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FGF signals from the nasal pit are necessary for normal facial morphogenesis

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ABSTRACT

Fibroblast growth factors (FGFs) are required for brain, pharyngeal arch, suture and neural crest cell development and mutations in the FGF receptors have been linked to human craniofacial malformations. To study the functions of FGF during facial morphogenesis we locally perturb FGF signalling in the avian facial prominences with FGFR antagonists, foil barriers and FGF2 protein. We tested 4 positions with antagonist-soaked beads but only one of these induced a facial defect. Embryos treated in the lateral frontonasal mass, adjacent to the nasal slit developed cleft beaks. The main mechanisms were a block in proliferation and an increase in apoptosis in those areas that were most dependent on FGF signaling. We inserted foil barriers with the goal of blocking diffusion of FGF ligands out of the lateral edge of the frontonasal mass. The barriers induced an upregulation of the FGF target gene, *SPRY2* compared to the control side. Moreover, these changes in expression were associated with deletions of the lateral edge of the premaxillary bone. To determine whether we could replicate the effects of the foil by increasing FGF levels, beads soaked in FGF2 were placed into the lateral edge of the frontonasal mass. There was a significant increase in proliferation and an expansion of the frontonasal mass but the skeletal defects were minor and not the same as those produced by the foil. Instead it is more likely that the foil repressed FGF signaling perhaps mediated by the increase in *SPRY2* expression. In summary, we have found that the nasal slit is a source of FGF signals and the function of FGF is to stimulate proliferation in the cranial frontonasal mass. The FGF independent regions correlate with those previously determined to be dependent on BMP signaling. We propose a new model whereby, FGF-dependent microenvironments exist in the cranial frontonasal mass and caudal maxillary prominence and these flank BMP-dependent regions. Coordination of the proliferation in these regions leads ultimately to normal facial morphogenesis.

Introduction

During ontogeny of the face, several initially separate buds of mesenchyme covered in epithelium known as the facial prominences grow out and merge together to give rise to the upper and lower jaws. The majority of the mesenchyme within the facial prominences is derived from cranial neural crest cells, with a smaller contribution from paraxial head mesoderm. The neural crest derived mesenchyme gives rise to all of the bone and cartilage in the face (Couly et al., 1996; Couly et al., 1993; Kontges and Lumsden, 1996; Noden, 1983). The majority of skeletal patterning information is carried in the neural crest-derived mesenchyme (Schneider and Helms, 2003; Tucker and Lumsden, 2004). Soon after neural crest cell migration ends, the facial prominences form around the primitive oral cavity. The frontonasal mass lies in the midline and is flanked by the nasal pits, the lateral nasal prominences are between the nasal pit and the eye, the maxillary prominence are at either side of the oral cavity, whereas the mandibular prominences lie below the maxillary prominences. The upper jaw is formed by the frontonasal mass with contributions from the maxillary and lateral nasal prominences. In contrast, the lower jaw is formed entirely by the mandibular prominences. To form the upper beak there must be contact between the corners (globular processes) of the frontonasal mass and maxillary prominences. Following growth and contact of the two prominences, a bilayered epithelial seam is formed and then is removed through apoptosis and epithelial–mesenchymal transformation (Sun et al., 2000). The degrading epithelium is invaded by mesenchyme from either side and residual grooves are filling out by proliferation.

Even though the bigger aspects of jaw identity are established prior to the formation of facial prominences (for example, distinguishing upper versus lower jaws), refinement of the basic pattern is still required to give species-specific morphology. Work carried out in avian embryos has shown that expression of certain growth factors such as Bone Morphogenetic Proteins (BMPs) is correlated with differences in beak shape (Abzhanov et al., 2004; Wu et al., 2004). For example, the shape of the early frontonasal mass is thought to be closely related to the final shape of the upper beak. The mechanism for modifying frontonasal mass morphology is thought to be by the positive influences of growth factors on proliferating cells within the

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facial mesenchyme (Wu et al., 2006, 2004). This idea is supported by the demonstration in several studies that antagonism of BMP signaling with Noggin reduces the proliferation and consequently the size of facial prominences (Ashique et al., 2002a; Foppiano et al., 2007; Wu et al., 2006, 2004).

In addition to BMPs, several other growth factor families are known to be important for growth of facial prominences. Wingless-related proteins (Wnt) constitute one class of signal required for frontonasal mass growth. In experiments where embryos were exposed to *Dkk1*, an antagonist of Wnt signaling, frontonasal mass growth was inhibited and clefts occurred (Brugmann et al., 2007). The epithelially-expressed gene *Sonic Hedgehog* is required at two times in development, early on to establish the facial midline and later to promote outgrowth of the frontonasal mass (Hu and Helms, 1999; Hu et al., 2003). BMPs are required to establish the *SHH* expression domain in the caudal edge of the frontonasal mass (Foppiano et al., 2007).

The contribution of FGF signaling to midfacial growth and the fusion of the upper lip has been addressed in several conditional deletions of *Fgf8*. In several lines, the mutant embryos have a truncated face (Macatee et al., 2003) and mandible (Trumpp et al., 1999), suggesting that FGF signaling may be required at specific times in development. In addition, there are intriguing data suggesting that prolonged expression of *FGF8* and maintenance of proliferation at higher levels at the edges of the frontonasal mass is one of the reasons why the duck has a wider beak than the chicken (Wu et al., 2006). However no one has rigorously studied the role of FGFs in facial morphogenesis.

There are 22 FGF ligands in mammals (Zhang et al., 2006), five of which are expressed in the mouse face *Fgf2*, *Fgf8*, *Fgf9, Fgf10, Fgf17* and *Fgf18* (Bachler and Neubuser, 2001; Crossley and Martin, 1995; Havens et al., 2006; Karabagli et al., 2002; Kettunen and Thesleff, 1998; Rice et al., 2004). In the chicken genome only 5 of these genes are present, including *FGF2, FGF8, FGF9, FGF10* and *FGF18*. Of these genes, *FGF2* is ubiquitously expressed, and the others are mainly expressed in the superficial ectoderm surrounding the nasal slit and lining the maxillo-mandibular cleft (Havens et al., 2006; Karabagli et al., 2002; McGonnell et al., 1998; Ohuchi et al., 2000; Richman et al., 1997). FGFs bind to three FGF receptors in the facial mesenchyme. *FGFR1* is expressed ubiquitously whereas *FGFR2*, is expressed in the medial frontonasal mass mesenchyme while *FGFR3* is restricted to the caudal edge of the frontonasal mass and medial edges of the maxillary prominences (Matovinovic and Richman, 1997; Wilke et al., 1997).

Our study focuses on the control of facial morphogenesis using gain or loss of function approaches that target the FGF pathway. Since there are so many FGF family members, to decrease signaling, we implanted beads soaked in a pan-antagonist of FGFRs, SU5402 (Mohammadi et al., 1997). To increase FGF receptor activation FGF2 protein was applied to discrete regions of the face. Our results revealed several novel FGF-dependent and independent regions in the frontonasal mass and maxillary prominence that together coordinate growth and contact of the facial prominences. Finally, to determine where the FGF signal originated, we implanted foil barriers to block directional signaling in the frontonasal mass.

Methods

Bead implantations and foil implants

White leghorn eggs were obtained from the University of Alberta and incubated at 38 °C. SU5402 (SUGEN, USA and EMD Biosciences, UK; Mohammadi et al., 1997) was dissolved in DMSO (dimethyl sulfoxide). AG1X2 beads (Formate form, Biorad) were soaked in 5 μl of SU5402 for 1 h with a drop of 0.01% Fast Green added for bead visualization. FGF2 beads were prepared by soaking 200–300 μm Affigel beads with either 1 mg/ml FGF2 or FGF8b protein (Peprotech). Control beads were soaked in DMSO or buffer. Beads were inserted into small incisions into the facial prominences and the final position was recorded.

Aluminium foil was inserted medial to the nasal slit in the frontonasal mass. Care was taken to position the foil cranially and not to interfere with the globular process.

Skeletal preparation and skull analysis

To study bone and cartilage morphology Hamburger and Hamilton stage 37–39 (E12–14) embryos were stained as described (Plant et al., 2000). Each process of the maxilla, premaxilla and palatine bones was compared to the normal, contralateral side. Bony processes were scored as normal, reduced or absent. The reduced category included bony processes that were greater than 50% shorter in length. The effect of SU5402 bead position on skeletal morphology was determined using χ^2 analysis (significance level set at $p<0.05$).

Fluorescence photography of whole heads and analysis of early phenotypes with FGF2 beads

Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight, stained in 1:5000 dilution of SybrSafe (Invitrogen) in PBS for 10 min, rinsed and photographed using the GFP filter on a Leica MZFLIII microscope.

BrdU analysis

Approximately 50 μl of 10^2 M BrdU (bromodeoxyuridine) was injected into the heart 2 h prior to collection. Embryos were fixed 12 h after bead implantation. Wax sections (7 μm) were treated with proteinase K, exonucleases and then incubated with neat primary antibody (Amersham, GE Healthcare). Alexa Fluor 488 (Molecular Probes, Invitrogen) labeled goat anti-mouse antibody (1:50) was incubated at room temperature for 30 min. Slides were coverslipped using Prolong Gold Antifade with DAPI (Molecular Probes, Invitrogen).

Proliferation index was calculated by dividing the number of BrdU positive cells by the DAPI positive (total cell number). Sections at least 14 μm apart were chosen so that different populations of cells would be represented. We used two WCIF Image J plugins for automated cell counting: ITCN (Image-based Tool for Counting Nuclei) for the BrdU positive cells and the Nucleus Counter for the DAPI stained nuclei.

To determine if there were differences in cell proliferation related to bead position we divided the lateral frontonasal mass into thirds. The cranial region lined up with the top edge of the nasal slit, the caudal region included the corner or globular process of the frontonasal mass. Each area was approximately 200 μm wide by 100 μm tall and contained approximately 800 cells. We compared each region counted, bead position and compared SU5402 treatment to DMSO using multifactorial ANOVA (MANOVA) with Fisher Least Significant Difference post hoc testing ($p<0.05$; Statistica). In the FGF2-treated embryos, changes in proliferation were qualitatively compared to the contralateral side or Tris-treated controls.

Cell death analysis

For Nile Blue Sulfate staining, bead implantation was performed without neutral red staining and embryos were collected 3, 6, and 9 h (Song et al., 2004). For TUNEL, embryos were collected 6 and 16 h after bead implantation and sections were stained as described (Buchtová et al., 2007). Apoptotic bodies in nearly adjacent sections were counted in the same regions that were used in the BrdU analysis plus an additional region in the medial frontonasal mass. Since apoptosis results in loss of cell integrity we placed specimens into one of three categories: 0–5; 6–10; 11–50 apoptotic bodies (Table S3).

Whole mount in situ hybridization and immunohistochemistry

Whole mount in situ hybridization was performed as described (Song et al., 2004). Section in situ hybridization was performed with antisense ^{35}S-labeled antisense probes as described (Wilke et al., 1997). The following individuals generously provided gallus cDNAs: G. Martin, *SPRY2*; P. Francis-West, *BMP4*; M. Kessel, *DLX5*; S. Wedden, *MSX1, MSX2*; O. Pourquie, intronic and exonic *FGF8*; S. Keyse, *PYST1*. The *SPRY4* probe was obtained from the MRC Geneservice (UK; Clone ID: 603786019F1).

Embryos for wholemount phosphorylated MAPK staining were stained as previously published (Corson et al., 2003) with the Phospho-p44/42 Map Kinase antibody (Cell Signaling #9101; 1:350 dilution). An additional proteinase K step for antigen retrieval was included.

Results

Our study uncovered differential gene expression patterns within the frontonasal mass and other regions of the face that led us to hypothesize there were differences in FGF signaling within the developing face. In order to understand the endogenous FGF signals taking place at the time of fusion we used bead implantation to locally block or stimulate FGF signaling. We focused our study on stage 26–28 chicken embryos, a time when key differences in proliferation have been noted (MacDonald et al., 2004; McGonnell et al., 1998; Wu et al., 2006, 2004). These stages are also just prior to the major morphogenetic changes such as fusion of the lip and beak outgrowth. Our