuroepithelium. Application of an inhibitor of FGF signalling led to severe and specific downregulation of _Krox20_ and _mafB/kr_ in the hindbrain neuroepithelium and neural crest. These data indicate that FGFs are involved in the control of regional induction and/or maintenance of _Krox20_ and _mafB/kr_ expression, thus identifying a novel function for these factors in hindbrain development, besides their proposed more general role in early neural caudalisation.

Key words: Hindbrain patterning, *Krox20, mafB/kr,* Rhombomere, FGF, Chick

INTRODUCTION

Establishment of anteroposterior (AP) identity within the vertebrate hindbrain has been shown to involve a segmentation process leading to the formation of 7-8 successive AP territories, the rhombomeres (r). The rhombomeres behave as units of metamerism and specific gene expression (reviewed by Lumsden and Krumlauf, 1996). A number of these AP-restricted genes encode transcription factors and signalling molecules that have been implicated in hindbrain patterning, either in the delimitation and maintenance of specific territories (proper segmentation), or in the specification of their positional identity, or both (reviewed by Schneider-Maunoury et al., 1998). These genes are thought to be organised in a regulatory network and, indeed, genes involved in the segmentation process (*Gbx2*, *Krox20*, *Hoxa1*, *mafB/kr*) encode transcription factors shown to act upstream of Hox positional identity genes (reviewed by Schneider-Maunoury et al., 1998). However, much remains to be learned about the regulation of the segmentation genes themselves and how their restricted AP expression patterns are established.

The study of the regulation of *Krox20* and *mafB/kr* is likely to provide valuable information on upstream mechanisms governing hindbrain development: these genes are among the earliest ones to be expressed with an AP restricted pattern within the hindbrain and are required for the development of their territories of expression, r3 and r5 for *Krox20,* and r5 and r6 for *mafB/kr* (Frohman et al., 1993; Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993; McKay et al., 1994; Schneider-Maunoury et al., 1998; Manzanares et al., 1999). Recently, based on the analysis of *Krox20* and *mafB/kr* expression, we have provided evidence of plasticity in the caudal hindbrain, implying anteriorization mechanisms (Marin and Charnay, 2000). Prospective r6 or r7 are induced to express *Krox20* or *mafB/kr* respectively when grafted rostralwards into the otic/preotic (r2-r6) hindbrain. These data were consistent with the existence of signals in the r2-r6 region involved in the induction of *Krox20* and *mafB/kr*.

In the present work, we have investigated the possibility that fibroblast growth factors (FGFs) may play a role in this induction process. These molecules constitute a family of structurally related peptide growth factors involved in multiple developmental processes (review by Szebenyi and Fallon, 1999). FGFs functions have been extensively studied in early *Xenopus* development, where they have been implicated in mesoderm induction and posteriorization (Amaya et al., 1991), and in neural induction (Lamb and Harland, 1995; Streit et al., 2000). In addition, with respect to AP neural patterning, FGFs have been assigned a posteriorizing activity at gastrulation and early neurulation stages (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Holowacz and Sokol, 1999). Similarly, in the chick embryo, exogenous FGFs (either FGF2, FGF4, FGF8 or FGF9) can promote neural induction in the epiblast of stage 3-4 embryos, the induced neural tissue

predominantly presenting a posterior character (Henrique et al., 1997; Alvarez et al., 1998; Storey et al., 1998). In recombinant explants, the posteriorizing activity of FGF was shown to be mediated through mesoderm (Muhr et al., 1997) and its blocking led to exclusive formation of anterior, prosencephalic neural tissue (Muhr et al., 1999). This involvement of FGFs in neural tube posteriorization is consistent with their continuous expression at the caudal end of the embryo, in the region underlying the gastrulation process (Mahmood et al., 1995; Storey et al., 1998; Shamim and Mason, 1999; Streit and Stern, 1999). Finally, FGF8 has also been shown to participate in the isthmic organising activity involved in patterning the midbrain and r1 (Crossley et al., 1996; Irving and Mason, 2000).

The r2-r6 region and adjacent tissues constitutes another area of FGF expression. In the chick, *FGF3* is expressed from the 1-somite stage in prospective r4-r6. This pattern evolves so that at the 16- to 19-somite stage expression appears in inter-rhombomeric boundaries from r2 to r6. In addition, from the 5-somite stage onwards *FGF3* is expressed with a dynamic pattern in the ectoderm and endoderm of the branchial region (Mahmood et al., 1995). *FGF4* is expressed transiently in prospective r4-r6 approximately and in the adjacent paraxial mesoderm around the 1-somite stage, while from the 15-somite stage it is expressed in the endoderm of the branchial arches (Shamim and Mason, 1999). *FGF8* is expressed in the cardiogenic mesoderm (underlying the r2-r6 region) around the 6-somite stage and from the 11-somite stage onwards in the ectoderm and endoderm of the branchial arches (Wall and Hogan, 1995; Shamim et al., 1999). Although these FGF expression patterns are highly dynamic, a constant feature of the 1- to 19-somite stages is their restriction rostral to the somitic region.

To investigate the involvement of FGFs in the regulation of *Krox20* and *mafB/kr*, we first tried to mimic the effect of FGF signalling by applying exogenous FGF2, FGF4 or FGF8 onto 4- to 7-somite chick embryos, targeting principally the somitic hindbrain (r7 and r8) since this region is devoid of FGF expression as indicated above. We have found that the different FGFs have the capacity to induce ectopic *Krox20* and *mafB/kr* expression, affecting preferentially the neuroepithelium or the neural crest, depending on the given FGF. Furthermore, we have observed that application of a specific FGF receptor inhibitor leads to a strong reduction of the normal expression of these two genes. These data therefore suggest that FGFs participate in the regional control of *Krox20* and *mafB/kr* expression and of hindbrain patterning, in addition to their proposed more general posteriorization function at early stages.

MATERIALS AND METHODS

Grafting of FGF-beads

Fragments of heparin acrylic beads (Sigma H5263) were soaked for 1-2 hours in a solution containing 1 mg/ml of FGF in PBS. For control cases, the beads were soaked in PBS. We used human FGF2 (Sigma F0291), human FGF4 (R&D Systems 235-F4) and mouse FGF8b (R&D Systems 423-F8). The FGF-soaked bead fragments (FGF-beads) were grafted onto chick embryos in ovo (4- to 7-somite stages). The beads were inserted through the neuroepithelium and mesoderm (Fig. 1A,B). Embryos were reincubated for 16 hours (unless otherwise indicated), reaching stages of 15-19 somites. In some cases the embryo was split into rostral and caudal halves just before bead grafting (Fig. 1C).

In situ hybridisation

After reincubation, embryos and explants were fixed and processed for in situ hybridisation as described (Nieto et al., 1996), but eliminating the proteinase K treatment and subsequent fixation in paraformaldehyde-glutaraldehyde. We used antisense probes for *Krox20*, *mafB/kr* and *Hoxb1* (respective gifts from Drs D. Wilkinson, M. Sieweke and A. Lumsden). Embryos were photographed in toto, and then included in paraffin wax and cut in 20 or 10 μm thick sections.

Explants culture

For culture in presence of FGF2, explants were prepared from 4- to 6-somite stage chick embryos as shown in Fig. 3A. Explants including only neuroepithelium were dissected in Ca^{2+} and Mg^{2+} free Hanks solution. They were cultured in collagen gels according to the method of Dale et al. (1997). The medium consisted of OPTIMEM 1 plus glutamine (Gibco), antibiotic-antimycotic solution (Gibco) and 10% foetal calf serum (Techgen). Explants were grown for 16 hours at 37.5°C in an atmosphere of 5% CO_2 and subsequently fixed and processed for in situ hybridisation. For exposure to SU5402, portions of 4- to 6-somite embryos, including the whole head reaching caudally to the 4th-6th somite, were explanted and cultured as mentioned above. SU5402 (Calbiochem) was added to the medium at a final concentration of 20 μM. Explants were fixed after 16-20 hours of reincubation, and processed for in situ hybridisation.

Quail-chick chimeras

Grafts were performed from quail to chick embryos at the 4- to 6-somite stage as described by Marin and Charnay (2000). Chimeric embryos were reincubated for 16 hours, reaching the 15- to 19-somite stage. They were fixed, processed for in situ hybridisation and subsequently for immunohistochemistry with the QCPN monoclonal antibody (Hybridoma Bank), using a secondary antibody coupled to peroxidase, and developed with diaminobenzidine.

RESULTS

FGF2 induces ectopic *Krox20* and *mafB/kr* expression in the neural crest

In a first set of experiments we grafted FGF2-soaked bead fragments (FGF2-beads) at the level of the first somite (either rostrally or caudally) in 4- to 7-somite embryos (Fig. 1A,B). 7 control embryos were grafted with PBS-beads. 4 of them were processed for *Krox20* RNA detection and 3 for *mafB/kr* RNA detection. In these cases, normal *Krox20* and *mafB/kr* expression patterns were observed, with *Krox20* mRNA detected in r3 and r5 and in neural crest cells emigrating from r5 and r6 (Fig. 1D and Nieto and collaborators, 1995) and *mafB/kr* mRNA in r5 and r6, and the derived neural crest, as well as in the prospective choroideal plexus of the otic/preotic hindbrain (Fig. 2A and Eichmann and collaborators, 1997).

Nineteen embryos were grafted with FGF2-beads. Nine of them were processed for *Krox20* RNA detection and 10 for *mafB/kr* RNA detection. In one case of the latter group the embryo received two beads instead of one, inserted respectively rostrally and caudally to the first somite (Fig. 2C). In every case, ectopic *Krox20* or *mafB/kr* expression was observed in the somitic neural crest (snc in Figs 1E,F, 2B,C). The positive cells were located either close to the bead (Figs

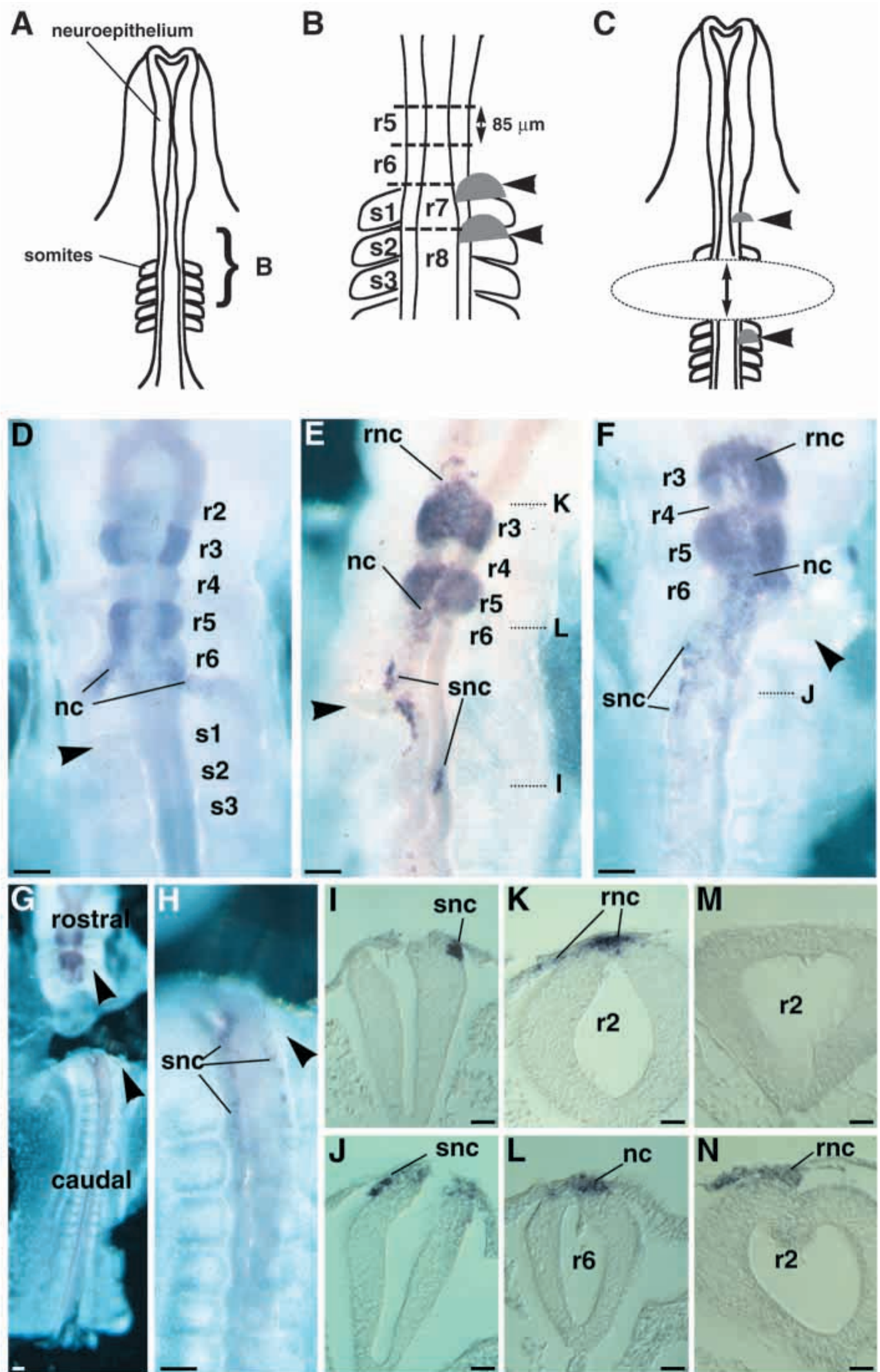

Fig. 1. Effects of FGF2 on *Krox20* expression analysed by whole-mount in situ hybridisation. (A) Drawing of a 5-somite embryo. The bracket indicates the area involved in most of the experiments. (B) Higher magnification view of the experimental area. The dashed lines indicate the limits of prospective rhombomeres 5, 6 and 7, according to Grapin-Botton and collaborators (1995) and Marín and Charnay (2000). PBS- or FGF-beads (arrowheads) were inserted through neuroepithelium and mesoderm. (C) In another set of experiments, the embryo was split at the limit between first and second somites, cutting through to the yolk, so that both halves remained separated. Beads were grafted respectively at the r5 level in the rostral half, and between the second and third somites in the caudal half (arrowheads). (D) Control embryo grafted with a PBS-bead (arrowhead). (E) Embryo in which a FGF2-bead was grafted caudally to the first somite (arrowhead). Note the abnormal presence of positive neural crest at the r2/r3 (rnc) and somitic levels (snc), in contact with or far away from the bead; in the dorsal edge of r5 and r6, the normally *Krox20*-positive neural crest (nc) remains stacked. (F) Another embryo, in which the FGF2-bead (arrowhead) was implanted rostrally to the first somite, shows effects similar to those in E. Note the reduced size of r4, principally on the side of the bead. (G) Embryo split into rostral and caudal halves, as in C, with FGF2-beads grafted in each of them (arrowheads). (H) Results of G, showing *Krox20* induction in the neural crest of the caudal half (snc). (I,J) Transverse sections of the embryos in E and F at the levels indicated by the dashed lines, showing staining in neural crest cells at somitic levels (snc). (K) Transverse section of the embryo in E showing positive neural crest cells (rnc) at the level of the r2/r3 interface. (L) Transverse section of the same embryo at the level of r6, showing the presence of positive neural crest cells (nc) which have delaminated from the neuroepithelium but have failed to migrate. (M,N) Transverse sections of control (M) and FGF2-treated (N) embryos at the r2 level showing presence of *Krox20*-positive neural crest cells (rnc) in the FGF2-treated case. Scale bars: D-H, 100 μm; I-N, 25 μm.

1E, 2C), relatively far away or on the contralateral side (Figs 1F, 2B). The AP location of the *Krox20*-positive cells varied between embryos, but they were always within the region of the first four somites (Fig. 1E,F). *mafB/kr*-positive cells were observed more caudally, down to the level of somites 5 to 10 (Fig. 2B). In most cases the bead was found detached from the neuroepithelium and/or shifted from the intended position, but this did not affect ectopic gene expression. Transverse sections confirmed that the positive cells were delaminating neural crest cells (snc in Figs 1I,J, 2H,I). *mafB/kr* was also expressed in the roof plate (rp in Fig. 2H,I). Finally, implantation of FGF2-beads was frequently associated with another site of *Krox20* ectopic expression, consisting of delaminating neural crest cells at the level of r3 and caudal r2 (n=7/9, rnc in Fig. 1E,F; sections in Fig. 1K,M,N). This expression may result from de novo activation of *Krox20* or, alternatively, from failure to downregulate its expression in r3-derived neural crest. Since the FGF2-beads were implanted at the first somite level,

modification of gene expression in r2-r3 crest represents a very long range effect. This may result from diffusion of FGF2 within the ventricle and/or the extraembryonic space.

In 4 out of the 9 embryos processed for *Krox20* RNA detection, we noticed an additional effect of FGF2 exposure: the neuroepithelial domain of *Krox20* expression, which in control embryos corresponds to r3 and r5, was enlarged in comparison to r4 (Fig. 1E,F and data not shown). This widening was more evident in embryos exposed to FGF4 or FGF8 (see below).

We performed additional experiments to exclude the possibility that the caudal *Krox20*- or *mafB/kr*-positive neural crest cells may have originated only by migration from the r5-r6 region. Before grafting the beads, the embryos were cut between the first and second somites and the two portions were separated (Fig. 1C). Beads were grafted at the r5 level and between the second and third somites. In 5 control cases we grafted PBS-soaked beads, and processed 3 of them for *Krox20* RNA detection and 2 for *mafB/kr* RNA detection. A normal expression pattern was observed in each case (Fig. 2D,E and data not shown). When FGF2-beads were grafted, induction of *Krox20* (n=4/4) and *mafB/kr* (n=4/4) expression was observed in the somitic neural crest (Figs 1G,H, 2G), although these cells cannot originate from the r5-r6 region. These data therefore demonstrate that ectopic activation of *Krox20* and *mafB/kr* occurs in the somitic neural crest.

Since *Krox20* is an immediate early response gene to growth factors, we addressed the possibility that FGF2 may have rapid effects on its expression. For this purpose, a time-course analysis was performed. Embryos were grafted with FGF2-beads at the level of the first somite and analysed for *Krox20* expression after 90 minutes (n=8) or 8 hours (n=9) incubation. After 90 minutes, no ectopic *Krox20* expression was detected, while after 8 hours a few neural crest cells were found positive in the vicinity of the beads (data not shown). These data indicate that induction of *Krox20* by FGF2 is a slow process which may require intermediate steps.

Beside ectopic activation of *Krox20* and *mafB/kr*, the presence of FGF2-beads led to the disappearance of the normal stream of *Krox20*- or *mafB/kr*-positive neural crest originating from r5 and r6. Among the 9 cases processed for *Krox20* RNA detection, 8 showed a bilateral disappearance (Fig. 1E,F) and one case was unilateral (data not shown). Among the 10 cases processed for *mafB/kr* expression, 7 showed a bilateral effect (Fig. 2B) and 3 were unilateral (Fig. 2C). Nevertheless, both *Krox20*- and *mafB/kr*-positive neural crest cells were present at the r5-r6 level, but they appeared stacked on the dorsal edge of the neural tube (nc in Figs 1E,F,L, 2B,F,J).

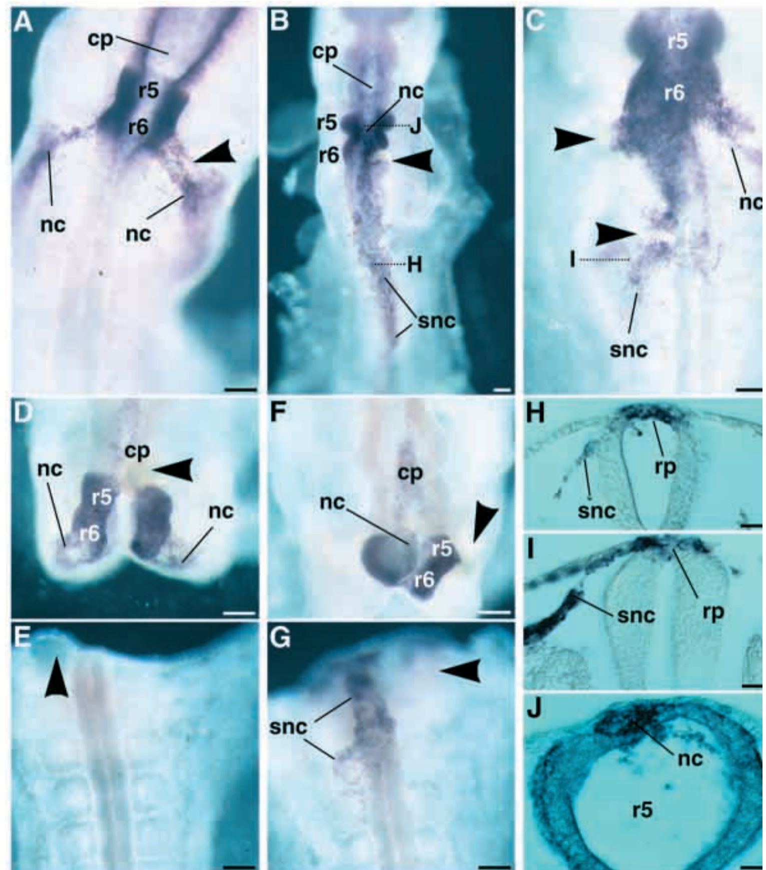

Fig. 2. Effects of FGF2 on *mafB/kr* expression. (A) Control embryo grafted with a PBS-bead (arrowhead). (B) Embryo grafted with a FGF2-bead (arrowhead). Note the absence of the neural crest streams originating from the r5-r6 region and the strong ectopic expression along the somitic region (snc). (C) Embryo grafted with one FGF2-bead rostrally and one caudally to the first somite. Note the ectopic induction in the neural crest of the somitic region (snc) and the inhibition of r5-r6 neural crest migration on the grafted side. (D) Rostral and (E) caudal halves of a split embryo (as shown in Fig. 1C), with PBS-beads (arrowheads) grafted on each half. Note the normal neural crest (nc) streaming from r5 and r6, while the caudal somitic region is negative. (F) Rostral and (G) caudal halves of a split embryo with FGF2-beads grafted on each side (arrowheads). In F note that the positive neural crest originating from r5-r6 (nc) remains stacked at the dorsal edge, while the expression in the prospective choroideal plexus (cp) is normal. In G note the ectopic expression in migrating neural crest cells in the somitic region (snc). (H,I) Transverse sections at the somitic level of the embryos in B and C respectively, showing ectopic *mafB/kr* expression in the roof plate (rp) and the neural crest (snc). (J) Transverse section of the embryo in B, showing the stacking of neural crest cells (nc) at the r5 level. Scale bars: A-G, 100 µm; H-J, 25 µm.

Dose-dependent induction of *mafB/kr* expression by FGF2 in cultured explants

To further investigate *mafB/kr* gene activation by FGF2, we cultured different types of explants from 4- to 6-somite embryos in the presence or absence of FGF2. In a first series of experiments, rostral (r1-r7) and caudal explants (posterior to the first somite), including the neighbouring non-neural tissues, were prepared (Fig. 3A). Rostral explants were taken as controls for normal *mafB/kr* expression. Caudal explants cultured in absence of FGF2 were negative for *mafB/kr* expression (Fig. 3B; n=6/6). When exposed to FGF2 concentrations ranging from 10 to 200 ng/ml, these explants expressed *mafB/kr* in a dose-dependent manner (10 ng/ml, n=5, Fig. 3C; 50 ng/ml, n=2, Fig. 3D; 100 ng/ml, n=4, data not shown; 200 ng/ml, n=6, Fig. 3E).

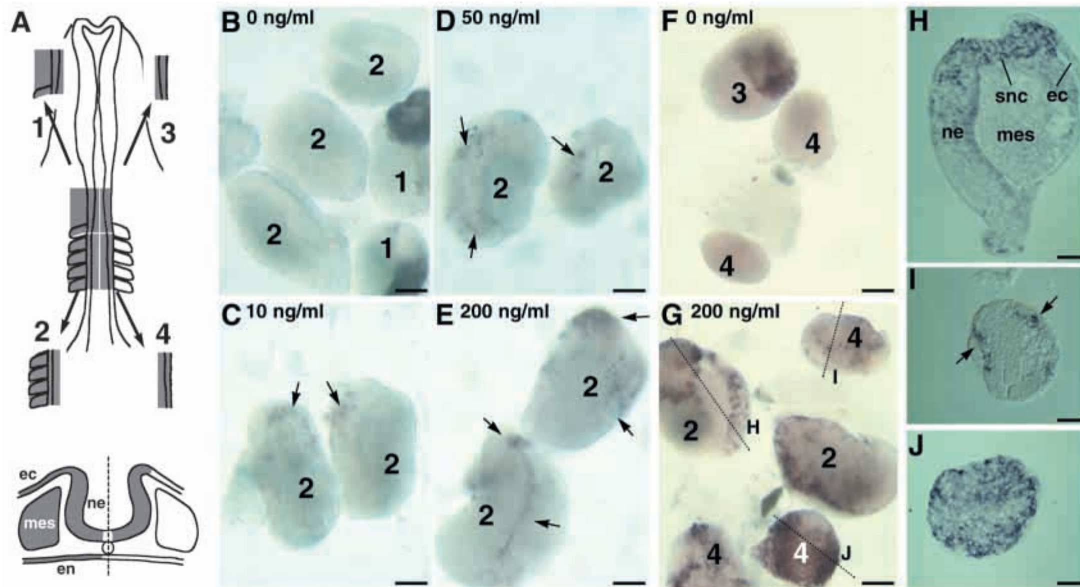

Fig. 3. FGF2 induces *mafB/kr* expression in vitro. (A) Upper: drawing of an embryo showing the different types of explants: (1), rostral hindbrain and adjacent mesoderm, ectoderm and endoderm, extending to the level between first and second somites; (2), caudal hindbrain (somites 2-5 or 2-4) including neuroepithelium and adjacent tissues; (3) and (4), same rostrocaudal regions as (1) and (2) respectively, but including only the neuroepithelium and the dorsal edge of the ectoderm. Lower: transverse view, showing the extension of the explants of type 1 and 2 (left side) and of type 3 and 4 (right side). (B-E) Effects of different concentrations of FGF2 on *mafB/kr* expression on caudal type 2 explants. Note the increased expression (arrows) in relation to the concentration. (F,G) Induction by FGF2 occurred in caudal neuroepithelium either in the presence or absence of adjoining somites (type 2 and 4 explants, respectively). (H-J) Examples of sections of explants shown in G. H corresponds to a type 2 explant and I and J to type 4 explants. In H induction can be observed in neuroepithelium (ne) and/or in neural crest cells (snc). In I, positive cells are indicated by arrows. ec, ectoderm; mes, mesoderm; ne, neuroepithelium. Scale bars: B-G, 100 µm; H-J, 25 µm.

To test for a possible involvement of non-neural tissues, in particular the somites, in *mafB/kr* induction in the neural tube, explants of caudal hindbrain were dissected free from non-neural tissues, leaving just the edge of ectoderm contacting the neuroepithelium (Fig. 3A). These explants showed no *mafB/kr* expression when cultured without FGF2 (*n*=4/4; Fig. 4F). When cultivated in the presence of 200 ng/ml of FGF2, a clear induction was observed (*n*=7; Fig. 3G-J) and no difference in the induction level was found with the explants containing somitic tissue. These experiments indicate that in these in vitro conditions FGF2 can induce *mafB/kr* expression in the neural tissue of somitic levels and can act directly on this tissue.

FGF4 and FGF8 induce *Krox20* and *mafB/kr* expression in both the neuroepithelium and the neural crest

To determine whether the effects of FGF2 could be mimicked by other members of the FGF family, 4- to 6-somite embryos were implanted with beads soaked into FGF4 or FGF8 and, following incubation, processed for *Krox20* or *mafB/kr* RNA detection. These experiments involved either the grafting of one bead at the level of the first somite, or of two beads at the r2 and first somite levels. The second variation was introduced to study effects at the r2-r3 level, since preliminary experiments showed a shorter range of action of FGF4 or FGF8 than FGF2.

Krox20

FGF4 and FGF8 led frequently to ectopic induction of *Krox20* in the caudal neural crest (*n*=5/13 and 5/12 respectively; snc in Fig. 4A,B,G). In contrast to the long distance induction observed with FGF2, *Krox20* expression was restricted to areas close to the grafted bead. *Krox20*-positive neural crest was also observed at the r2-r3 level when a bead had been inserted at the r2 level (*n*=5/7 for FGF4 and *n*=3/4 for FGF8; rnc in Fig. 4A,B,H). FGF4 and FGF8 also led to an extension of the domain of expression of *Krox20* in the neuroepithelium, which was more evident in the side receiving the beads. This effect included an enlargement of the r5 domain (compare the size of r5 between experimental and control sides in Fig. 4B-E,H), and/or the formation of *Krox20*-positive patches within r4 or r6 (arrows in Fig. 4D,E,I), which in one case almost covered the entire r6 territory (arrow in Fig. 4G). A possible extension of the r3 domain was apparent in some experiments (compare in Fig. 4G the size of r3 between experimental and control sides), but was more difficult to assess. In parallel with the extension of r5 and possibly of r3, the size of the r4 *Krox20*-negative domain was significantly reduced, principally on the side receiving the beads (Fig. 4A-E,H).

mafB/kr

Exogenous FGF4 led to ectopic *mafB/kr* expression in the neural crest of the somitic region in areas close to the grafted bead (*n*=9/13; Fig. 4F and cross sections not shown), with no significant extension of the r5-r6 domain in the neuroepithelium. In contrast, FGF8 led to systematic induction (*n*=7/7) both in the neural crest and in r7 neuroepithelium, possibly extending into r8. This induction in the neural tube was unilateral in 5 cases (Fig. 4J-L), and bilateral in the other two (data not shown). The embryos in which a second FGF4-

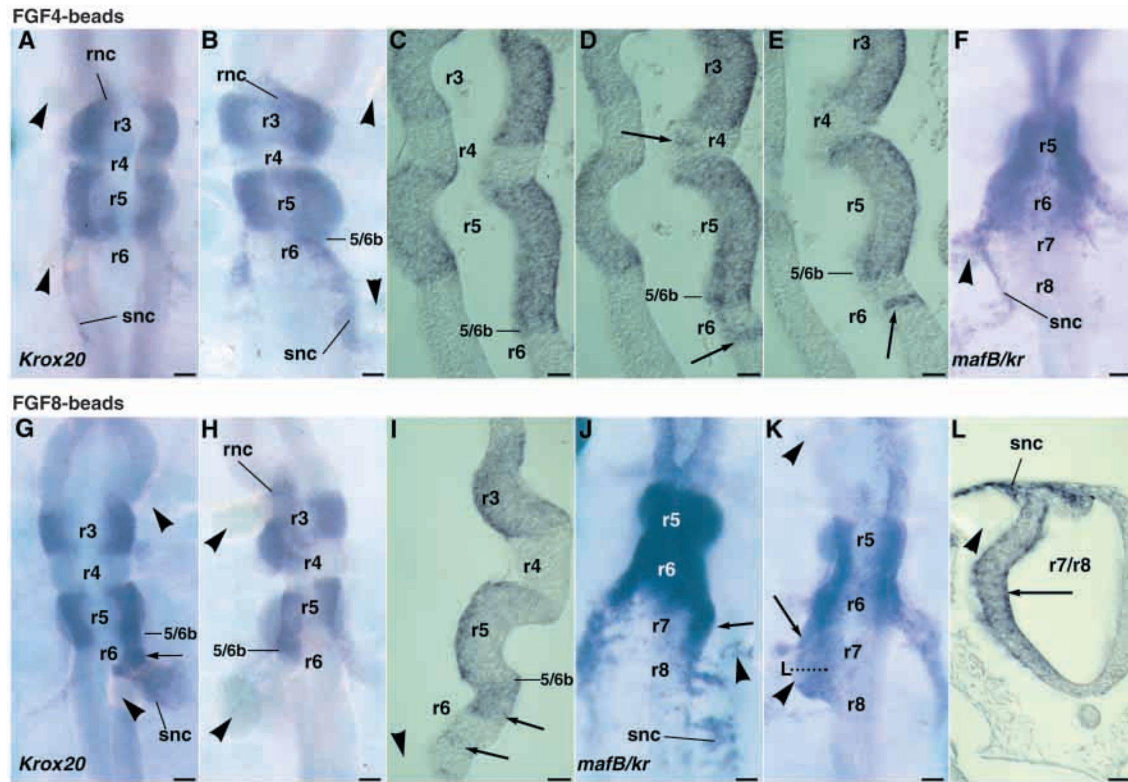

Fig. 4. Effects of FGF4 and FGF8 on *Krox20* and *mafB/kr* expression. (A,B) Two embryos grafted with FGF4-beads (arrowheads) at the levels of r2 and of the first somite were processed for *Krox20* RNA detection. Note the presence of positive neural crest at r2/r3 (rnc) and somitic (snc) levels, and the rostrocaudal widening of the neuroepithelial positive areas (r3 and r5). This enlargement of r3 and r5 is accompanied by a reduction of the size of the r4 territory (bilaterally in A; ipsilaterally in B). In B the caudal limit of r5 in the grafted side (5/6b) is shifted caudalwards. (C-E) Horizontal sections of the case shown in B, ordered from dorsal to ventral levels. Note the reduction of r4 in the grafted side, the caudalwards shifting of the r5/6 boundary and the formation of ectopic *Krox20*-positive patches (arrows) within r4 and r6. (F) Embryo grafted with a FGF4-bead (arrowhead) and processed for *mafB/kr* RNA detection. Note the ectopic *mafB/kr* expression in the caudal neural crest (snc) on the grafted side. (G) Embryo grafted with two FGF8-beads (arrowheads), at the r2 and first somite levels and processed for *Krox20* detection. There is ectopic expression of *Krox20* in r6 (arrow) and in the somitic neural crest (snc). (H) Embryo grafted with two FGF8-beads (arrowheads). Note the induction of *Krox20* in the r2-r3 neural crest (rnc), the reduction of r4 on the grafted side (left) and the enlargement of r5 by a caudalwards shifting of the r5/6 boundary (5/6b) on the grafted side. (I) Horizontal section of the embryo in H, showing ectopic *Krox20* expression in the r6 territory (arrows). (J,K) Two embryos grafted with FGF8-beads (arrowheads) and processed for *mafB/kr* mRNA detection. In J one bead was grafted at the first somite level, and in K two beads were inserted at the r2 and first somite levels. Note in both cases the ectopic expression in r7 and probably r8 neuroepithelium (arrows), and in the somitic neural crest (snc). (L) Transverse section of the embryo presented in (K), showing expression in the neuroepithelium (arrow) and the neural crest (snc) at the r7-r8 level. Scale bars: A,B,F-H,J,K, 50 μm; C-E,I,L, 25 μm.

or FGF8-bead was grafted at r2-r3 level showed no alteration of *mafB/kr* expression in this area (Fig. 4K and data not shown). In some cases a reduction of the stream of neural crest emigrating from r5-r6 was observed (see for example the experimental side in Fig. 4A,J), but this effect was less evident and reproducible than with FGF2.

In conclusion, these experiments indicate that, like FGF2, exogenous FGF4 and FGF8 can lead to ectopic induction of *Krox20* and *mafB/kr* in the developing hindbrain. However, they differ in that their range of action is reduced, yet they exert a stronger effect on the neuroepithelium. The observed extension of the r3 and r5 territories may occur in part at the expense of r4.

Reduction of the size of r4 following FGF treatment

The reduction in size of the *Krox20*-negative domain at the level of r4 after exposure to exogenous FGF led us to study the expression of an r4 molecular marker, *Hoxb1,* after implantation of FGF beads at the r2-r3 and first somite levels. An almost systematic reduction of the size of the r4 *Hoxb1*-positive territory was observed with FGF2 (4 cases out of 6), FGF4 (5 cases out of 5) and FGF8 (4 cases out of 5), together with an increase of r3 and r5 (Fig. 5). However, in the remaining *Hoxb1*-positive r4 territory, the level of expression was not significantly affected as compared to control embryos, and the caudal domain of expression of *Hoxb1*, including ectoderm, endoderm and neural tube, was not affected (Fig. 5 and data not shown).

In conclusion, FGF treatment leads to a reduction of the size of r4 in parallel to the extension of the *Krox20*- and *mafB/kr*-positive territories, while the level of expression of *Hoxb1* in the remaining r4 territory is not significantly affected. These data support the idea that the extension of r5 and r3 may occur by recrutment of r4 cells following activation of *Krox20*.

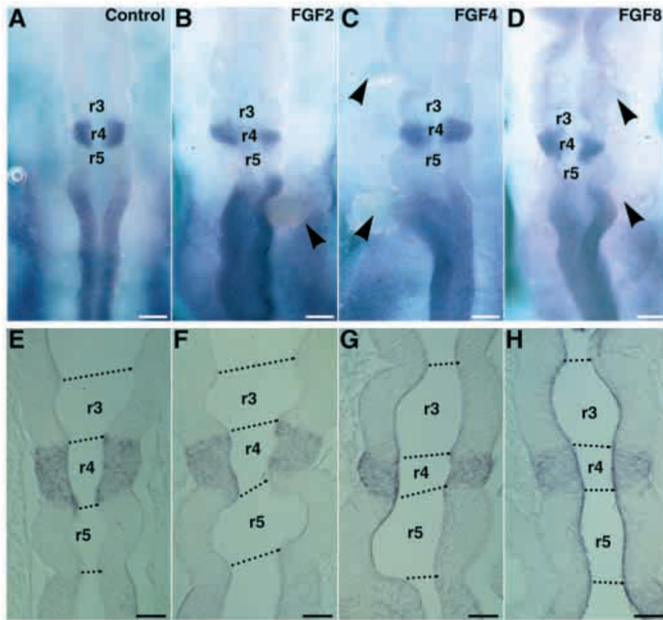

Fig. 5. Effects of FGFs on *Hoxb1* expression. (A) Control embryo showing normal *Hoxb1* expression in the r4 neuroepithelium and in the somitic region. (B-D) Embryos that received FGF2-, FGF4- and FGF8-beads respectively at the levels indicated by the arrowheads. (E-H) Horizontal sections of the above embryos. Note the rostrocaudal reduction of r4 and the size increase of r3 and r5. Scale bars: A-D, 100 μm; E-H, 50 μm.

Induction of *Krox20* and *mafB/kr* expression in the caudal neural crest in transplantation experiments

Consistent with the effects of FGFs described above and with the presence of FGFs in the r2-r6 region, we have previously shown that *Krox20* and *mafB/kr* can be induced in r6 and r7 neuroepithelium respectively, upon contact with a rostral environment (Marin and Charnay, 2000). We have also found that neural crest derived from r8 (adjacent to the second somite) can be driven to express these genes after rostral grafting. Since the present work indicates that more caudal neural crest also has the plasticity to express these genes, we investigated whether this induction can be reproduced upon rostral grafting.

Prospective quail neural crest from different caudal somitic levels was grafted at the level of prospective chick r5 (Fig. 6A). In the two cases processed for *Krox20* detection, in which the neural crest originated from the level of the third somite, a large portion of the grafted neural crest appeared positive for *Krox20* (snc, Fig. 6B,C). In the three cases processed for *mafB/kr* detection, in which the neural crest originated respectively from the third, fourth and fifth somites, grafted neural crest was largely positive for *mafB/kr* (snc, Fig. 6D,E).

We also grafted a lateral portion of the embryo, including endoderm, mesoderm and ectoderm, from otic/preotic into somitic levels (Fig. 6F). Two cases were processed for *Krox20* and two for *mafB/kr* RNA detection. In every case we found that these genes were induced in a population of neural crest cells migrating from the adjacent host chick neuroepithelium into the grafted tissue (snc, Fig. 6G-J). These experiments indicate that similar results are obtained by application of exogenous FGF or by contact with a region likely to be a physiological source of FGFs.

SU5402 inhibits *Krox20* and *mafB/kr* expression in the hindbrain

To investigate whether FGF signalling is required for normal expression of *Krox20* and *mafB/kr* in the hindbrain, we made use of SU5402, an inhibitor of the tyrosine kinase activity of FGF receptors (FGFRs) (Mohammadi et al., 1997; Muhr et al., 1999). The head portion of 4- to 6-somite embryos (to somites 4-6) was explanted and incubated in the presence or absence of 20 μM SU5402 for 16-20 hours. Control explants processed for *Krox20* RNA detection (*n*=10) showed normal expression in r3 and r5 (Fig. 7A), while SU5402-treated whole-mount explants (*n*=11) all appeared negative (Fig. 7B). Sections through the latter explants however revealed a very low level staining at the expected r3 and r5 levels (data not shown). Concerning the neural crest, a *Krox20*-positive stream originating from r5-r6 was apparent in 4 out of the 10 control cases (nc, Fig. 7A), but in none of the SU5402-treated cases.

mafB/kr presented a normal expression pattern in all control explants (*n*=7), including r5, r6, the derived neural crest and the choroideal plexus (Fig. 7C,E). In all SU5402-treated explants (*n*=9), expression in r5-r6 and the derived neural crest was eliminated (Fig. 7D,G) or was reduced to very low levels (Fig. 7F). Strikingly, expression in the choroideal plexus appeared unaffected (cp, Fig. 7D-G). In addition, in some embryos it was possible to observe a caudal staining which, according to the established pattern of expression (Eichmann et al., 1997), may correspond to the mesonephros (m, Fig. 7C,D). Mesonephros staining was observed in 3 out of 7 control embryos and in 4 out of 9 SU5402-treated embryos, suggesting that *mafB/kr* expression in this region, like in the choroideal plexus, was not affected by the FGF inhibitor.

To investigate whether SU5402 was generally altering gene expression in otic/preotic hindbrain, we studied its effect on *Hoxb1* expression. Control explants (*n*=23) showed the expected expression in the neural tube in r4 and *Hoxb1* RNA was also detected in the endoderm (Fig. 7H). However, in these in vitro conditions, the r5-r6 region did not downregulate *Hoxb1* expression as happens in normal embryos (Fig. 7H). 22 out of 25 SU5402-treated explants showed the same qualitative pattern, with a slight reduction in the level of expression in the neural tube and a more severe reduction in the endoderm (Fig. 7I). Only 3/25 SU5402-treated explants showed a general inhibition of *Hoxb1* expression (data not shown).

In conclusion, SU5402 strongly inhibits *Krox20* and *mafB/kr* expression in the neuroepithelium and the neural crest, while it has only a marginal effect on *Hoxb1* expression in r4 and no visible effect on *mafB/kr* expression in the choroideal plexus and the mesonephros. This suggests that FGF signalling is involved in the normal regulation of *Krox20* and *mafB/kr* expression in the developing hindbrain.

DISCUSSION

The present work indicates that FGF signalling is involved in the establishment of the normal pattern of expression of *Krox20* and *mafB/kr* in the developing hindbrain, both in the neural crest and in the neuroepithelium. This identifies a novel

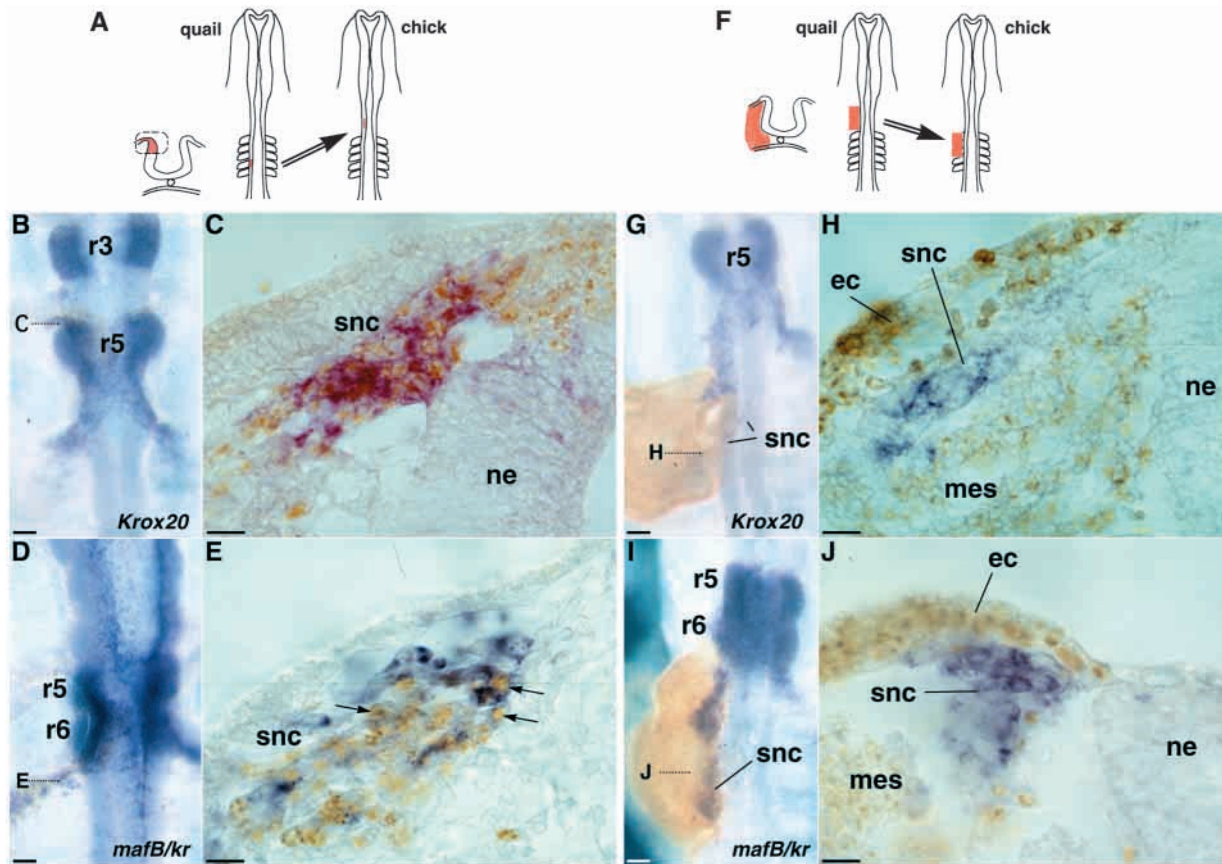

Fig. 6. Induction of *Krox20* and *mafB/kr* expression in the somitic neural crest in quail-chick transplantations. (A) Diagram of neural crest grafts from caudal (third to fifth somite) to rostral (r5-r6) levels. The transverse section shows the region taken as prospective neural crest in red. (B,C) Chimera processed for *Krox20* RNA detection (blue-red precipitate) and quail tissue detection (brown precipitate). (B) Whole-mount embryo, (C) transverse section. A large portion of the grafted quail neural crest appears positive for *Krox20*. (D,E) Whole-mount view and transverse section of a chimera processed for *mafB/kr* RNA and quail tissue detection, showing induction in the grafted neural crest (snc). Arrows indicate some of the cells that are positive for both markers (brown nucleus and blue cytoplasm). (F) Diagram to show grafts of mesoderm, ectoderm and endoderm from otic into somitic regions. (G,H) Whole-mount view and transverse section of a chimera processed for *Krox20* RNA and quail tissue detection. There is an ectopic stream of *Krox20*-positive cells (snc) originating from the host chick neuroepithelium adjacent to the graft and migrating into it. (I,J) Whole-mount view and transverse section of a chimera processed for *mafB/kr* RNA and quail tissue detection, showing induction of this gene in caudal neural crest of the host (snc). Scale bars: B,D,G,I: 50 μm; C,E,H,J, 25 μm.

role for FGFs in hindbrain patterning, besides their postulated more general function in the posteriorization of the neural anlage.

FGFs are involved in *Krox20* and *mafB/kr* induction in the neural crest

FGF signalling has been implicated in the generation of the prospective neural crest at the limits between neural and epidermal anlagen (reviewed by Baker and Bronner-Fraser, 1997; Streit and Stern, 1999). We provide evidence for a novel function of FGFs in relation to the rostrocaudal molecular patterning of the neural crest. Addition of exogenous FGFs in vivo led to ectopic induction of *Krox20* and *mafB/kr* expression in the somitic neural crest. Furthermore, exposure to an inhibitor of FGF signalling prevented normal neural crest expression of these genes. Also grafting experiments indicating that *Krox20* and *mafB/kr* can be induced in the somitic neural crest placed in contact with tissues from the r4-r6 region, which may constitute an endogenous source of FGFs. Together these data suggest that FGFs are involved in the rostrocaudal specification of the incipient neural crest by inducing or maintaining *Krox20* and *mafB/kr* expression.

The known patterns of expression of FGF genes in the hindbrain and branchial region are complex and dynamic (see Introduction). However, a constant characteristic is a limitation to the regions rostral to r7, with no expression in the somitic hindbrain and adjacent tissues. We propose that neural crest along the entire hindbrain, including somitic levels, is competent to express *Krox20* and *mafB/kr* but requires FGF signalling for induction or maintenance. FGF produced in the prospective branchial region between the 10- and 19-somite stages may thus be involved in maintaining the expression of these genes in r5-r6-derived neural crest. This interpretation would fit with recent work showing that the environment can impose a phenotype on migrating neural crest, instead of the latter having a definitive predetermination (Ferguson et al., 2000; Trainor and Krumlauf, 2000). However, the known distribution of FGFs cannot alone account for the precise spatial expression of *Krox20* and *mafB/kr*. Thus activation of these genes may require the synergistic actions of FGFs and

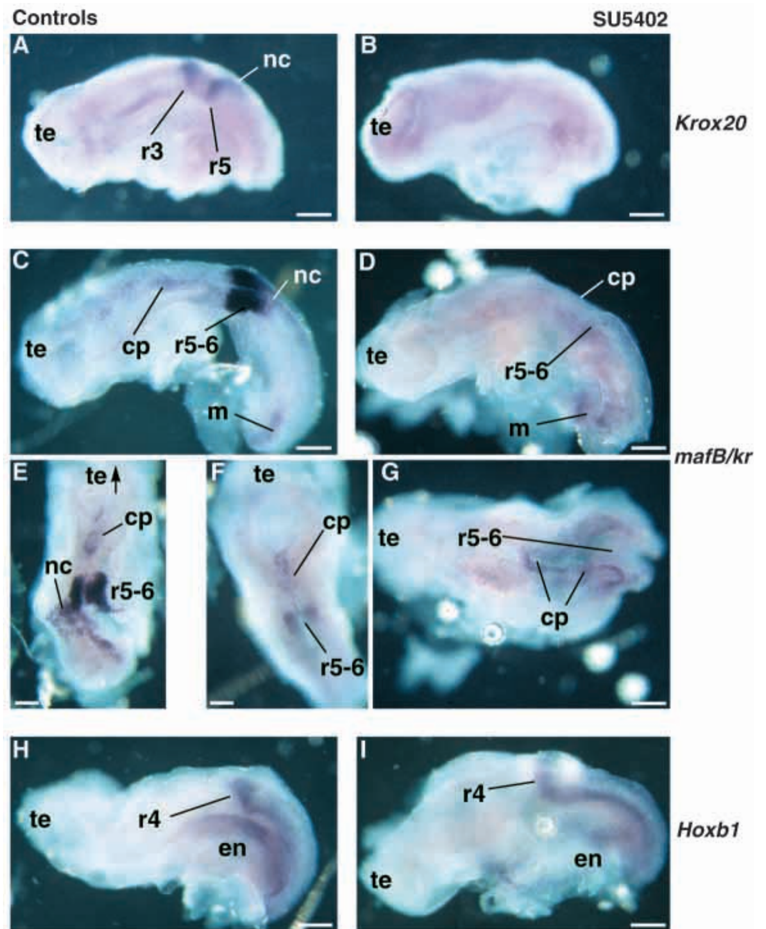

Fig. 7. SU5402 inhibits *Krox20* and *mafB/kr* expression. Embryo explants including the whole head region and the anterior somitic region, extending to somites 4-6 were incubated in the presence (B,D,F,G,I) or absence (A,C,E,H) of SU5402 and processed for *Krox20*, *mafB/kr* or *Hoxb1* RNA detection as indicated. (A-D,H,I) Lateral views of embryos, (E-G) dorsal views. The telencephalon (te) is indicated in each case as a rostral reference point. (A,B) Analysis of *Krox20* expression. Note the inhibition of *Krox20* expression in the SU5402-treated embryo. (C-G) Analysis of *mafB/kr* expression. Note the normal expression in the r5-r6 region and derived neural crest as well as in the choroideal plexus region (cp) of two control embryos (C,E). Two SU5402-treated embryos, D, G and F, show no labelling or a faint staining in the r5-r6 region, while expression in the choroideal plexus and in the presumptive mesonephros (m) is not affected. (H,I) Analysis of *Hoxb1* expression. Expression in the neural tube (including r4 and the somitic region) is similar in control (H) and treated (I) embryos, with a slight reduction in the latter. A more dramatic reduction is observed in the endoderm (en) of the SU5402-treated embryo. Scale bars: 200 μm.

other factors. Alternatively, or in addition, FGF-inducing activity may be subject to antagonism by other factors. For instance, TGFβ-family signalling have been shown to antagonise FGF activity in several other systems (Niswander and Martin, 1993; Ericson et al., 1998). The appearance of *Krox20*-positive neural crest in r2-r3 upon exposure to FGF may indicate that, when present at high concentration, FGFs are able to bypass the requirement for other factors or to override antagonistic activities. At the somitic levels, the absence of FGF would prevent expression of *Krox20* or *mafB/kr* and FGFs may therefore participate in the determination of their posterior borders of expression in the neural crest.

We have also observed that exposure to exogenous FGFs prevents normal neural crest migration from r5-r6. This may result from a direct effect on the neural crest cells themselves, as a vast excess of FGF could interfere with their guidance system or migratory capacity. In this respect, it is interesting to note that FGF2 is a chemoattractant for migrating neural crest in the midbrain (Kubota and Ito, 2000). Alternatively, FGF may modify the surrounding mesodermal cells, making them non-permissive for neural crest migration.

FGFs participate in the patterning of the hindbrain neuroepithelium

In the neuroepithelium, we have observed that exposure to exogenous FGFs leads to a relative enlargement of the *Krox20*- or *mafB/kr*-positive domains as compared to the negative ones and the induction of the expression of these genes in cell patches within normally negative rhombomeres (r4 and r6 for *Krox20* and r7 for *mafB/kr*). Furthermore, blocking of FGF signalling inhibits normal expression of these genes in the hindbrain. The two latter observations suggest that FGFs are required, as in the neural crest, for the induction and/or the maintenance of *Krox20* and *mafB/kr* expression in the neuroepithelium. Similar to the situation in the neural crest, the non-expression of these genes in the somitic hindbrain would be due to the absence of FGFs in this region, although other signalling pathways are likely to participate in the determination of the posterior limit of expression. Retinoids, which are known to affect *Krox20* and *mafB/kr* expression (Grapin-Botton et al., 1998; Dupe et al., 1999; Gale et al., 1999), may co-operate with FGFs in this process. The determination of the limits of the *Krox20* and *mafB/kr* expression domains in more anterior regions would rely, again as in the case of the neural crest, on additional influences, although the rostrocaudal domains of action of these would be different than in the neural crest.

The enlargement of the r3 and r5 bulges relative to r4 following exogenous FGF exposure can be interpreted in two ways. (1) It may be due to the ectopic induction of *Krox20* and *mafB/kr* expression as discussed above. This would result in the recruitment into r3 or r5 of territories normally fated to belong to r4 or r6. The observed reduction in absolute size of r4 is in favour of this possibility. In addition, our laboratory has recently demonstrated the capacity of ectopic *Krox20* expression to initiate a change of molecular identity consistent with such a modification of fate (F. Giudicelli, P. Gilardi-Hebenstreit and P. C., unpublished data). (2) FGF may specifically stimulate the proliferation of *Krox20*-positive cells, leading to differential expansion of positive and negative territories. This possibility would fit with the known mitogenic effect of FGFs, but in this case it would be restricted to a specific cell population. The two hypotheses, which may also

apply to the extension of the *mafB/kr* expression domain after FGF8 exposure, are non-exclusive and their combination may actually explain the observed phenomenon.

FGFs play multiple successive roles in neural AP patterning

As indicated in the Introduction, previous studies have led to the conclusion that FGFs are part of the posteriorizing or transforming activity postulated in the model of Nieuwkoop (1952). In contrast, the present work demonstrates that FGFs have the capacity to induce the expression of anterior markers in caudal tissues. Our conditions differ from those in previous work in that we are dealing with much later (postneurulation) stages of development. At these stages, in the hindbrain, we propose that FGFs are no longer involved in establishing a general AP pattern, but rather, together with other signalling systems, in the delimitation of specific AP domains and determination of their regional identity. In this respect, the hindbrain domain of FGF expression, including neural and extra-neural tissues, may play a role similar to other secondary centres involving FGF signalling, such as the isthmus which is involved in the patterning of midbrain and r1 territories (Martinez et al., 1999; Irving and Mason, 2000) and the anterior neural ridge required for forebrain patterning (Shimamura and Rubenstein, 1997). The absence of significant modifications in *Hoxb1* expression in r4 upon FGF exposure or inhibition is consistent with this idea of a local role of FGF signalling at this stage of hindbrain development, in contrast to a more general posteriorization activity of retinoids (Gould et al., 1998; Grapin-Botton et al., 1998; Dupe et al., 1999; Gale et al., 1999).

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