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Disrupting the intrinsic growth potential of a suture contributes to midfacial hypoplasia

Jingtao Li [a,c,1], Chelsey A. Johnson [b,c,1], Andrew A. Smith [c], Benjamin Salmon [c,d,e], Bing Shi [a], John Brunski [c], Jill A. Helms [c,*]

[a] Department of Oral and Maxillofacial Surgery, West China Stomatology Hospital, Chengdu, China 610041
[b] College of Medicine, University of Arizona, Tucson, AZ 85721, USA
[c] Division of Plastic and Reconstructive Surgery, Department of Surgery, Stanford School of Medicine, Stanford, CA 94305, USA
[d] Dental School, University Paris Descartes PRES Sorbonne Paris Cité, EA 2496, Montrouge, France
[e] AP-HP Odontology Department Bretonneau, Hôpitaux Universitaires Paris Nord Val de Seine, Paris, France

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ABSTRACT

Children with unoperated cleft palates have nearly normal growth of their faces whereas patients who have had early surgical repair often exhibit midfacial hypoplasia. Surgical repair is responsible for the underlying bone growth arrest but the mechanisms responsible for these surgical sequelae are poorly understood. We simulated the effect of cleft palate repair by raising a mucoperiosteal flap in the murine palate. Three-dimensional micro-CT reconstructions of the palate along with histomorphometric measurements, finite element (FE) modeling, immunohistochemical analyses, and quantitative RT-PCR were employed to follow the skeletal healing process. Inflammatory bone resorption was observed during the first few days after denudation, which destroyed the midpalatal suture complex. FE modeling was used to predict and map the distribution of strains and their associated stresses in the area of denudation and the magnitude and location of hydrostatic and distortional strains corresponded to sites of skeletal tissue destruction. Once re-epithelialization was complete and wound contracture subsided, the midpalatal suture complex reformed. Despite this, growth at the midpalatal suture was reduced, which led to palatal constriction and a narrowing of the dental arch. Thus the simple act of raising a flap, here mimicked by denuding the mucoperiosteum, was sufficient to cause significant destruction to the midpalatal suture complex. Although the bone and cartilage growth plates were re-established, mediolateral skeletal growth was nonetheless compromised and the injured palate never reached its full growth potential. These data strongly suggest that disruption of suture complexes, which have intrinsic growth potential, should be avoided during surgical correction of congenital anomalies.

1. Introduction

Sutures are moveable joints in the craniofacial region that unite the bones of the face and skull [1]. Sutures have numerous functions: they act as articulation sites that allow minor movements of the craniofacial bones and thus protect bones from fracture [2], and as growth sites (reviewed in [3]), allowing the expansion of the skull to accommodate the growing brain [4] and face [5]. Disruptions to the sutures, caused by congenital defects, physical injury, or surgical intervention, can therefore have serious consequences. For example, premature fusion of the craniofacial sutures during early childhood (i.e., congenital craniosynostoses) causes significant morphologic abnormalities including hypoplasia of the midface, a compromised airway, and compression of the growing brain [6,7]. Trauma to suture regions in the craniofacial skeleton can also lead to growth arrest of the involved skeletal elements [8,9].

Surgical interventions can also cause an arrest in growth of the facial skeleton if they involve facial sutures [10–13]. For example, the vast majority of young (6–12 month old) patients who have undergone cleft palate repair show evidence of midfacial growth arrest [14–16]. In contrast, young patients who have undergone soft palate repair exhibit little observable impact on midfacial growth [17]. The growth arrest is not due to an inherent deficit in growth potential either, as cleft palate patients who do not undergo surgical repair typically exhibit normal dimensions to their dental arch, normal maxillary projection, and a normal Class II occlusion [15,16,18,19].

Together these findings imply that surgical perturbation of a suture will likely result in skeletal growth arrest. Precisely what aspect of surgical repair is most likely causing midfacial growth arrest, however, is unclear. Investigators have largely focused on mucoperiosteal denudation as being the culprit [20–23]. This procedure involves elevation of

* Corresponding author at: 257 Campus Drive, Stanford, CA 94305, USA.
E-mail address: jhelms@stanford.edu (J.A. Helms).
[1] Authors contributed equally to this work.

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the palatal mucoperiosteum, medial rotation of the flap to provide soft tissue coverage of the defect, and a resulting denudation of the palatine processes, which heals by secondary intention. Some groups have investigated the sites of these palatal bone denudations and demonstrated that the scar tissue covering this region is comprised of myofibroblasts [24] that appear to render the tissue "inelastic" [25]. A direct, causal relationship between the palatal scarring and midfacial growth arrest, however, has not been demonstrated [26,27].

We asked how mucoperiosteal denudation could have such a profound effect on facial growth. We first created a mouse model of mucoperiosteal denudation that specifically involved the midpalatal suture complex then used histology, immunohistochemistry, finite element (FE) modeling, micro-CT analyses, and quantitative molecular readouts to draw a direct link between the surgical procedure, the healing response, and the resulting palatal growth inhibition. In doing so we gained critical insights into how a commonly employed surgical procedure could have the unintended consequence of impeding midfacial development.

2. Materials and methods

2.1. Animal surgeries

All procedures were approved by the Stanford Committee on Animal Research. Gas anesthesia was delivered to post-natal day 7 (P7) C57BL/6 pups, and the palatal mucoperiosteal denudation was performed before awakening. With the use of a dissecting microscope, a 1 mm diameter full thickness punch was made in the middle of the hard palate and the mucoperiosteum was removed with forceps; care was taken to leave the underlying skeletal tissues intact. The anterior border of the punch is made immediately posterior to the first pair of discontinuous palatal rugae (Supplemental Fig. 1B). The wound healed by secondary intention. Age-matched littermates that were unoperated served as controls.

2.2. Histology and immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde at 4 °C overnight, decalcified in 19% EDTA at room temperature for 10 days, and dehydrated for paraffin embedding. Coronal sections were cut at a thickness of 8 μm. Histology was performed using Gomori Trichrome, Movat's Pentachrome, and Safranin O/Fast Green/Hematoxylin staining following standard staining procedures [28]. Picrosirius red staining was completed and imaged under polarized light as described [28]. For alkaline phosphatase (ALP) staining, slides were pre-incubated in NTMT buffer for 15 min and then stained in ALP solution containing 2 mL NTMT, 10 μl NBT (Roche), and 7.5 μl BCIP (Roche) for 30 min at 37 °C. Tartrate resistant acid phosphatase (TRAP) staining was performed using a Leukocyte Acid Phosphatase Kit (Sigma, St. Louis, MO). Immunohistochemistry for Ki67, Osteopontin, collagen I, collagen II, and X was carried out as described [28]. In brief, slides were immersed in 0.2% Triton for 5 min then incubated in Antigen Unmasking Solution (Vector Laboratories, diluted 1:100) at 95 °C for 20 min. After returning to room temperature slides were immersed in 3% hydrogen peroxide for 5 min and blocked in 5% goat serum for 30 min. Slides were incubated in corresponding primary antibodies (Ki67 rabbit polyclonal antibody, Thermo Scientific, diluted 1:100; Osteopontin rabbit polyclonal, Abcam, diluted 1:2000; Collagen I rabbit polyclonal antibody, Calbiochem, diluted 1:500; Collagen II rabbit polyclonal antibody, Millipore, diluted 1:50; Collagen X rabbit polyclonal antibody, Calbiochem, diluted 1:500) overnight at 4 °C. For Ki67 and Osteopontin detection, slides were incubated in biotinylated anti-rabbit antibody (Vector Laboratories, diluted 1:200) for 30 min, ABC (Vector Laboratories) for 30 min and developed with DAB (Vector Laboratories). For collagen I, II, and X detection, slides were incubated in Alexa Fluor 555, 488, and 555 goat anti-rabbit antibody (Invitrogen, diluted 1:500), respectively, for 30 min and then mounted with DAPI mounting medium (Vector Laboratories). To detect cell death, TUNEL (In Situ Cell Death Detection Kit, Roche) was performed as described by the manufacturer. Imaging of the stained tissue sections was performed with a Leica DM 5000B fluorescent microscope and a Leica DFC 500 digital camera.

2.3. Quantitative RT-PCR

Mucoperiosteal denudation was performed and animals were sacrificed at 7, 14, 21, and 28 days (Table 1). Tissues from all mice were harvested by microscopic dissection from 8 injured and 8 control mice for each of the four time points reported, for a total of 64 mice (Table 1). The epidermis was removed and the midpalatal suture complex, which included the medial edges of the palatine bones, its growth plates, and the fibrous interzone, was collected then homogenized in TRIzol (Invitrogen). RNA was quantified, and qRT-PCR was performed (Quantace Bioline, Taunton, MA). Expression levels were calculated using the 2^{Δ}-(ddCt) method, normalized to GAPDH [29], and converted to fold-expression. The following primer sets were used: *GAPDH*, acccagaagactgtggatgg and ggatgcagggatgatgttct; *Sox9*, agaacaagccac acgtcaag and cagcagcctccagagctt. *ALP*, accttgactgtggttactgc and catata ggatggccgtgaagg; *OPN*, catgaagagcggtgagtctaag and ttccagacttggttcatccag.

2.4. Micro-CT analyses and quantification of growth arrest

Micro-CT scanning (Imtek/Siemens MicroCAT II/SPECT system, 52 μm resolution) was performed using six injured and six age-matched control mice on PID28. Scanning results were exported into DICOM format and Osirix software version 5.8 (Pixmeo, Bernex, Switzerland) was employed to render the 3D multiplanar reconstruction in order to evaluate coronal sections across the midpalatal suture complex at exactly the same axis for each sample. Distances between left and right palatine foramen were measured and reported as interforaminal width. These skeletal landmarks were used as fiducials to assess the effect of the mucoperiosteal denudation injury on mediolateral expansion of the hard palate.

For histomorphometric analyses, tissues from 6 injured and 6 control mice were used for each of the four time points reported, for a total of 48 mice (Table 1). The palate was sectioned at 8 μm thickness/section and collected from the area bound by the first and second molars, corresponding to the middle region of the injury. Each slide contained two tissue sections. From the resulting ~30 slides, 6 slides were chosen (one every fifth slide) in order to perform the following quantifications. Tissue sections were stained with Ki67, Safranin O, or TUNEL protocols. Regions of interest (ROI) were defined by centering the midpalatal suture complex at 20× magnification and photographed using the Leica DFC 500 digital camera. A minimum of 6 images per sample and 6 separate samples were used.

To quantify the area of the growth plate composed of cartilage (which stains red after Safranin O/Fast Green staining), images were imported into Adobe PhotoShop. The area of the Safranin O stained cartilage growth plates was measured in a double-blinded manner by two independent investigators.

To quantify the extent of cell proliferation and cell death within the midpalatal suture complex, a standard process was employed [30–33] where regions of interest (ROI) were photographed using a minimum of 6 images per sample, and 6 separate samples. In the cases of TUNEL

Table 1
Distribution of animal groups.

Experimental group	Time points analyzed			
	Day 7	Day 14	Day 21	Day 28
Intact, qRT-PCR	8	8	8	8
Intact, histology/IHC	6	6	6	6
Injured, qRT-PCR	8	8	8	8
Injured, histology/IHC	6	6	6	6