Gene Ther Mol Biol Vol 9, 423-430, 2005

Craniosynostosis: current treatment and future therapy

Review Article

Matthew D. Kwan, Derrick C. Wan, Michael T. Longaker*

Children's Surgical Research Program, Division of Plastic and Reconstructive Surgery, Department of Surgery, Stanford University School of Medicine

---

***Correspondence**: Michael T. Longaker, MD, MBA, Stanford University School of Medicine, 257 Campus Drive, Stanford, CA 94305-5148; Phone: 650-736-1707; Fax: 650-736-1705; Email: Longaker@stanford.edu

Key words: Craniosynostosis, Gene Therapy, Fibroblast Growth Factors, Transforming Growth Factor-β, Bone Morphogenetic Proteins

Abbreviations: bone morphogenetic proteins (BMP); fibroblast growth factor receptors (FGF-R); fibroblast growth factors (FGF); long terminal repeats (LTR); posterior frontal (PF); quantitative real time reverse transcriptase polymerase chain reaction, (QRT-PCR); RNA-induced silencing complex (RISC); sagittal (SAG); transforming growth factor-β (TGF-β)

Received: 14 November 2005; Accepted: 21 November 2005; electronically published: November 2005

Summary

Craniosynostosis, the premature fusion of cranial sutures, is one of the most common congenital craniofacial conditions. It can lead to severe neurological and cognitive deficits in children. Surgery, with its inherent risks, is currently the only therapeutic modality. In the investigation of growth factors involved in cranial suture biology, including the fibroblast growth factors (FGF), transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMP), and BMP antagonists, potential targets for biologically based therapy are identified. With new developments in nucleic-acid based therapeutics, it is conceivable that these growth factors can be targeted with gene therapy to serve as an adjunct to minimally invasive surgery.

I. Introduction

With recent strides in medical genetics and molecular biology, it is evident that advances in the treatment of craniosynostosis are dependent upon elucidating the molecular events leading to this condition. Craniosynostosis, defined as the premature fusion of cranial sutures, is one of the most common congenital craniofacial abnormalities, with an overall incidence of 1:2000 live births world-wide (Cohen, 1979; Lajeunie et al, 1995).

Craniosynostosis is associated with a host of morphologic and functional abnormalities. Premature ossification of one or more cranial sutures results in dysmorphic craniofacial features. Of greatest concern is the elevated intracranial pressure that craniosynostosis can lead to, resulting in sequelae such as deafness, blindness, seizures, and cognitive deficits (Marchac and Renier, 1989). There are countless variants of craniosynostosis, dictated morphologically by the number and type of cranial sutures involved. When a suture fuses prematurely, compensatory growth of the skull will advance in directions parallel to the affected suture, while growth perpendicular to the affected suture is interrupted (Cohen, 2005).

Cranial sutures, consisting of dense fibrous articulations between the bone plates of the calvarium, are central to the discussion of craniosynostosis (Cohen, 2005). The cranial suture complex is composed of dura mater underlying the suture, the osteogenic fronts of the calvarial bone plates, the intervening cranial suture mesenchyme, and the overlying pericranium. Sutures provide a malleable quality to the neonate's skull, allowing for passage of the head through the birth canal. Cranial sutures also provide separation of the cranial bones during the period of brain growth (Panchal and Uttchin, 2003). The osteogenic fronts of the apposed bone plates are areas of high proliferation during cranial vault growth. At a cellular level, cranial suture fate may be dependent on the balance between osteoblast proliferation and differentiation in this region (Mathy et al, 2003). The interwoven relationships between the elements of the cranial suture complex interact together to mold the ultimate morphology of the skull.

On a molecular level, interactions between growth factors, their receptors, and transcription factors are an integral part of cranial suture biology. Only 8% of all cases of craniosynostosis occur in a familial pattern, but at least 150 syndromes have been identified with specific mutations resulting in craniosynostosis (Cohen, 2000).

The identification of these genetic alterations has not only underscored the molecular mechanisms fueling this abnormality, but has also provided targets for further investigation. Many of the syndromes, such as Apert, Pfeiffer, and Crouzon syndromes, can be traced to gain of function mutations in fibroblast growth factor receptors (FGF-R) (Wilkie, 1997). Craniosynostosis has also been found to result from mutations of *TWIST*, a helix-loop-helix transcription factor, in Saethre-Chotzen syndrome, and *MSX2*, a member of the homeobox gene family, in the Boston-type craniosynostosis (Jabs et al, 1993; el Ghouzzi et al, 1997; Howard et al, 1997; Ishii et al, 2003).

Significant investigation is still required to understand the exact interplay between these molecules, and to identify other factors in the etiopathogenesis of craniosynostosis. Advances in the molecular realm will be crucial to developing novel treatments for craniosynostosis. This article will provide a brief summary of the current therapeutic approach to craniosynostosis. We will also discuss pertinent findings in cranial suture biology, and the potential applications of these findings for developing biologically based treatments for craniosynostosis.

II. Current treatment

Current treatment of craniosynostosis consists of surgical modalities. Surgical approaches have evolved to excise the prematurely fused suture and to remodel dysmorphic skulls via various cranial vault remodeling and advancement procedures. This requires not only strip craniectomies of the affected sutures, but often extensive osteotomies and repositioning of bony plates to remodel the cranial vault. The goal of surgery is to increase intracranial volume and to prevent elevations of intracranial pressure. As a result, surgical correction of craniosynostosis is best begun within the first three to six months of life. Delay in correcting craniosynostosis can exacerbate associated facial skeletal abnormalities such as facial asymmetry, malocclusion, and strabismus (Panchal and Uttchin, 2003). The exact operations required depend on the sutures involved. For example, metopic synostosis is marked by a restriction in the volume of the anterior cranial fossa. This is treated by performing a sagittal advancement of the fronto-orbital bar, with particular focus on the lateral regions (Panchal and Uttchin, 2003). Midface hypoplasia and other craniofacial dysmorphisms often accompany syndromic forms of craniosynostosis and are usually addressed at 4-5 years of age.

Despite advances made in craniofacial surgery for the treatment of craniosynostosis, the operations are not without their own morbidities. These operations are usually performed in the young infant, a time when they are most susceptible to physiologic insults. Infection, optic nerve ischemia, seizures, bleeding, and the need for blood transfusions are not insignificant events in the infant or young child (Whitaker, 1979; Grabb et al, 1991). It has been estimated that 80-100% of patients undergoing frontoorbital advancement procedures for craniosynostosis require blood transfusions (Meara, 2005). This is compounded by the need for further surgery, whether for suture re-fusion after strip craniectomy or for correction of secondary defects. Mortality rates have been described to be as high as 1.5-2% overall (Whitaker, 1979). For these reasons, the development of minimally invasive, biologically based-therapies offer a very attractive option to be pursued for the treatment of craniosynostosis.

III. Cranial suture biology

For a variety of reasons, the murine model has proven to be instrumental in the study of craniosynostosis. In rats and mice, the posterior frontal (PF) suture fuses in a predictable manner, while all other cranial sutures remain patent. This natural juxtaposition allows for in depth analysis of factors dictating cranial suture biology. Because craniosynostotic tissues from humans are often limited and represent post-fusion samples, studying sutures in mice or rats obviates this concern. Investigations using the murine model have allowed for the process of suture fusion to be analyzed along a time continuum. And with the advent of transgenic mice, these models have also allowed for the function of genes and their interaction with each other to be evaluated *in vivo*.

Within the cranial suture complex, the underlying dura mater and its paracrine signaling has been shown to be critical in determining suture fate. Opperman and colleagues demonstrated in E19 and P1 rats that only coronal sutures with associated dura mater were able to maintain patency when transplanted into the center of parietal bones. In contrast, sutures transplanted without dura mater displayed suture fusion. Interestingly, the presence or absence of periosteum had no effect on suture fate in this model (Opperman et al, 1993). Our laboratory has also highlighted the paracrine role of the underlying dura mater, by performing a strip craniectomy in rats along the sagittal (SAG) and PF sutures. Leaving the underlying dura mater undisturbed, the suture complex was then rotated 180 degrees and reimplanted so that the SAG suture was positioned over the PF dura mater and the PF suture over the SAG dura mater. After reversal of this strip of calvarium, the PF suture, remained patent, while the SAG suture abnormally fused (Levine et al, 1998).

These studies all point to the paracrine signaling between the cranial suture and its associated suture complex. A host of molecules are known to mediate this communication. Much work has been devoted to examining the roles of fibroblast growth factors (FGF), transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), and BMP antagonists in this signaling network.

A. TGF-β

TGF-β describes a superfamily of signaling molecules with roles in a broad range of processes, including embryonic development, wound healing, cellular migration, extracellular matrix synthesis, and of particular relevance to craniosynostosis, osteoblast biology (Javelaud, 2005). The superfamily includes the TGF-β isoforms, BMPs, activin, and nodal. The ligands bind to a heterotetramer transmembrane receptor complex and activate a serine/threonine protein kinase signaling cascade, ultimately effecting changes in gene transcription

through phosphorylated Smad protein intermediates (Zavadil, 2005). In humans, TGF-β consists of three isoforms (β1, β2, β3), which are potent growth regulatory molecules. Importantly, TGF-β molecules have been found to mediate proliferation and differentiation of osteoblasts (Poisson et al, 2004).

TGF-β isoforms have been shown to play a critical role in regulating cranial suture patency. Analyzing immunoreactivity for the three TGF-β isoforms in suture samples from patients with craniosynostosis, TGF-β2, in particular, was found to be heavily expressed in fusing sutures (Roth et al, 1997a). Similarly, Poisson and colleagues compared the distribution of TGF-β isoforms between cranial sutures from wild type rabbits and a lagamorph strain with familial, coronal craniosynostosis. Immunohistochemistry revealed that premature fusion of coronal sutures in this strain of rabbits was associated with increased expression of TGF-β2, and decreased levels of TGF-β1 and -β3 (Poisson et al, 2004). Coronal suture fusion was prevented in this lagamorph model with the exogenous application of TGF-β3 in a dose-dependent fashion (Chong, 2003). These findings paralleled the proposal by Opperman and colleagues that TGF-β3 inhibits the activity of TGF-β2 and prevents suture fusion by down regulating expression of TGF-β receptors (Opperman et al, 2002).

Studies have demonstrated similar findings with TGF-β distribution in the murine model. Immunohistochemistry revealed TGF-β, especially the β2 isoform, to display relatively stronger immunoreactivity in fusing PF sutures compared to patent SAG sutures. Furthermore, with addition of exogenous TGF-β2 to the SAG sutures of neonatal rats, abnormal fusion was observed (Roth et al, 1997b). Northern analysis of dura mater and quantitative real time reverse transcriptase polymerase chain reaction (QRT-PCR) of the suture complex found TGF-β expression to be significantly higher in the fusing PF suture than the patent SAG suture (Greenwald et al, 2000; Nacamuli, 2004).

In an experiment that confirmed the necessity of TGF-β's role in suture fusion, murine PF sutures were transfected with an adenovirus encoding the dominant negative TGF-β receptor. These sutures, with attenuated expression of TGF-β receptors, demonstrated interrupted fusion of the PF suture when compared to those treated with viral vehicle alone (Mehrara et al, 2002). Sutures transfected with the dominant negative TGF-β receptor also revealed a down regulation of collagen I expression. Furthermore, blocking TGF-β signaling inhibited proliferation of osteoblasts *in vitro* (Song et al, 2004).

B. FGF

The FGF family consists of 24 highly conserved proteins, involved in a broad range of cell regulatory activities, including proliferation, differentiation, and migration (Dailey et al, 2005). The ligand transmits its effects via a family of receptors, consisting of four transmembrane tyrosine kinase proteins. The FGF ligands induce receptor homodimerization or heterodimerization, which in turn causes phosphorylation of intracellular tyrosine residues. The two major pathways for cytoplasmic signal transduction are activation of phopholipase C-γ or Crk proteins (Powers et al, 2000). The varying effects of FGF depend on temporal factors, tissue environment, as well as type of receptor activation (Ornitz and Itoh, 2001). Because the majority of craniosynostosis syndromes, such as Apert, Crouzon, Pfeiffer, and Jackson-Weiss syndromes, are associated with gain of function mutations in FGF receptors, this family of messenger molecules has been extensively studied in the context of cranial suture biology (Warren and Longaker, 2001; Spector et al, 2005).

Mounting evidence suggests FGF-2, the most abundant ligand, to be a mediator of cranial suture fate. There are significant data from the murine model suggesting that FGF-2 is secreted by the dura mater and subsequently acts in paracrine fashion to promote fusion (PF) of the overlying posterior frontal suture. Immunohistochemical staining of rat PF and SAG sutures revealed relatively increased immunoreactivity of FGF-2 in the dura mater underlying the PF suture prior to and during the period of fusion (Mehrara et al, 1998).

The role of FGF signaling in dictating cranial suture fate has been further demonstrated by adenoviral mediated delivery of a dominant negative FGF-R1 construct to the dura mater underlying the PF suture in embryonic rats. Subsequent interruption of FGF signaling prevented normal PF suture fusion. Conversely, fusion of the coronal suture was induced by *in utero* infection of the underlying dura with an FGF-2 overexpressing adenovirus (Greenwald et al, 2001).

It appears that the FGF receptors also contribute to the regulation of cranial suture fate. In analysis of the FGF receptors, Iseki *et al.* found that FGF-R2, in embryonic mouse coronal sutures, was localized to regions of high proliferation and low differentiation, as evidenced by the absence of *osteopontin*, a marker of early osteoblastic differentiation. Of interest, however, FGF-R1 localized to regions of differentiation, as determined by expression of the osteoblastic differentiation markers, *osteopontin* and *osteonectin.* Similarly, in these regions of differentiation, high concentrations of FGF-2 were revealed as well (Iseki et al, 1999). Using QRT PCR, our laboratory demonstrated elevated levels of *FGF-R1* transcripts in the fusing PF suture as compared to the patent SAG suture. Of interest, the PF suture also displayed evidence of increased osteoblast differentiation, with higher levels of *runx2, osteopontin, and osteocalcin* transcripts than in the patent SAG suture (Nacamuli et al, 2004).

C. BMP and BMP antagonists

BMPs are part of the TGF-β superfamily and were originally found to induce both bone and cartilage formation (Wan, 2005). This large group of proteins, comprising nearly one-third of the TGF-β superfamily, has also been found to be involved in mesoderm induction, skeletal patterning, and limb development (Duprez et al, 1996). Immunohistological analysis of embryonic mouse sagittal sutures revealed expression of BMP-2 and BMP-4 at the osteogenic fronts, and BMP-4 in suture mesenchyme and dura mater. In the postnatal mouse, BMP-4 was found

to be equivalently expressed in the suture mesenchyme and osteogenic fronts of both fusing and patent sutures (Kim et al, 1998).

Our laboratory discovered similar findings of BMP expression in both fusing and patent sutures. This suggested the presence of BMP antagonist molecules that are differentially expressed to regulate BMP activity. Upon screening mRNA levels of BMP antagonists, *noggin* expression levels were found to be significantly higher in the patent coronal and sagittal sutures (Warren et al, 2003). Noggin is a secreted protein, originally described in the X*enopus* embryo, which inhibits BMP signaling through direct binding. Noggin binds with high affinity to BMP-2 and BMP-4 (Yanagita, 2005). Exogenous FGF-2 was found to inhibit Noggin expression, implying a mechanism for the various syndromic forms of craniosynostosis linked to gain of FGF-R function (Warren, et al, 2003).

Another BMP antagonist is BMP-3, which, in contrast to Noggin, does not bind directly to BMP ligands. BMP-3 has been shown to bind with Activin receptors and thus negate BMP signaling through competition for their shared intracellular signal transducers, the Smads (Daluiski et al, 2001). Using microarray analysis on rat PF and SAG cranial sutures, our laboratory found BMP-3 expression to increase over time in the patent SAG suture, while decreasing in the fusing PF suture. These findings all suggest BMP-3 as another osteogenic antagonist that plays a role in maintaining suture patency (Nacamuli et al, 2005).

IV. Future treatment

Paralleling recent work on cranial suture biology has been the explosion of nucleic acid-based therapeutics and the delivery mechanisms of these biological products. While only two DNA-based drugs, fomivirsen, an antisense oligonucleotide for treatment of CMV retinitis, and Gendicine, a p53 adenovirus for oncological applications, have been approved for use, countless other formulations are in various stages of clinical trials (Patil, 2005). Progress has been impeded predominantly by development of safe and effective techniques (Verma and Weitzman, 2005). Nevertheless, medicine sits on the cusp of an exciting revolution where diseases are targeted at the genomic level. The application of these developments to craniosynostosis presents exciting options to surgical intervention.

As the exact roles of FGF, TGF-β, BMP, BMP antagonists, and their respective receptors are being clarified in cranial suture biology, it is foreseeable that these molecules will be targets of therapy for craniosynostosis. With the increased use of microarray technology, other potential candidate genes will also be identified. The goal would be to suppress the expression of genes promoting suture fusion or to increase expression of those which support suture patency. While the direct application of exogenous growth factors or neutralizing antibodies is an option, shortfalls of such approaches include these short half-life, low bioavailability, enzymatic inactivation, and high cost of purification. With such considerations, gene therapy holds great promise for the future of craniosynostosis.

A. Gene therapy modalities

The armamentarium for effecting changes in gene expression is rapidly expanding to include plasmids, antisense oligonucleotides, aptamers, and more recently, short interfering RNA (siRNA). While efforts are being directed towards optimizing the efficiency of these various agents, the safety of these therapeutics for use in humans is at the forefront of considerations.

Plasmids represent one approach to introduce gene expression into target cells. Plasmids are high molecular weight, double-stranded DNA constructs containing transgene products. Upon gaining entry into the nucleus of the target cell, plasmids employ the cell's endogenous machinery to synthesize the transgene protein (Patil, 2005).

As a means of blocking gene expression, antisense oligonucleotides involve the introduction of short single-stranded nucleic acid segments into a cell which can subsequently bind with cytoplasmic mRNA and block protein translation. Antigene techniques involve the introduction of oligonucleotides into the cell's nucleus to bind directly with double stranded DNA, inhibiting protein production at the transcriptional level. Chemical modifications to the backbone of oligonucleotides can be performed, making them less susceptible to degradation by endonucleases (Patil, 2005). Ribozymes provide yet another means to knockout gene expression. These are RNA molecules that selectively bind to target mRNA, forming a duplex, causing distortion of the target structure, and hence resulting in hydrolysis of the target mRNA. Because the backbone of a ribozyme complex is RNA, it is an inherently unstable molecule *in vivo*. DNAzymes are analogs of ribozymes, but are much more stable (Patil, 2005).

Aptamers represent a novel method for inhibiting protein production. They are either single- or double-stranded nucleic acids that behave similarly to antibodies by binding directly with proteins. Because of their specificity, nonimmunogenicity, and overall pharmacokinetic stability, they are often preferred to antibodies (Patil, 2005).

The development of RNA interference presents an exciting option for targeted gene knockout. Originally described in plants and subsequently demonstrated in other organisms, this system utilizes the cell's natural defense against double stranded RNA, usually in the form of an RNA virus, to suppress gene expression (Fire, 1998). When double stranded RNA is introduced intracellularly it is recognized by the RNAse III enzyme, Dicer, which cleaves it into double stranded small interfering RNA. A specialized protein, known as the RNA-induced silencing complex (RISC), then unwinds the double stranded siRNA and joins with the antisense strand. This complex then binds with a high degree of specificity to the target mRNA, eliciting cleavage (Lu et al, 2005). SiRNA technology is potentially superior to other forms of gene therapy because of its high degree of specificity, its efficiency at silencing gene expression, its relative

nonimmunogenic nature, and its high degree of resistance to ribonucleases (Patil, 2005). Because of its cytoplasmic activity, siRNAs circumvent the obstacle of transfer into the nucleus, providing for greater potential efficiency. In addition, siRNAs do not integrate into the host genome, eliminating safety concerns of insertional mutagenesis. Finally, because of their small size, the theoretical potential of simultaneously delivering multiple siRNAs would allow for targeting of multiple genes (Patil, 2005).

There is increasing interest in developing siRNA into a therapeutic modality in medicine, as reflected by the numerous *in vivo* trials currently. Initially, most *in vivo* trials focused on local delivery of the siRNA to suppress gene expression. For example, Bitko and colleagues demonstrated prevention of RSV pulmonary infection in mice with intranasal delivery of siRNA as an antiviral agent (Bitko, 2005). For treatment of retinal neovascularization, knockout of VEGF expression in a murine model was achieved by local delivery of siRNA (Kim et al, 2004). Dorn and colleagues demonstrated in rats decreased pain responses after intrathecal infusion of siRNA targeted at the pain-related cation channel gene (Dorn et al, 2004).

SiRNAs have also been introduced systemically in the murine model. Soutschek and colleagues introduced siRNA via the tail vein in mice, targeting the apolipoprotein B gene. They were able to demonstrate decreased plasma levels of apolipoprotein B and total cholesterol subsequent to systemic administration of the siRNA (Soutschek, 2004). Recentl efforts have been directed at increasing the specificity of siRNA delivery *in vivo*. Song and colleagues, utilized systemic introduction of siRNAs against HIV infected cells. HIV infected cells were specifically targeted by attaching the siRNA to protamine, which enhanced intracellular entry, and to an anti-HIV envelope protein antibody, which provided the siRNA with cellular specificity. (Song et al, 2005). Work has also been directed towards improving efficiency of siRNA *in vivo*. Morrissey and colleagues packaged siRNA directed against the hepatitis B virus in a liposome and demonstrated improved efficiency of delivery compared to the naked siRNA in the mouse model (Morrissey, 2005).

B. Delivery systems

Delivery of gene products using non-viral techniques includes direct injection of DNA, liposomes, electroporation, and particle bombardment. Direct injection of naked plasmid DNA containing the *luciferase* gene was first demonstrated in 1990 by Wolff using rodent muscle, with resultant luciferase activity (Wolff et al, 1990). This technique is attractive because of its simplicity, lack of systemic effects, and effectiveness on non-dividing cells. Concerns, however, have been raised over the ability to reach internal organs and the low transfection efficiencies (Tepper and Mehrara, 2002). Liposomes consist of phospholipids which assemble themselves in a bilayer, encapsulating the genetic material to be delivered (Hashida et al, 2005). Liposomes, which utilize the cell's phagocytic mechanisms to gain intracellular entry, are attractive because of their large carrying capacity, ease of assembly, and relative lack of immunogenicity. However, work remains to improve the nonspecific targeting of liposomes, as well as its relatively low transfection efficiency (Tepper and Mehrara, 2002). Electroporation, which uses brief electric pulses to make the plasma membrane of cells permeable to exogenous DNA molecules, is not toxic and can allow delivery of larger size DNA molecules. Electroporation can also be used to transfect nonreplicating cells. This delivery system is not optimal because of its low efficiency and the need for applying an electric current to the tissue of interest (Tepper and Mehrara, 2002). Finally, non-viral delivery of gene products can be achieved with a "gene gun." This involves bombarding target tissue with DNA coated, microscopic gold particles. Also known as particle bombardment, this technique has been applied to muscle, skin, and liver (Tepper and Mehrara, 2002).

Viral vectors can be classified in broad terms to those which integrate into the host genome, such as retroviruses and lentiviruses, and those which remain episomal, such as adenoviruses. Retroviruses allow one to selectively infect proliferating cells, an attractive attribute when targeting malignancies (Tepper and Mehrara, 2002). Retroviruses are composed of a RNA genome with three essential genes, *gag*, *pol*, and *env*, and flanked by long terminal repeats (LTR). All or portions of these genes are deleted and replaced in designing recombinant retroviral vectors. Lentiviruses also integrate their transgene products into the host genome. However, these vectors have the ability to infect dividing and non-dividing cells, allowing for stable, long-term transgene expression (Thomas et al, 2003). As with retroviruses, there is the concern for insertional mutagenesis with lentiviral vectors. Finally, adenoviruses infect both dividing and non-dividing cells with high efficiency, but do not incorporate their DNA into the host cell chromosome (Thomas et al, 2003).

The surgical literature has already demonstrated experience with gene therapy via adenoviral vectors for osseous defects. Lindsey utilized a BMP-2 adenoviral vector to reengineer critical size defects in the nasal bone of athymic nude rats (Lindsey, 2001). In the field of craniofacial surgery, adenoviral constructs have also been used to deliver BMP-2 and BMP-9 to critical size mandibular defects, with increased healing compared with controls (Alden et al, 2000).

Our laboratory has demonstrated the ability to use local adenoviral treatment of sutures to control expression of target molecules and, hence, effect changes in suture fate. Using an adenovirus to deliver *noggin* to the PF sutures of mice, we were able to prevent fusion of the PF suture. After adenoviral infection, increased *noggin* expression was demonstrated *in vitro*, in a suture organ culture. In the *in vivo* model, local delivery of the *noggin* adenovirus to the PF sutures of 3 day old mice, resulted in not only histological characteristics of widely patent PF sutures, but also anatomic attributes, including short broad snouts and widely spaced eyes (Warren et al, 2003).

Adenoviral delivery of a dominant-negative form of the TGF-β receptor II to prevent fusion of the mouse PF suture in organ culture has also been shown by our laboratory (Mehrara et al, 2002; Song et al, 2004). Further studies demonstrated that interference of TGF-β signaling,

via the induction of a dominant-negative TGF-β receptor, suppressed expression of certain markers of osteoblast differentiation, including *MSX2* and *osteopontin*, and of collagen IaI, an extracellular matrix molecule (Mehrara et al, 2002; Song et al, 2004).

Finally, our laboratory has shown that using local adenoviral infection to block FGF signaling *in vivo* translated into alterations of suture fate. Infection of rat PF sutures *in utero* with a dominant-negative FGF-R1 construct resulted in prevention of physiologic suture fusion. Conversely, adenoviral infection of the normally patent coronal suture with a FGF-2 construct resulted in pathologic suture fusion (Greenwald et al, 2001).

Much remains to be learned about the molecular and cellular mechanisms of cranial suture biology. However, it is conceivable that gene therapy can be targeted at manipulating expression of key molecules to prevent pathologic suture fusion. One approach would be to inhibit expression of molecules that promote suture ossification, such as FGF-2, TGF-β2, MSX-2, and NELL-1. Gene therapy could also be directed at increasing expression of molecules which foster suture patency, such as the BMP antagonists, Noggin and BMP-3. Significant investigations are still required to determine which gene or group of genes would serve as the optimal target. Although, biological treatments of craniosynostosis have only been tested in animal experiments, it portends promise as a therapy modality in humans. Methods of delivery, dosing, and timing are all factors that will need to be clarified, but perhaps the best approach would be to use gene therapy as an adjuvant treatment combined with minimally invasive surgery to prevent suture re-fusion post-operatively.

V. Conclusion

Craniosynostosis is a common congenital disease with dramatic consequences on craniofacial morphology, as well as neurologic and cognitive development. Current treatment entails extensive, surgical remodeling of the infant's skull at a relatively young age. Research, however, continues to be directed towards identifying new growth factors involved in craniosynostosis and decoding the role of known elements in regulating calvarial osteoblast biology and cranial suture fusion. With the simultaneous burgeoning of gene therapy, we will be able to identify potential genes and target them for suppression or increased expression in order to prevent pathologic suture fusion. Future work continues to be directed at improving the efficacy of gene therapy. As gene therapy technology improves, we are hopeful that it can be applied in humans to the treatment of craniosynostosis. However, ensuring patient safety, is at the forefront of all considerations.

Acknowledgments

This work was supported by National Institutes of Health grant R01 DE13194 and a grant from the Oak Foundation to Michael T. Longaker.

References

Alden TD, Beres EJ, Laurent JS, Engh JA, Das S, London SD, Jane JA, Hudson SB and Helm GA **(2000)** The use of bone morphogenetic protein gene therapy in craniofacial bone repair. **J Craniofac Surg** 11, 24-30.

Bitko V, Musiyenko A., Shulyayeva O, Barik S **(2005)** Inhibition of respiratory viruses by nasally administered siRNA. **Nat Med** 11, 50-55.

Chong S, Mitchell R, Moursi AM, Winnard P, Losken HW, Bradley J, Ozerdem OR, Azari K, Acarturk O, Opperman LA, Siegel MI, Mooney MP **(2003)** Rescue of coronal suture fusion using transforming growth factor-β3 (Tgf-β3) in rabbits with delayed-onset craniosynostosis. **Anat Rec A Discov Mol Cell Evol Biol** 274, 962-71.

Cohen MM **(1979)** Craniosynostosis and syndromes with craniosynostosis: incidence, genetics, penetrance, variability and new syndrome updating. **Birth Defects Orig Artic Ser** 15, 13-63.

Cohen MM **(2000)** Craniofacial disorders caused by mutations in homeobox genes MSX1 and MSX2. **J Craniofac Genet Dev Biol** 20, 19-25.

Cohen MM **(2005)** Editorial: perspectives on craniosynostosis. **Am J Med Genet A**136, 313-326.

Dailey L, Ambrosetti D, Mansukhani A and Basilico C **(2005)** Mechanisms underlying differential responses to FGF signaling. **Cytokine Growth Factor Rev** 16, 233-47.

Daluiski A, Engstrand T, Bahamonde M E, Gamer LW, Agius E, Stevenson SL, Cox K, Rosen V and Lyons KM **(2001)** Bone morphogenetic protein-3 is a negative regulator of bone density. **Nat Genet** 27, 84-8.

Dorn G, Patel S, Wotherspoon G, Hemmings-Mieszczak M, Barclay J, Natt FJ, Martin P, Bevan S, Fox A, Ganju P, Wishart W and Hall J **(2004)** siRNA relieves chronic neuropathic pain. **Nucleic Acids Res** 32, e49.

Duprez D, Bell EJ, Richardson MK, Archer CW, Wolpert L, Brickell PM and Francis-West PH **(1996)** Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. **Mech Dev** 57, 145- 57.

el Ghouzzi V, Le Merrer M, Perrin-Schmitt F, Lajeunie E, Benit P, Renier D, Bourgeois P, Bolcato-Bellemin AL, Munnich A and Bonaventure J (**1997)** Mutations of the TWIST gene in the Saethre-Chotzen syndrome. **Nat Genet** 15, 42-46.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC **(1998)** Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. **Nature** 391, 806-811.

Grabb WC, Smith JW and Aston SJ **(1991)** Plastic surgery, 4th edn. Little, Brown.

Greenwald JA, Mehrara BJ, Spector JA, Warren SM, Crisera FE, Fagenholz PJ, Bouletreau PJ and Longaker MT **(2000)** Regional differentiation of cranial suture associated dura mater *in vivo* and *in vitro*: implications for suture fusion and patency. **J Bone Miner Res** 15, 2413-30.

Greenwald JA, Mehrara BJ, Spector JA, Warren SM, Fagenholz PJ, Smith LE, Bouletreau PJ, Crisera FE, Ueno H and Longaker MT **(2001)** *In vivo* modulation of FGF biological activity alters cranial suture fate. **Am J Pathol** 158, 441-452.

Hashida M, Kawakami S and Yamashita F **(2005)** Lipid carrier systems for targeted drug and gene delivery. **Chem Pharm Bull (Tokyo)** 53, 871-80.

Howard TD, Paznekas WA, Green ED, Chiang LC, Ma N, Ortiz de Luna RI, Garcia Delgado C, Gonzalez-Ramos M, Kline AD and Jabs EW **(1997)** Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. **Nat Genet** 15, 36-41.

Iseki S, Wilkie AO and Morriss-Kay GM **(1999)** Fgfr1 and Fgfr2 have distinct differentiation- and proliferation-related roles in

the developing mouse skull vault. **Development** 126, 5611-20.

Ishii M, Merrill AE, Chan YS, Gitelman I, Rice DP, Sucov HM and Maxson RE **(2003)** Msx2 and Twist cooperatively control the development of the neural crest-derived skeletogenic mesenchyme of the murine skull vault. **Development** 130, 6131-42.

Jabs EW, Muller U, Li X, Ma L, Luo W, Haworth IS, Klisak I, Sparkes R, Warman ML and Mulliken JB (**1993)** A mutation in the homeodomain of the human MSX2 gene in a family affected with autosomal dominant craniosynostosis. **Cell** 75, 443-450.

Javelaud D, Mauviel A **(2005)** Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-β: implications for carcinogenesis. **Oncogene** 24, 5742-5750.

Kim B, Tang Q, Biswas PS, Xu J, Schiffelers RM, Xie FY, Ansari AM, Scaria PV, Woodle MC, Lu P and Rouse BT **(2004)** Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. **Am J Pathol** 165, 2177-85.

Kim H J, Rice DP, Kettunen PJ and Thesleff I **(1998)** FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. **Development** 125, 1241-51.

Lajeunie E, Le Merrer M, Bonaiti-Pellie C, Marchac D and Renier D **(1995)** Genetic study of nonsyndromic coronal craniosynostosis. **Am J Med Genet** 55, 500-4.

Levine JP, Bradley JP, Roth DA, McCarthy, JG and Longaker MT **(1998)** Studies in cranial suture biology: regional dura mater determines overlying suture biology. **Plast Reconstr Surg** 101, 1441-1447.

Lindsey WH **(2001)** Osseous tissue engineering with gene therapy for facial bone reconstruction. **Laryngoscope** 111, 1128-36.

Lu PY, Xie F and Woodle MC **(2005)** *In vivo* application of RNA interference: from functional genomics to therapeutics. **Adv Genet** 54, 117-42.

Marchac D and Renier D **(1989)** Craniosynostosis. **World J Surg** 13, 358- 365.

Mathy JA, Lenton K, Nacamuli RP, Fong KD, Song HM, Fang TD, Yang GP and Longaker MT **(2003)** FGF-2 stimulation affects calvarial osteoblast biology: quantitative analysis of nine genes important for cranial suture biology by real-time reverse transcription polymerase chain reaction. **Plast Reconstr Surg** 112, 528-39.

Meara J, Smith EM, Harshbarger RJ, Farlo JN, Matar MM, Levy ML **(2005)** Blood conservation techniques in craniofacial surgery. **Ann Plast Surg** 54, 525-529.

Mehrara BJ, Mackool RJ, McCarthy JG, Gittes GK and Longaker MT **(1998)** Immunolocalization of basic fibroblast growth factor and fibroblast growth factor receptor-1 and receptor-2 in rat cranial sutures. **Plast Reconstr Surg** 102, 1805-17; discussion 1818-20.

Mehrara BJ, Spector JA, Greenwald JA, Ueno H and Longaker MT **(2002)** Adenovirus mediated transmission of a dominant negative transforming growth factor-β receptor inhibits *in vitro* mouse cranial suture fusion. **Plast Reconstr Surg** 110, 506-14.

Morrissey D, Lockridge JA, Shaw L, Blanchard K, Jensen K, Breen W, Hartsough K, Machemer L, Radka S, Jadhav V, Vaish N, Zinnen S, Vargeese C, Bowman K, Shaffer CS, Jeffs LB, Judge A, MacLachlan I, Polisky B **(2005)** Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNAs. **Nat Biotechnol** 23, 1002-1007.

Nacamuli RP, Fong KD, Lenton KA, Song HM, Fang TD, Salim A and Longaker MT **(2005)** Expression and possible mechanisms of regulation of BMP3 in rat cranial sutures. **Plast Reconstr Surg** 116, 1353-62.

Nacamuli RP, Song HM, Fang TD, Fong KD, Mathy JA, Shi YY, Salim A, Longaker MT **(2004)** Quantitative transcriptional analysis of fusing and nonfusing cranial suture complexes in mice. **Plastic Reconstr Surg** 114, 1818-1825.

Opperman LA, Galanis V, Williams AR and Adab K **(2002)** Transforming growth factor-β3 (Tgf-β3) down-regulates Tgf-β3 receptor type I (Tβr-I) during rescue of cranial sutures from osseous obliteration. **Orthod Craniofac Res** 5, 5-16.

Opperman LA, Sweeney TM, Redmon J, Persing JA, Ogle RC **(1993)** Tissue interactions with underlying dura meter inhibits osseous obliteration of developing cranial sutures. **Dev Dyn** 198, 312-22.

Ornitz DM and Itoh N **(2001)** Fibroblast growth factors. **Genome Biol** 2, REVIEWS3005.

Panchal J and Uttchin V **(2003)** Management of craniosynostosis. **Plast Reconstr Surg** 111, 2032-48.

Patil S, Rhodes DG, Burgess DJ **(2005)** DNA-based therapeutics and DNA delivery systems: a comprehensive review. **AAPS J** 7, 61-77.

Poisson E, Sciote JJ, Koepsel R, Cooper GM, Opperman LA and Mooney MP **(2004)** Transforming Growth Factor-β Isoform Expression in the Perisutural Tissues of Craniosynostotic Rabbits. **Cleft Palate Craniofac J** 41, 392-402.

Powers CJ, McLeskey SW and Wellstein A **(2000)** Fibroblast growth factors, their receptors and signaling. **Endocr Relat Cancer** 7, 165-97.

Roth DA, Gold LI, Han VK, McCarthy JG, Sung JJ, Wisoff JH and Longaker MT **(1997a)** Immunolocalization of transforming growth factor β 1, β 2 and β 3 and insulin-like growth factor I in premature cranial suture fusion. **Plast Reconstr Surg** 99, 300-309.

Roth DA, Longaker MT, McCarthy JG, Rosen DM, McMullen HF, Levine JP and Gold LI **(1997b)** Studies in cranial suture biology: Part I. Increased immunoreactivity for transforming growth factor-β (β1, β2, β3) during rat cranial suture fusion. **J Bone Miner Res** 12, 311-321.

Song E, Zhu P, Lee SK, Chowdhury D, Kussman S, Dykxhoorn DM, Feng Y, Palliser D, Weiner DB, Shankar P, Marasco WA, Lieberman J **(2005)** Antibody mediated *in vivo* delivery of small interfering RNAs via cell-surface receptors. **Nat Biotechnol** 23, 709-717.

Song HM, Fong KD, Nacamuli RP, Warren SM, Fang TD, Mathy JA, Cowan CM, Aalami OO and Longaker MT **(2004)** Mechanisms of murine cranial suture patency mediated by a dominant negative transforming growth factor-β receptor adenovirus. **Plast Reconstr Surg** 113, 1685-97.

Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Röhl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliansky V, Limmer S, Manoharan M, Vornlocher HP **(2004)** Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. **Nature 432**, 173-178.

Spector JA, Mathy JA, Warren SM, Nacamuli RP, Song HM, Lenton K, Fong KD, Fang DT and Longaker MT **(2005)** FGF-2 acts through an ERK1/2 intracellular pathway to affect osteoblast differentiation. **Plast Reconstr Surg** 115, 838-52.

Tepper OM and Mehrara BJ **(2002)** Gene therapy in plastic surgery. **Plast Reconstr Surg** 109, 716-34.

Thomas CE, Ehrhardt A and Kay MA **(2003)** Progress and problems with the use of viral vectors for gene therapy. **Nat Rev Genet** 4, 346-58.

Verma IM and Weitzman MD **(2005)** Gene therapy: twenty-first century medicine. **Annu Rev Biochem** 74, 711-38.

Wan M, Cao X **(2005)** BMP signaling in skeletal development. **Biochem Biophys Res Commun** 328, 651-657.

Warren SM and Longaker MT **(2001)** The pathogenesis of craniosynostosis in the fetus. **Yonsei Med J** 42, 646-59.

Warren SM, Brunet LJ, Harland RM, Economides AN and Longaker M T **(2003)** The BMP antagonist noggin regulates cranial suture fusion. **Nature** 422, 625-9.

Whitaker L, Munro IR, Salyer KE, Jackson IT, Ortiz-Monasterio F, Marchac D **(1979)** Combined report of problems and complications in 793 craniofacial operations. **Plast Reconstr Surg** 64, 198-203.

Wilkie AO **(1997)** Craniosynostosis: genes and mechanisms. **Hum Mol Genet** 6, 1647- 56.

Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A and Felgner PL **(1990)** Direct gene transfer into mouse muscle *in vivo*. **Science** 247, 1465-8.

Yanagita M **(2005)** BMP antagonists: Their roles in development and involvement in pathophysiology. **Cytokine Growth Factor Rev** 16, 309-317.

Zavadil J, Böttinger EP **(2005)** TGF-β and epithelial-to-mesenchymal transitions. **Oncogene** 24, 5764-5774.

Michael T. Longaker