The role of FGF2 in craniofacial skeletogenesis

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Summary

Evidence that the major craniosynostosis syndromes are caused by mutations in fibroblast growth factor receptors (FGFRs) has suggested that this signalling pathway plays a major role in skull development. Our studies on the effect of FGF2 treatment and transfection of constitutively active FGFRs on premigratory neural crest and cranial vault mesenchyme indeed support the view that fine modulation of the FGF signalling pathway is crucial for normal skull formation.
The limited capacity of bone to heal and regenerate, particularly in the craniofacial region, represents a serious clinical problem. Several issues need to be thoroughly addressed in order to establish therapeutic approaches resulting in effective and extensive reconstruction of bone. A key step is the identification of the molecules that play major roles in survival and differentiation of skeletogenic precursors in order to eventually be able to appropriately modulate their expression or mimic their function in a clinical context. Growth factors and extracellular matrix components are of particular interest because they can be more easily manipulated than intracellular proteins. Among the various animal models that can be used to address some of these issues, the chick embryo presents several advantages in that it is easily accessible, hence amenable to embryonic manipulation in ovo, and in this species the cellular origins of the craniofacial skeleton have been thoroughly mapped (Couly et al., 1993).

In the chick head, most of the skeletal tissues originate from the cranial neural crest. Following emigration from the dorsal aspect of the neural tube, these cells give rise to cartilage, endochondral bone and membrane bone (Couly et al., 1993). In this paper we will briefly review our recent results on the role of FGF signalling in skeletogenic differentiation of the premigratory neural crest and of the mesenchyme of the cranial vault in the developing avian embryo. Our interest in FGF signalling stems from the observation that mutations which constitutively activate fibroblast growth factor receptors (FGFRs) result in craniosynostosis (Muenke and Schell, 1995; Burke et al., 1998; Wilkie, 1997). These patients display severe craniofacial abnormalities due to premature fusion of the sutures of the cranial vault. It was proposed that excessive FGF signalling leads to precocious bone differentiation. Our work and other in vitro and in vivo studies using a variety of models support this hypothesis (Iseki et al., 1997, 1999; Lomri et al., 1998; Montero et al., 2000; Moore at al., 2001; Petiot et al., 2001; Sarkar et al. 2001).

In a recent study we have found that neural crest responsiveness to FGF in avians exists before migration and that it is required for neural crest cell survival (Sarkar et al. 2001). In addition, we have shown that low concentrations (0.1 and 1 ng/ml) of FGF2 induce cell proliferation in neural crest explants from quail embryos at stage HH 8/9 (Hamburger and Hamilton, 1951). In contrast, at high concentrations (10 ng/ml) FGF2 induces only a limited proliferative effect (Fig. 1). This high
FGF2 concentration, however, promotes the formation of cartilage nodules, which are easily identifiable in 10 day old cultures (Fig. 2A). Such chondrogenic differentiation does not appear to occur in neural crest cells cultured for the same length of time in the presence of 1 ng/ml FGF2, and cells grown under these conditions retain their fibroblastic morphology.

The occurrence of chondrogenic differentiation in cultures treated with 10 ng/ml FGF2 has been confirmed at the morphological level by alcian blue staining, a histochemical marker for cartilaginous matrix, and transmission electron microscopy (TEM). TEM analysis of these cultures has clearly demonstrated the presence of cells with the typical morphology of chondrogenic cells, that are rounded, with scalloped plasma membrane profiles and surrounded by large areas of extracellular
matrix. Mature chondrocytes embedded in lacunae within the extracellular matrix are also present in these cultures (Fig. 2B). Further confirmation that high concentrations of FGF2 induce chondrogenic differentiation comes from the analysis of cartilage markers, such as type II collagen and Sox9, that has been carried out both at the mRNA and protein level (Sarkar et al., 2001; Petiot et al., 2001). These markers of chondrogenic differentiation are indeed up-regulated in response to treatment with 10 ng/ml FGF2, but not to treatment with 1 ng/ml FGF2 (Fig. 3A).

**Fig. 2** Differentiation of quail neural crest cells grown in the presence of 10 ng/ml FGF2. A) A cartilage nodule in a 12 day old culture is indicated by the white arrow. B) Transmission electron microscopy of a representative mature chondrocyte enclosed in its own lacuna (arrowheads). C) Separated sheets of bone (b, alizarin red staining) and cartilage (c, alcian blue staining) suggestive of membrane bone formation are observed in 28 day old cultures. D) Inclusion of bone (red), indicated by arrowheads, within cartilage (blue) suggests that endochondral bone forms in these cultures. Scale bars are: 100 µm in A, 5 µm in B, 1 mm in C and 0.1 mm in D.
Fig. 3 Semi-quantitative RT-PCR analysis of Col2a1 (Collagen 2a1) and osteopontin in neural crest cells grown in the presence of either 1 ng/ml or 10 ng/ml FGF2 for different lengths of time. A) Col2A1 expression is higher in 10 ng/ml than in 1 ng/ml FGF2-treated cultures both at 5 and 26 days. B) Osteopontin expression is higher in FGF2-treated cultures than in controls after 26 days in culture but not at 5 days.

In addition to their ability to undergo chondrogenic differentiation, neural crest cultures maintained for 28 days in the presence of 10 ng/ml FGF2 undergo osteogenic differentiation when the culture medium is supplemented from days 11 to 28 with ascorbate, β-glycerophosphate and dexamethasone, factors that are permissive for bone mineralisation. Osteogenesis can be detected both by morphological analysis (Fig. 2C-D) and by up-regulation of the osteogenic marker osteopontin (Fig. 3B). Very interestingly, both regions of endochondral and membranous ossification are observed in these cultures, as indicated by the presence of nodules which are stained both for bone (alizarin red-positive staining) and cartilage matrix (alcian blue-positive staining), and areas where alizarin red-positive tissue is clearly separated from alcian blue stained cartilage (Fig. 2C-D).

From these studies it emerges that FGF-2 has a concentration-dependent effect on cranial neural crest cell proliferation and differentiation. Furthermore, it appears that FGF2 can promote both chondrogenic and osteogenic responses from mesencephalic neural crest cells. The fact that FGFR1,
2, and 3 are expressed by neural crest progenitors \textit{in vivo} and by cultured neural crest cells is consistent with their ability to respond to FGF stimulation.

We have also assessed whether high FGF2 concentration does indeed mimic the effects of constitutively active FGFRs, since activating mutations in these receptors are the primary cause of several autosomal dominant craniosynostoses. For this purpose, we have transfected neural crest cells with mutated forms of FGFRs using electroporation \textit{in vivo} prior to explanting and growing them in culture (Petiot et al., 2001). The efficiency of transfection was high, as indicated by electroporation of neural crest cells with marker genes. Twelve days after transfection by electroporation with the mutant FGFR1-K656E neural crest explants express chondrogenic (Col2a1, 57%) and osteogenic (osteopontin, 52%) markers. In addition, formation of cartilage nodules is observed. Therefore, a high concentration of FGF2 does indeed mimic the effects of constitutively active FGF receptors. Occurrence of excessive differentiation is consistent with early suture closure in patients with craniosynostosis.

To extend and complement the \textit{in vitro} studies, we have used Chorio-Allantoic Membrane (CAM) grafting of cranial mesenchyme as a convenient model for the manipulation of FGF2 levels in the developing cranial vault (Moore et al., 2001). This experimental system has allowed us to assess growth, differentiation and even to some extent morphogenesis of the CAM-grafted embryonic crania. We carried out a series of experiments where beads soaked either with FGF2 or with a blocking anti-FGF2 antibody were implanted in the dissected region containing the presumptive frontal and parietal bones and occipital cartilage prior to grafting. We have found a dose-dependant effect of the anti-FGF2 antibody on the availability of FGF2 and that a reduction in the levels of endogenous FGF2 in chick cranial vault results in significantly enlarged CAM-grafts (Fig. 4), indicating that the neural crest-derived mesenchymal cells switch from a differentiative to a proliferative mode. This has also been confirmed by analysis of the proliferation marker PCNA in anti-FGF2 antibody-treated grafts, which contain a significantly higher number of proliferating mesenchymal cells than control grafts. In contrast to the effects observed when the levels of FGF2 available are reduced, administration of FGF2 does not have any effect on graft growth. This suggests that FGF2 is already present in cranial tissues in “excess” and that regulation and specificity of this signalling pathway under physiological conditions may be mainly dependent on
receptor rather than ligand availability. This view is consistent with the fact that application of FGF-2 to coronal sutures can induce local up-regulation of osteopontin only when FGF2 is used at the very high dose of 400 mg/ml (Iseki et al., 1997). Overall these results demonstrate that cranial skeletal tissue, like cranial neural crest cells, responds to FGF2 in a concentration-dependent fashion.

Fig. 4 Implantation of a single anti-FGF2 bead results in a massive increase in the size of CAM-grafted cranial tissue. A) Cranial tissue implanted with an anti-FGF2-soaked bead. Note the significant increase in size (2-3 fold) as compared to the control (B). B) Cranial tissue implanted with a PBS-soaked bead (control). C) Cranial tissue implanted with a PBS-soaked bead (control) and stained for bone (alizarin red) and cartilage (alcian blue). Note that notwithstanding the significant increase in size in the treated graft as compared to the control (D), osteogenic differentiation is largely normal. D) Cranial tissue implanted with an anti-FGF2-soaked bead and stained for bone and cartilage. Arrows denote the site of bead implantation. Scale bar: 2 mm.
In conclusion, changes in the level of FGF signalling both *in vitro* and *in vivo* can shift the balance between proliferation and skeletogenic differentiation both in neural crest cells and in mesenchymal cells of the cranial vault. This indicates that a fine tuning of FGF signalling, and possibly of other growth factors implicated in cranial morphogenesis, is required for the progression of skeletogenic differentiation. The work discussed in this chapter, together with the work from other groups (Iseki *et al.*, 1997, 1999), points to a key role for FGF signalling in determining the skeletogenic fate of neural crest cells and in modulating the skeletogenic differentiation of neural crest-derived tissues. Finally, it clearly indicates that induction of bone regeneration either using growth factors, or cell therapy, or a combination of the two, is most likely to succeed if such mechanisms are thoroughly understood and strategies to reproduce them devised.
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References


