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Molecular interactions coordinating the development of the forebrain and face

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Abstract

From an architectural point of view, the forebrain acts as a framework upon which the middle and upper face develops and grows. In addition to serving a structural role, we present evidence that the forebrain is a source of signals that shape the facial skeleton. In this study, we inhibited Sonic hedgehog (Shh) signaling from the neuroectoderm then examined the molecular changes and the skeletal alterations resulting from the treatment. One of the first changes we noted was that the dorsoventral polarity of the forebrain was disturbed, which manifested as a loss of *Shh* in the ventral telencephalon, a reduction in expression of the ventral markers *Nkx2.1* and *Dlx2*, and a concomitant expansion of the dorsal marker *Pax6*. In addition to changes in the forebrain neuroectoderm, we observed altered gene expression patterns in the facial ectoderm. For example, *Shh* was not induced in the frontonasal ectoderm, and *Ptc* and *Gli1* were reduced in both the ectoderm and adjacent mesenchyme. As a consequence, a signaling center in the frontonasal prominence was disrupted and the prominence failed to undergo proximodistal and mediolateral expansion. After 15 days of development, the upper beaks of the treated embryos were truncated, and the skeletal elements were located in more medial and proximal locations in relation to the skeletal elements of the lower jaw elements. These data indicate that a role of Shh in the forebrain is to regulate *Shh* expression in the face, and that together, these *Shh* domains mediate patterning within the frontonasal prominence and proximodistal outgrowth of the middle and upper face.
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Introduction

The facial skeleton is an agglomeration of bones and cartilages that arise from distinct facial prominences, each of which is unique in terms of the epithelia that surround them and the rostrocaudal origins of the neural crest which comprise the skeletogenic mesenchyme (Couly and Le Douarin, 1990; Thorogood, 1988, 1993). The mandibular prominence gives rise to the lower jaw skeleton, and studies in a variety of animal models indicate that the pharyngeal epithelium influences the patterning and growth of the mandibular skeleton (Couly et al., 2002; Kimmel et al., 2001; Miller et al., 2000). The maxillary and lateral nasal prominences give rise to lateral parts of the upper jaw skeleton, and epithelial–mesenchymal interactions are also important for their proper morphogenesis (Depew et al., 2002; Ozeki et al., 2004). The central portion of the upper jaw skeleton is derived from the frontonasal prominence, and in this structure, the frontonasal epithelium is required for morphogenesis (Hu et al., 2003).

The forebrain neuroectoderm also participates in craniofacial patterning, but in ways that are not clearly defined. The forebrain acts as a structural support for facial development, as exemplified by the clinical condition of holoprosencephaly (HPE) (Cohen and Sulik, 1992; Muenke, 1994; Muenke and

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Cohen, 2000). Impaired midline cleavage of the forebrain typically results in alterations of facial elements derived from the frontonasal prominence. Likewise, craniofacial defects arising as a result of disruptions in the activity of growth factors that are required by the neuroectoderm, such as retinoids (Niederreither et al., 1999) and fibroblast growth factors (Creuzet et al., 2004), may be attributable to a collapse of the forebrain scaffold, due to apoptosis in this tissue layer (Creuzet et al., 2004; Schneider et al., 2001).

Facial malformations may also arise because signals emanating from the forebrain provide instructional information to the tissues of the face. If such a scenario were true, then the loss of forebrain signals would compromise normal facial development, despite an intact supporting framework. To explore whether the forebrain acted as more than supporting framework for facial morphogenesis, we examined how Sonic hedgehog (Shh), which has a well-documented role in forebrain development (Chiang et al., 1996; Ericson et al., 1995a; Macdonald et al., 1995; Ye et al., 1998), affects craniofacial development. Shh is produced by multiple epithelia in the head, including forebrain neuroectoderm (Echelard et al., 1993), frontonasal and maxillary ectoderm (Helms et al., 1997), and pharyngeal endoderm (Bitgood and McMahon, 1995). Disruptions in Shh signaling, or an inability of neural crest cells to respond to Hedgehog signaling, results in a range of craniofacial dysmorphologies. Most of these experimental approaches either eliminated an entire tissue (Hu and Helms, 1999), inhibited the ability of Shh to bind to its receptor (Ahlgren and Bronner-Fraser, 1999; Cordero et al., 2004; Hu and Helms, 1999), eliminated Shh from all tissues (Chiang et al., 1996), or eliminated the ability of specific tissues to respond to Shh (Jeong et al., 2004). We wanted to discriminate the function of Shh in the forebrain neuroectoderm from its role in the other head epithelia, and to pinpoint which aspect of craniofacial morphogenesis was regulated by this particular source of Shh. We used an experimental approach that allowed us to selectively disrupt Shh signaling originating from the forebrain neuroectoderm and examined the morphological, cellular, and molecular consequences of this perturbation on forebrain and facial patterning, on craniofacial morphogenesis, and on development and maturation of the craniofacial skeleton. In doing so, we uncovered a Shh-dependent signaling center within the forebrain that regulates *Shh* expression in the face. Furthermore, we show that Shh signaling within the forebrain is not required for the initiation of skeletogenesis, but rather for elaborating the proximodistal and mediolateral axes of the middle and upper facial skeleton.

Materials and methods

In situ hybridization and immunohistochemistry

Patterns of gene expression were analyzed by in situ hybridization using radiolabeled riboprobes as previously described (Albrecht et al., 1997). Subclones of *Shh*, *Ptc1*, *Gli1*, *Fgf8*, *Pax6*, *Otx2*, *Dlx2*, *Nkx2.1*, *AP2*, *Msx1*, and *BarX1* were linearized to transcribe ^{35}S- or DIG-labeled riboprobes. Images of radioactive in situ hybridization assays are pseudo-colored superimpositions of the in situ hybridization signal and a blue nuclear stain (Hoescht Stain, Sigma) that are made using Adobe Photoshop. Briefly, two separate images were captured in Adobe Photoshop. One image was a fluorescent image of the nuclei, and the other was a dark field image of the in situ hybridization signal. These images were then superimposed as different layers within Photoshop. The "colorize" tool was used to add a contrasting color to everything within the layer containing the in situ hybridization signal. No changes in threshold intensities were made; however, slight adjustments to the contrast and brightness were performed to accurately reflect what was observed with the microscope. The image was then flattened for importation into Adobe Illustrator, where the final figures were assembled.

The detection of the 5E1 and 40-1A antibodies was accomplished on sections that were adjacent to those used for in situ hybridization. De-paraffinized, rehydrated sections were incubated for 10 min at room temperature in 3% H_2O_2 to quench endogenous peroxidase activity and then washed 3 times in wash buffer (PBS supplemented with 3 mg/ml BSA and IGEPAL detergent (0.1%)). Anti-mouse IgG conjugated to horse-radish peroxidase ((HRP) diluted 1:200) was applied to sections in wash buffer containing 10% normal goat serum and incubated overnight at 4°C. Sections were washed 3 times in wash buffer followed by a final wash in PBS. HRP was visualized by the application of diaminobenzidine supplemented with nickel and cobalt. The sections were counterstained with eosin and cover-slipped before viewing on a Leica DMRB brightfield microscope.

Preparation of embryos and inhibition of Shh signaling in the neuroectoderm

Fertilized chicken eggs (*Gallus gallus*, Rhode Island Red Chickens from Petaluma Farms, Petaluma, CA) were prepared for surgical manipulations as follows. A small hole was made in the shell directly over the embryo after removing 1.0 ml of albumin, and embryos were visualized by applying neutral red (Gibco, diluted 1:20 in Hanks Balanced Salt Solution, Sigma) using a blunt glass rod. To gain access to the embryo, the entire vitelline membrane was removed from over the embryo.

The inhibition of Shh signaling originating within the neuroectoderm was achieved by injecting hybridoma cells that expressed either the immunoneutralizing anti-Shh antibody (i.e., 5E1; Ericson et al., 1995b), or the control, anti-β-galactosidase antibody (i.e., 40-1A). Approximately 0.15 μl of media containing Trypan blue and cells (40×10^6 cells/ml) was injected into the anterior neural tube of stage 10 (Hamburger and Hamilton, 1951) chicken embryos. Both antibodies are IgG1 isotypes (Developmental Studies

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