Developmental Biology 325 (2009) 200–210

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Unique organization of the frontonasal ectodermal zone in birds and mammals

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ARTICLE INFO

Article history:
Received for publication 6 June 2008
Revised 10 October 2008
Accepted 13 October 2008
Available online 31 October 2008

Keywords:
Sonic hedgehog
Fibroblast growth factor 8
Bone morphogenetic protein
Bmp-2
Bmp-4
Bmp-7
Frontonasal ectodermal zone (FEZ)
Mouse-chick chimera

ABSTRACT

The faces of birds and mammals exhibit remarkable morphologic diversity, but how variation arises is not well-understood. We have previously demonstrated that a region of facial ectoderm, which we named the frontonasal ectodermal zone (FEZ), regulates proximo-distal extension and dorso-ventral polarity of the upper jaw in birds. In this work, we examined the equivalent ectoderm in murine embryos and determined that the FEZ is conserved in mice. However, our results revealed that fundamental differences in the organization and constituents of the FEZ in mice and chicks may underlie the distinct growth characteristics that distinguish mammalian and avian embryos during the earliest stages of development. Finally, current models suggest that neural crest cells regulate size and shape of the upper jaw, and that signaling by Bone morphogenetic proteins (Bmps) within avian neural crest helps direct this process. Here we show that *Bmp* expression patterns in neural crest cells are regulated in part by signals from the FEZ. The results of our work reconcile how a conserved signaling center that patterns growth of developing face may generate morphologic diversity among different animals. Subtle changes in the organization of gene expression patterns in the FEZ could underlie morphologic variation observed among and within species, and at extremes, variation could produce disease phenotypes.

Introduction

Formation of the face in vertebrates occurs through development of homologous facial primordia. In part, the initial divergence of facial morphologies in birds and mammals results from unique patterns of growth within the facial primordia that will form the upper jaw. In chicks the middle and upper face is derived from the frontonasal process (FNP). In mice, initial growth is enhanced in lateral regions of the face. Instead of an FNP, this part of the mammalian face is comprised of paired median nasal processes (MNP). In mice, as the right and left median nasal processes grow, the mesenchymal tissues forming the medial edge of each MNP merge and form the FNP. Therefore, understanding mechanisms that regulate the establishment of these different growth zones will illuminate the regulation of patterning and variation in the shape of the upper jaw.

A series of reciprocal signaling interactions among the forebrain, the neural crest, and the surface ectoderm controls morphogenesis of the upper jaw. For example, signals from the brain and neural crest regulate gene expression within the adjacent surface ectoderm (Marcucio et al., 2005; Schneider and Helms, 2003). In turn, the ectoderm signals back to the mesenchyme. In the cephalic ectoderm of avian embryos, we defined a boundary between cells expressing *Sonic hedgehog* (*Shh*) and *Fibroblast growth factor 8* (*Fgf8*), and we named this the tissue the frontonasal ectodermal zone (FEZ; Hu et al., 2003). We showed that transplantation of the FEZ to ectopic locations within the FNP induces expression of down-stream targets of the SHH pathway (i.e., *Ptc* and *Gli1* (Dahmane et al., 1997; Goodrich and Scott, 1998; Lee et al., 1997)) in neural crest cells, and induces duplications of the upper beak. Interestingly, this work also revealed that the FEZ induces duplications of the lower jaw when transplanted onto the mandibular process, but does not affect the morphology of the hyoid arch (Hu et al., 2003). These results indicate that the FEZ does not specify regional anatomical identity of the underlying neural crest cells. Rather, the FEZ controls dorso-ventral polarity and proximo-distal extension of the FNP by evoking intrinsic responses from the mesenchyme in chicks. However, the extent to which the FEZ is a conserved signaling center that could participate in generating unique avian and mammalian growth zones is not known.

Growth of facial primordia is regulated by a variety of signaling molecules including: Bone morphogenetic proteins (Bmps), Fibroblast growth factors (Fgfs), Sonic hedgehog (Shh), Retinoids, and Wingless family members (Wnts) (Depew and Simpson, 2006; Hu and Helms, 1999; Lee et al., 2001; Mina et al., 1994, 2002; Richman et al., 1997, 2006; Song et al., 2004; Wilke et al., 1997). Of these pathways, the role that Bmp signaling plays in regionalizing domains of cell proliferation in the upper jaw anlagen has been characterized. Differences in proliferation between avian species are associated with Bmp signaling in neural crest mesenchyme in the FNP (Wu et al., 2006). Furthermore, differential expression of *Bmp-2* and *-4* in neural crest mesenchyme correlates with variation in shape and size of the upper beak observed among Darwin's finches (Abzhanov et al., 2004), and activation of Bmp

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doi:10.1016/j.ydbio.2008.10.026

signaling creates localized growth zones that transform the shape of the cartilage elements that comprise the upper beak (Abzhanov et al., 2004; Wu et al., 2004, 2006). Together, these results underscore the importance of signaling by Bmps in neural crest mesenchyme.

In this work we examined the ectoderm covering the median nasal processes for evidence of a FEZ in mice. While many studies have examined the expression patterns of *Fgf8* and *Shh* in the mouse (e.g., Jeong et al., 2004; Kawauchi et al., 2005), the distinct relationship between these two genes and other molecules expressed by the mouse FEZ has not been illustrated. Further, there has been no direct comparison of these expression patterns between mammals and birds. Therefore, we compared the molecular constituents of the FEZ between mice, chicks, and ducks, and then we performed a functional analysis of the mouse FEZ. We demonstrate that the FEZ is conserved between mice and chicks. Thus, the FEZ may be a fundamental signaling center that participates in regulating development of the upper jaw in vertebrates, in part by regulating expression patterns of *Bmps* in neural crest mesenchyme. Overall, our results indicate that the unique molecular organization of the FEZ in mice and chicks correlates with the divergent facial characteristics that are apparent during the earliest stages of facial development in birds and mammals. The FEZ appears to be a source of patterning information that could contribute to variation in facial form, and at extremes, variation produced by the FEZ could create disease phenotypes such as those observed in *formes frustes* of Holoprosencephaly (HPE) or cleft lip and palate.

Materials and methods

Preparation of embryos and engraftment of the mFEZ

Fertilized chicken eggs (*Gallus gallus*, Petaluma Farms, Petaluma, CA) were prepared for surgical manipulations as follows. Embryos were incubated to Hamburger and Hamilton stage 10 (HH 10 (Hamburger and Hamilton, 1951)) and then a small hole was made in the shell directly over the embryo after removing 1.0 ml of albumin. Embryos were returned to the incubator until HH 21 or 25. At this time the ectoderm covering the dorsal region of the FNP was removed with a sharpened tungsten needle. Grafts were prepared from mouse embryos at e10 as described (Hu et al., 2003). Briefly, mouse embryos were dissected from *uteri* of euthanized dams and placed in ice cold PBS. Facial tissues were dissected from embryos and were placed in dispase (2.5 U/ml in PBS) for 20 min. Then putative FEZ ectoderm was removed, and the graft was transferred to the host and positioned to replace the removed ectoderm. The graft was secured with glass pins (Supplemental Fig. 1, and see Hu et al., 2003). Mouse embryos were collected at e9.5, e10, and e10.5 for in situ hybridization analysis. Animal procedures were approved by the UCSF IACUC.

Histology

Twenty-four hours after engraftment and at days 9, 12, and 13 of development embryos were collected, fixed in 4% paraformaldehyde, stained with ethidium bromide, and then photographed using epifluorescent or brightfield illumination on a Leica MZFLIII microscope connected to a computer. After documentation, chimeras were dehydrated, embedded in paraffin, and sectioned (10 μm). Sections were stained with Safranin-O/Fast Green to visualize cartilage (Lu et al., 2005), modified Milligan's Trichrome to visualize bone (Lu et al., 2005). Sections were imaged using a Leica DM5000B and Adobe Photoshop.

In situ hybridization

Patterns of gene expression in chimeras and normal mouse, chick, and duck embryos were analyzed on tissue sections and/or in whole mount via in situ hybridization using radiolabeled or digoxigenin-labeled riboprobes as previously described (Lu et al., 2005). Subclones of mouse B2 SINE (Bollag et al., 1999), *Shh*, *Fgf8*, *Bmp-2*, *Bmp-4*, and Bmp-7, and chick *Bmp-2*, *Bmp-4*, *Bmp-7*, *Shh*, *Fgf8*, *Msx1*, and *Msx2* were linearized to transcribe riboprobes. Images of in situ hybridization assays performed on tissue sections are pseudo-colored superimpositions of the in situ hybridization signal and a blue nuclear stain (bis-benzimide; Sigma). Whole mount in situ hybridizations were photographed using a Leica MFLZIII dissecting microscope.

Results

Ontogeny of the murine FEZ

We initially identified the FEZ in chick embryos based on the presence of a boundary between *Shh* and *Fgf8*-expressing cells in the ectoderm covering the FNP at HH 20 (Hu et al., 2003). To compare the relationship between *Shh* and *Fgf8* expression domains in the FEZ in birds and mammals, we performed whole mount in situ hybridization. In mice and chicks, *Fgf8* transcripts were detected in the ectoderm that spans the medio-lateral axis of the developing middle and upper face (Figs. 1A, B). However, expression of *Shh* in these animals is unique. In chick embryos *Shh* and *Fgf8* expressing cells form a boundary in the ectoderm covering the neural crest cells that comprise the avian FNP at Hamburger and Hamilton Stage 20 ((HH20) (Hamburger and Hamilton, 1951)), and *Shh* expression is continuous across the medio-lateral axis of the FNP (Figs. 1C, E (Hu et al., 2003)). This same pattern is also observed in duck embryos at (Supplemental Fig. 2A). In contrast to the avian FEZ, the murine FEZ is not a single signaling center. Rather *Shh* is expressed in domains on the right and left side of the mouse face creating left and right FEZs (Figs. 1D, F). In the lateral regions neural crest cells are present, but these cells are absent or greatly reduced at the midline. A bilateral pattern of *Shh* expression is also observed in human embryos in the upper jaw and is accompanied by reduced mesenchymal cells in the medial region (Odent et al., 1999) and the presence of median nasal processes rather than a Frontonasal Process. Thus, these unique patterns of *Shh* expression in the FEZ correlate with the morphology that distinguishes avian and mammalian embryos at these early times.

Our next step in characterizing the murine FEZ was to examine the ontogeny of *Shh* and *Fgf8* expression prior to and after outgrowth of the median nasal processes had begun. At e9.5 ($n=3$) *Shh* expression was detected in the forebrain epithelium but not in the surface ectoderm in either medial or lateral domains (Figs. 2A, B and see Supplemental Fig. 2). In contrast, *Fgf8* transcripts were detected in the forebrain, and in medial and lateral domains of the surface ectoderm (Figs. 2G, H). By e10.0 ($n=6$) *Shh* transcripts were not present in the medial region of the face (Fig. 2C, Supplemental Fig. 2), and concomitantly there were no neural crest cells in this region of the face. In contrast, *Shh* transcripts were present in the ectoderm adjacent to neural crest cells that formed the median nasal processes (Fig. 2D), and this domain forms a boundary with cells expressing *Fgf8* (Figs. 2I, J). The boundary between *Shh* and *Fgf8*-expressing cells persists in the left and right median nasal processes at e10.5 (Figs. 2F, L, K, $n=10$), but no *Shh* transcripts were detected in ectodermal cells located between the median nasal processes (Fig. 2E). Again, no neural crest cells were present in the midline of the mouse face.

In addition to *Shh* and *Fg8*, *Bmps* are also expressed in the FEZ of birds and mice. In chick embryos at HH 22 ($n=10$) *Bmp-2* (Fig. 3A), *Bmp-4* (Fig. 3B), and *Bmp-7* (Fig. 3C) transcripts were present in the FEZ. Again, the expression of these genes spanned the entire medio-lateral axis of the FNP. In mice, *Bmp-2* expression was not evident in the FEZ during the times we examined (e9–5, $n=3$; e10.0, $n=6$; e10.5, $n=10$, Figs. 3D, G and data not shown).