STUDIES IN CRANIAL SUTURE BIOLOGY

DISSERTATION

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By

Sundaralingam Premaraj, B.D.S., M.S.

The Ohio State University

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Dissertation Committee: Approved by

Dr. Sarandeep Huja, Adviser

Dr. Amr Moursi

Dr. Mark Mooney Adviser

Dr. Katherine Vig Oral Biology Graduate Program
ABSTRACT

Craniosynostosis is a disorder characterized by premature fusion of cranial sutures. Treatment of prematurely fused sutures typically involves a series of invasive surgical procedures beginning in infancy. In many cases, surgical sites show rapid resynostosis. This re-fusion requires additional surgical procedures which increase the patient’s risk of complications. It is increasingly clear that surgery cannot offer a “cure” for craniosynostosis but only a symptomatic improvement.

Quantifying gene expression patterns in cranial sutures is essential for the design of targeted adjunct therapies for craniosynostosis. Using real time RT-PCR in a rodent model of cranial suture biology, expression profiles of several growth factor ligands and receptors were quantitatively characterized. Suture mesenchyme and adjacent osteogenic fronts of posterior frontal and sagittal sutures were collected from Sprague-Dawley rats. Real time RT-PCR was performed for Bmp3, Bmp4, Bax, Bcl-2, Fgfr-1, Tβr-I, Tβr-II and Tβr-III. Results showed differences in the expression between posterior frontal and sagittal sutures and within each suture at different time points. These differences in expression could be exploited to develop a strategy to prevent re-fusion.
Administration of cytokines or genes that correct the underlying pathological process may arrest the ossification process. Exogenous application of transforming growth factor-beta3 (Tgf-β3) cytokine has been shown to inhibit suture fusion. However, use of cytokines as localized therapeutic agents is limited by the lack of a satisfactory delivery system.

Release kinetics and bioactivity of a simplified cytokine/collagen gel system designed to achieve extended, local delivery of bioactive cytokines at sites of premature cranial suture fusion were investigated. Gels loaded with Tgf-β3 produced a sustained release, determined by ELISA, over 14 days with a pattern of initial large release followed by a gradual reduction in the amount released over the time. Mink lung epithelial cell proliferation assay results indicated that Tgf-β3 released from the collagen gel retained its bioactivity following incorporation into the collagen gel and release into the media. This bioactivity was further illustrated by a decreased alkaline phosphatase activity measured in osteoblasts cultured on the gels loaded with Tgf-β3. In calvaria implanted with collagen gel containing Tgf-β3 protein, a statistically significant reduction in mean percent bridging of posterior frontal sutures was seen compared to control groups. The above findings confirm that collagen gel/cytokine delivery system can retain and release bioactive cytokine over a prolonged period.

Delivery of cytokines is limited by short half-life, degradation of the proteins during incorporation or following the release from the vehicle by moisture, temperature, or change in chemical environment. Gene transfer can avoid some of these limitations.
Therefore, suitability of a dense collagen gel as a vehicle for sustained delivery of non-viral plasmid DNA to sutural tissues was investigated. Structural integrity, bioactivity and transfectivity of released plasmids were established in rat osteoblast and calvarial organ cultures. Data revealed collagen gel can provide sustained release of plasmid DNA. The plasmid DNA retained its transfectivity as demonstrated by the prolonged osteoblast transfection and elevated growth factor production. The ability to rescue cranial suture fusion by released Tgf-β3 plasmid was tested in vitro and in vivo. Data from in vitro studies demonstrated that Tgf-β3 plasmid treated group was found to have 77% to 85% less bony bridging than collagen control and untreated groups. Mean percent bridging of the Tgf-β3 plasmid treated group was 3.59 ± 3.21 (mean ± S.D). Similarly in animals treated with Tgf-β3 plasmid, there was a significant reduction in suture fusion in the middle region of the posterior frontal sutures when compared to control groups. In this region Tgf-β3 plasmid treated group was found to have 70% to 75% less bony bridging than control groups. These histomorphometric evaluations of suture fusion were validated by micro CT imaging.

Possible mechanisms by which increased production of Tgf-β3 interferes with suture fusion were investigated by immunohistochemistry. Plasmid-mediated upregulation of Tgf-β3 protein production lead to an upregulation of pro-apoptotic Bax expression, down regulation of anti-apoptotic Bcl-2 expression and differential distribution of Tgf-β2 and Tβr-I in sutural cells. Therefore, Tgf-β3 induced suture maintenance is
possibly mediated by upregulation of apoptosis and down regulation of Tgf-β2 and Tβr-I.

These studies demonstrated a non-viral plasmid carrying a therapeutic gene can be delivered in a collagen gel vehicle, to produce a localized and sustained, overproduction of Tgf-β3 recombinant cytokine to prevent the fusion of cranial sutures in rats. This is one of the first studies to demonstrate the ability of a non-viral plasmid in vivo to prevent programmed cranial suture fusion.
Dedicated to my father late Ponniah Sundaralingam and
my mother Punithavathy Sundararalingam
I would like to thank my advisor, Dr. Amr Moursi, for his guidance, support and understanding, which made this research possible, and for his patience in correcting my scientific errors and writing style. Many thanks to the members of my dissertation committee, Drs. Sarandeep Huja, Mark Mooney and Katherine Vig for their help and support. I am grateful to Dr. Sarandeep Huja especially for allowing me to use his lab, sharing wisdom, showing encouragement and enthusiasm in all my endeavors. I would like to thank all past members of the Moursi lab without whose intellectual and technical assistance my research would not have progressed. Specifically, I am grateful to Bethany Mundy, Jennifer Parker Barnes and Phillip Winnard for all their help. Many thanks to Dr. Kichoon Lee for teaching me real time PCR and allowing me to use his lab for real time PCR studies. Thanks to Dr. Bing Li for assisting me in RNA extractions and statistical analysis. I wish to thank Dr. Michael Beck for his help in statistical analysis. Many thanks to Mary Lloyd from the Section of Oral Pathology for preparing the histological sections.

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VITA

1990…………………………………………..Bachelor of Dental Surgery (B.D.S)  
University of Peradeniya  
Sri Lanka

1996………………………………………… Master of Surgery (M.S) (Orthodontics)  
University of Colombo  
Sri Lanka

1990 – 2001………………………………….Lecturer/Senior Lecturer  
Division of Orthodontics  
University of Peradeniya  
Sri Lanka

2002 – 2006 ………………………………   Graduate Research and Teaching  
Associate, The Ohio State University

PUBLICATIONS

1. Premaraj S, Mundy B, Morgan D, Winnard PL, Mooney MP, Moursi AM.  
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FIELDS OF STUDY

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CHAPTER 1

INTRODUCTION

1.1. Overview

Cranial sutures are a thin layer of mesenchyme and fibrous tissue which unite the osteogenic fronts of the bones of the skull (Wagemans et al., 1988). Sutures play a major role in the postnatal growth of the skull (Moss, 1958b; Moss, 1960; Moss, 1972; Sicher, 1952). In addition, sutures allow the passage of the head through a narrow birth canal by sutural overlap and parietal bone deformation (Cohen, 2005). The timing and possibly also the mechanism of sutural closure differs between different sutures of calvaria (Morriss-Kay, 2001). Formation, development and closure of cranial sutures are regulated by many factors and disturbance in any of these factors may result in suture malformation.

Craniosynostosis is a disorder characterized by premature cranial suture fusion (Cohen, 2000a). The incidence of an isolated suture fusion is about 1 in 2000 live births (Shuper et al., 1985; Singer et al., 1999). The majority of craniosynostoses are isolated. However, premature fusion occurs as part of more than 150 genetic syndromes (Cohen,
Premature fusion of sutures prevents further growth of calvaria in a direction perpendicular to the affected sutures and excessive compensatory growth at other sutures give rise to skull distortion (Cohen, 1993). These extensive secondary craniofacial deformities are often associated with an elevated intracranial pressure (Hudgins et al., 1998; Mooney et al., 1999), altered intracranial volume (Gault et al., 1990; Gault et al., 1992), and dilation of the subarachnoid spaces (Chadduck et al., 1992). Left untreated, these may result in papilladema, blindness, and mental retardation (Kapp-Simon et al., 1993; Miller, 2000). These problems in craniofacial growth, vision and cognitive development necessitate extensive and recurrent clinical and surgical management (Panchal and Uttchin, 2003; Persing et al., 1989; Posnick and Ruiz, 2000).

Surgical management of craniosynostosis typically involves: 1) the surgical release of the synostosed suture in infancy, 3 to 12 months; 2) correction of midfacial deformities at 4 to 12 years; and 3) orthognathic surgery to correct jaw discrepancies at 14 to 18 years (Jane and Persing, 2000). The timing of surgery is based on the number of fused sutures, the severity of the secondary deformities, and the functional and psychological needs of the patient (Jane and Persing, 2000; Panchal and Uttchin, 2003).

While these procedures can result in normal craniofacial growth, the suturectomy sites reossify in 30% to 100% of reported cases (McCarthy et al., 1995; Moreira-Gonzalez et al., 2003; Pollack et al., 1996; Williams et al., 1997). Resynostosis can be very rapid, as early as 6 months postoperatively, particularly in cases where simple suturectomies or linear craniectomies are performed (Pollack et al., 1996). Re-operation of resynostosis
dramatically increases patient morbidity and mortality (Jane and Persing, 2000; Williams et al., 1997). To overcome such reossification problems a number of clinical strategies have been utilized. Wrapping the bony margins with a barrier proved ineffective because bone rapidly overgrew the barriers and re-ossified the suturectomy site (Babler et al., 1982). Attempts to chemically damage the dura and reduce its osteogenic potential maintained suturectomy site patency (Anderson and Johnson, 1956) however, seizures and neurological problems were typically a consequence (Jane and Persing, 2000). Another approach involved the radical repositioning of the calvarial bones to avoid reapproximation and resynostosis (Jane and Persing, 2000; Panchal and Uttchin, 2003). Although resynostosis was reduced, there was an increase in the rates of other complications and re-operation rates (Moreira-Gonzalez et al., 2003; Williams et al., 1997) from these high risk procedures. Major complications included, osseous relapse and instability; severe intra-operative blood loss, convulsions, infections, conjunctival chemosis /facial swelling and soft tissue necrosis (Jane and Persing, 2000; Panchal and Uttchin, 2003; Persing et al., 1989; Posnick, 2000). The findings described above indicate that there is a strong need for improved surgical management of craniosynostosis.

1.2. Craniosynostosis: Etiology and Pathogenesis

Both genetic and environmental factors contribute to craniosynostosis. While great progress has been made in identifying the genetic etiologies of craniosynostoses associated with a number of syndromes, the pathogenesis of primary craniosynostosis still remains poorly understood (Cohen, 2000c). Abnormal mechanical
forces (external pressure or deficiency in brain growth) may be a predisposing cause in some cases (Graham et al., 1980; Graham et al., 1979; Koskinen-Moffett and Moffett, 1989). Altered progression of osteogenesis is believed to be the basis of the premature fusion observed in craniosynostosis. The disruption of osteogenesis observed in craniosynostosis can occur from syndromic genomic deletions or from inherited or spontaneous mutations. Although the specific gene mutations are not known in all cases, several specific gene defects have been identified. Specific mutations in the genes encoding Fibroblast growth factor (Fgf) receptors have been associated with Crouzon, Crouzonodermoskeletal, thanatophoric dysplasia type 2, Pfeiffer, Jackson-Weiss, Apert, Beare-Stevenson and Muenke syndromes, all of which have craniosynostosis as a prominent feature (Jabs, 2002; Lajeunie et al., 1999; Wilkie, 1997).

1.3. Growth Factor Regulation of Suture Morphogenesis

The etiology of craniosynostosis is thought to involve a complex cascade of signaling molecules, receptors and transcription factors (Opperman, 2000). Recent studies have shown that changes in the expression of specific transcription factors, growth factors and their receptors such as, transforming growth factor-betas (Tgf-βs), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Noggin, Msx2 and Twist are responsible, in part, for the regulation of suture patency and fusion (Opperman et al., 1997; Opperman and Ogle, 2002; Roth et al., 1997b). Growth factors play an important role in the bone remodeling (Mundy, 1996; Mundy et al., 2003). Enhanced osteogenesis noted in craniosynostotic individuals may occur, in part, due to alterations in growth factor production (Cohen, 2000c; Opperman and Ogle, 2002). A number of studies have
demonstrated that heparin binding, soluble growth factors, secreted by the dura mater, were required for normal suture maintenance and eventual fusion (Bradley et al., 1996a; Kim et al., 1998; Opperman et al., 1993; Opperman et al., 1994; Opperman et al., 1995; Roth et al., 1996). A significant body of literature supports a role for Tgf-βs in the regulation of suture fusion.

The Tgf-β isoforms; Tgf-β1, 2 and 3 are potent growth regulatory molecules that influence craniofacial development in early embryonic stages and in subsequent stages of mesenchymal cell differentiation (Roelen and Dijke, 2003; Waite and Eng, 2003). The biological effects of Tgf-βs are exerted via specific binding interactions involving three distinct membrane bound receptors known as Type I, II and III (Attisano and Wrana, 2002). Tgf-β isoform-null mice show distinct craniofacial and somatic phenotypes depending on which isoform is absent (Sanford et al., 1997). Differential distribution of Tgf-β isoforms has been observed in the peri-sutural tissues of human infants and rodent models as well as in the rabbit model of human craniosynostosis. Roth and coworkers and Opperman and co workers studied spatial and temporal expression of the three Tgf-β isoforms in developing cranial sutures in the rat (Opperman et al., 1997; Roth et al., 1996; Roth et al., 1997b). These isoforms mediate proliferation and differentiation of osteoblastic suture cells and affect cranial suture fusion in vivo (Opperman et al., 1997; Opperman et al., 1999; Opperman et al., 2000; Opperman and Ogle, 2002; Roth et al., 1997a; Roth et al., 1997b). Expression of Tgf-β1, β2 and β3 isoforms are differentially expressed in rat posterior frontal suture during its programmed suture fusion (Most et al., 1998; Roth et al., 1997b). In an immunohistochemical study of developing posterior
frontal and sagittal sutures in the rat, Opperman and coworkers demonstrated a declining Tgf-β3 and continued reactivity for Tgf-β1 and Tgf-β2 during the programmed fusion of posterior frontal sutures (Opperman et al., 1997). Similarly, an abnormal pattern of reduced Tgf-β3 production has been noted in humans with familial, non-syndromic craniosynostosis (Lin et al., 1997; Roth et al., 1997a). Also in craniosynostotic rabbits, it is has been shown that low Tgf-β3 production was associated with fusing sutures (Poisson et al., 2004). In a murine model of intra-uterine head constraint, constraint-induced synostosis was associated with a reduction of dural Tgf-β3 immunoreactivity (Kirschner et al., 2002). In conclusion, Tgf-β3 is down-regulated in fusing sutures and abundant in patent sutures (Lin et al., 1997; Opperman et al., 1997; Poisson et al., 2004; Roth et al., 1997a; Roth et al., 1997b).

1.4. Apoptosis and Cranial Suture Fusion

Apoptosis is a regulated process of genetically programmed cell death and involves the integration of many pathways and intracellular regulators in the initiation, progression, and completion of cell death (Reed, 2000). Mitochondria play an essential role in regulating apoptotic activity within a cell by releasing apoptogenic factors into the cytoplasm that can mediate down stream execution of programmed cell death (Zimmermann et al., 2001). The Bcl-2 family of proteins plays a critical role the mitochondrial apoptotic pathway. The members of this family include anti-apoptotic genes such as Bcl-2 and proapoptotic genes such as Bax and Bak (Zimmermann et al., 2001). Bcl-2 is a membrane protein localized to the nuclear membrane, inner surface of mitochondria and the endoplasmic reticulum. The Bcl-2/Bax ratio controls the relative
susceptibility of cells to stimuli which induce apoptotic cell death (Korsmeyer et al., 1993). Overexpression of Bcl-2 increases the viability of cells under various adverse circumstances including cytokine withdrawal, loss of cell adhesion as well as γ-irradiation (Sorenson, 2004).

Despite reports claiming that apoptosis plays a key role in the development of cranial sutures, the role of programmed cell death in cranial suture biology remain poorly understood and controversial (Agresti and Gosain, 2005; Chen et al., 2003; Fong et al., 2004; Furtwangler et al., 1985; Rice et al., 1999). There are studies in the literature to support either an increase or decrease in apoptosis with premature suture fusion (Bourez et al., 1997; Dry et al., 2001; Lemonnier et al., 2001; Mansukhani et al., 2000; Mathijssen et al., 2000; Mathijssen et al., 2001; Opperman et al., 2000). Apoptotic bodies have been observed at the osteogenic fronts during bony apposition in fetal and newborn mice (Furtwangler et al., 1985). Apoptotic cells have been reported in mouse cranial sutures but the nature of the cells has not been described (Bourez et al., 1997; Furtwangler et al., 1985). In mice, apoptosis appears more active in patent sagittal sutures than in fused posterior frontal sutures on day 45 (Agresti and Gosain, 2005). In calvarial osteoblasts derived from new born and postnatal day 10 mice, increased expression of fibroblast growth factor (FGF)-2 inhibited DNA synthesis and increased apoptosis (Mansukhani et al., 2000). In osteoblast cultures established from cranial sutures of aborted Apert fetuses, Lemonnier and coworkers demonstrated a increased apoptosis (Lemonnier et al., 2001). Chen and coworkers have demonstrated that craniosynostosis in a Fgfr2-Ser250Trp mutant mice is accompanied by decreased bone formation due to
increased Bax expression and apoptosis (Chen et al., 2003). They hypothesized that abnormally increased apoptosis in the mutant mice decrease the distance between two opposing osteogenic fronts and eventually result in premature fusion of sutures (Chen et al., 2003). Additional studies are needed to determine the exact role of apoptosis in the maintenance of suture patency.

1.5. Cytokine Therapy for Craniosynostosis

Involvement of several cytokines and growth factors in cranial suture morphogenesis and patency provides a tremendous opportunity to use these proteins in the treatment of craniosynostosis (Mooney et al., 2004). Development of a cytokine-based therapeutic approach as an adjunct therapy will allow for earlier and less extensive surgical interventions. This approach could decrease complications inherent in high risk surgical procedures, prevent post-surgical resynostosis and eliminate the need for multiple surgeries for children with craniosynostosis.

Use of cytokines as therapeutic agents poses certain difficulties due to their short half lives, poor tissue penetration and large molecular size (Babensee et al., 2000). Even though systemic bolus delivery of cytokines is technically simple, their distribution throughout the body and subsequent rapid degradation may lead to undesirable effects on non-targeted tissues and an insufficient local concentration for the required time frame, respectively (Chen and Mooney, 2003). Polymeric vehicles can be used for controlled, localized delivery of growth factors for a required time frame. This is achieved by implantation of a polymer matrix incorporated with cytokines. Several elaborate delivery
systems have been studied using polymers or hydrogels as vehicles to deliver cytokines for therapeutic and tissue engineering purposes (Babensee et al., 2000; Friess, 1998; Parker et al., 2002; Saltzman and Olbricht, 2002). These vehicles differ in their chemical and structural properties and in the method by which the cytokine is incorporated. Many of these vehicles may require very sophisticated techniques and equipment for preparation (Holland et al., 2003; Maire et al., 2005a; Maire et al., 2005b). Release dynamics of cytokines from these polymer vehicles can be controlled by factor diffusion, polymer degradation, amount of factor loaded in the system and the composition of the polymer (Chen and Mooney, 2003). Both natural and synthetic polymers are being tested as vehicles for growth factor delivery.

1.6. Biomaterials for Cytokine Delivery

1.6.1. Collagen

Collagen is the most abundant and ubiquitous structural protein in the body and may be readily purified from both animal and human tissues with an enzyme treatment and salt/acid extraction (Wallace and Rosenblatt, 2003). The suitability of collagen as a biomaterial rests largely on the view that it is a natural material of low immunogenecity and is therefore recognized by the body as a normal constituent rather than a foreign matter. Collagen can be processed into a number of forms such as sheets, tubes, sponges, injectable solutions and dispersions, all of which have found use in medical practice. Collagen implants also have the benefit of being resorbable; they degrade through a sequential attack by lysosomal enzymes (Bonadio et al., 1999).
A collagen gel with a density much greater than conventional hydrogels was used as a vehicle in the studies documented in this dissertation. This highly purified, bovine Type-I collagen gel (NeuColl Inc., Campbell, CA) was an attractive candidate for use as a vehicle because it did not promote osteoblast proliferation, migration or mineralization (Premaraj et al., 2006). The antigenic N- and C-terminal domains have been removed from the collagen molecule by pepsin digestion thereby, greatly reducing the likelihood of inducing an immune response (DeLustro et al., 1986; Ellingsworth et al., 1986).

Although highly purified, this formulation retains the native fibrillar conformation of the collagen molecule as opposed to other commercially available degraded collagen gels (Rosenblatt et al., 1989). In addition, the injectable material is semi-viscous at room temperature and gels at 37° C, making it ideal for placement into surgical sites. This collagen gel is most widely used clinically as a FDA-approved, intra-dermal injection for soft tissue augmentation where it will resorb in approximately six months to a year (Pachence, 1996). Previous studies have clearly shown the ability of collagen gel to deliver therapeutic proteins in vitro and in vivo (Moursi et al., 2002; Moursi et al., 2003).

Its retention and rate of resorption was tested in a wild-type rabbit. Biotinylated collagen gel (100 µl) was injected sub-periosteally over the coronal suture in normal New Zealand white rabbits (27 days old). The collagen gel was slowly resorbed with time, although it could still be visualized histologically up to 63 days post-implantation (Mooney et al., 2004). There was no evidence of an inflammatory response, immune reaction or fibrosis. These results indicated that the collagen gel is localized to the suture site, physiologically incorporated into the perisutural tissue and slowly resorbed.
Although, growth factor therapy can be successful in vivo, it is limited by a half life which may be too short to be effective in preventing synostosis clinically, where weeks or months of sustained release and bioactivity are necessary. Degradation of the proteins during incorporation or following the release from the vehicle can be caused by moisture, temperature, or change in chemical environment (Fu et al., 2000). Consequently, high, and possibly toxic, concentrations are needed to produce an effect. Also, because of the limited duration of effect, the timing of treatment becomes critical. Even a large amount of a normally potent protein, given either before or after the optimal biological window, will result in an ineffective therapy (Fang et al., 1996).

Gene transfer can overcome the many limitations associated with the direct administration of cytokines or growth factors such as low bio-availability, systemic toxicity, in-vivo instability, high hepatic and renal clearance rates and the high cost of production of recombinant proteins and growth factors. In recent years, gene therapy has emerged as an effective approach to delivering therapeutic factors in a physiological and sustainable manner. Non-syndromic craniosynostosis is a particularly appropriate disorder in which to test this type of gene therapy because synostosis may simply reflect an acceleration of normal physiological sutural maturation as opposed to a severe disruption of complex fusion mechanisms (Cohen, 2000c; De Pollack et al., 1996; Roth et al., 1997b). Thus, this disorder may be more likely than others to respond to overproduction of an individual signaling molecule.
1.7. Gene Therapy

Gene therapy or gene transfer is a relatively new concept in the treatment of human disease. In its current definition, gene therapy is defined as the use of nucleic acid transfer, either RNA or DNA, to treat or prevent a disease (Cha and Boden, 2003) and the scope of gene therapy has expanded beyond its initial application as a method of replacing genetic defects.

Gene transfer may represent the next step in the evolution of therapies to prevent resynostosis in the management of craniosynostosis. In theory, one or more genes coding for cytokines or growth factors known to be associated with patent or nonfusing sutures could be introduced into the cranial suture environment. Once this genetic material was transfected into the cells, transduced cells will then secrete the cytokines extracellularly and release it into the local environment in physiologically appropriate doses for a sustained period of time. Successful application of gene therapy as an adjunct therapy in premature cranial suture fusion requires the consideration of several factors. A gene cassette must be constructed that directs the production of growth factors. This gene cassette should contain both the therapeutic gene and several other genetic elements, including introns, polyadenylation sequences and transcript stabilizers to control transcription, translation and protein stability and secretion from the host cells (Cha and Boden, 2003). A vector to deliver this genetic material into the cells needs to be selected. After a vector is selected, a vehicle or strategy for administration of this vector to the target cells or tissues needs to be identified.
Vectors can be defined as vehicles that are designed to deliver genetic material into a cell so that the cellular machinery can ultimately produce the therapeutic protein coded for by the introduced genetic material (Cha and Boden, 2003). An ideal vector should be safe, stable, easy to produce in large quantities, and capable of achieving efficient and tissue-specific gene expression when directly administered in vivo (Li and Ma, 2001). Vectors may be classified either as viral or non-viral. Viral vectors consist of modified, usually non-replicating, viral genomes carrying a specific transgene. Adenovirus, retrovirus and adeno-associated viruses are the most popular vectors in the current gene therapy protocols. For example, a viral vector was used by investigators to prevent suture fusion in vivo by producing an increase in noggin production using an adenoviral vector delivered in saline injection (Warren et al., 2003).

Viruses are well suited for gene delivery as they have adapted to infect specific tissues and cells, and manipulate the cell’s machinery to make viral proteins. However, they can be injected into a person only once or twice before the immune response they provoke poses a safety threat. This immune response can destroy the viral vector or the cells it infects, blocking production of the useful protein. The issues of safety and ease of application have lead to increasing efforts to develop non-viral transfection approaches.

Non-viral vectors utilize chemical substrates to facilitate entry of genetic material into the cell. Plasmid DNA is economical and relatively simple to manufacture (Evans and Robbins, 1995). Non-viral vectors utilize a variety of agents (lipids, polymers, and peptides) complexed with the plasmid or utilize the “naked” DNA alone (Maurer et al.,
Formulations of plasmids with cationic agents results in nanometer-sized particles that show improved transfection efficiency relative to naked plasmids (Stayton et al., 2000). Plasmid DNA has stable flexible chemistry that is compatible with established polymer-based drug delivery systems (Shea et al., 1999). Cationic liposomes introduce DNA into the cell by fusing with the cell membrane. Although a large amount of DNA can be introduced into the cell via this method, the process is less efficient than with viral vectors (Felgner et al., 1987).

Non-viral vector systems offer the advantages of low immunogenicity, simplicity of vector design and relative ease of large scale production (Cohen et al., 2000). Plasmid DNA avoids the possibility of a virally-mediated mutagenesis (Herweijer and Wolff, 2003). The major disadvantage of this approach is related to its lower efficiency of transfection.

Transfection efficiency of non-viral plasmids could be increased by slow and sustained plasmid DNA delivery, where the newly released genes provide replacement for those delivered previously that have ceased to function. This could be achieved by incorporation and immobilization of plasmid DNA within biocompatible and biodegradable polymer matrices. This approach allows for localized and sustained delivery of plasmids to promote gene expression over a desired time frame. Plasmid gene retention and expression at the site of matrix implantation is prolonged (weeks vs. hours) compared to recombinant protein delivery (Bonadio et al., 1999).
A local gene delivery system composed of biodegradable vehicles to deliver plasmid DNA is referred to as the Gene Activated Matrix (GAM) (Bonadio, 2000b). At its most basic, a GAM consists of two ingredients: plasmid DNA and a biodegradable structural matrix carrier. Site-specific delivery of plasmid DNA from the polymer matrix allows for subsequent transfection of cells, leading to transgene expression.

1.8. Biomaterials for Gene Delivery

The polymeric systems developed to deliver biologically active proteins have been adapted to deliver DNA in viral, non-viral and cell-based gene therapeutics. These DNA or gene-modified cells incorporated implants have been fabricated in a range of shapes and sizes: from microcapsules and microspheres to rods, films and three-dimensional matrices. Encapsulation of DNA within the polymer can protect against its degradation. These scaffolds act to increase the local concentration of DNA within the cellular microenvironment either by providing a sustained release of DNA or by maintaining the DNA locally. Gene transfer is enhanced by these polymer matrices *in vivo* possibly due to an increased plasmid concentration at the cell surface, which has been shown to enhance gene transfer in *vitro* (Luo and Saltzman, 2000). In addition, the matrices protect the DNA from the biological system until it is released. Also, plasmid diffusion from the delivery site should not cause systemic toxicity because of the high efficiency of DNA turnover in the blood stream (Lew *et al.*, 1995). Plasmid gene transfer from GAM is capable of yielding significant amounts of recombinant protein *in vivo*. In a canine bone defect model, 1.0 mg plasmid DNA yielded picogram amounts of recombinant peptide over 2-week period (Bonadio *et al.*, 1999). Studies in animal models have shown
that GAM plasmid gene transfer is capable to repair cells in bone (Fang et al., 1996), skin (Shea et al., 1999), heart and skeletal muscle (Labhasetwar et al., 1998).

Polymeric carrier matrices have been fabricated from a variety of both naturally derived materials (e.g., collagen) and synthetic (e.g., poly (lactide-co-glycolide) polymers. Naturally derived materials have the potential advantage of biological recognition. An advantage of synthetic polymers is reproducible large scale production with controlled properties of strength, degradation rate and microstructure. GAMs may take several forms (e.g., a lyophile implant or sponge, an injectable gel or paste, and a medical device coating) that can all be manufactured as off-the-shelf products for direct placement into the tissues.

1.8.1. Collagen-Based Gene Delivery System

Studies directed toward using collagen as a carrier for genes have yielded promising results (Bonadio et al., 1999; Fang et al., 1996). Collagen is available as either injectable or implantable formulations.

Collagen Sponges

Collagen sponges were originally developed as wound dressings and have been used widely in plastic surgery as matrices for tissue reconstruction after burns. Fang and coworkers were the first to explore and successfully demonstrate the possibility of using collagen sponges as implantable carriers for naked plasmid DNA (Fang et al., 1996). In animal models, collagen sponges loaded with DNA coding for bone morphogenetic
protein 4 (BMP-4) and/or parathyroid hormone enhanced the bone healing (Bonadio et al., 1999; Goldstein and Bonadio, 1998). Incorporation of plasmids into lyophilized collagen matrices stabilized the plasmid in vivo and allowed for prolonged delivery and expression. In an animal model of wound healing, lyophilized collagen pads containing either PDGF-A or PDGF-B DNA promoted a 4-5 fold increase in granulation tissue and in reepithelialization compared to controls (Chandler et al., 2000). In rabbits, topical application of lyophilized collagen matrices embedded with PDGF-B and PDGF-A plasmid DNA enhanced the healing of ischemic, full thickness dermal ulcer wounds (Tyrone et al., 2000). Mat-100 was the first GAM formulation used in the human subjects (Bonadio, 2000a). Mat-100 is a single application plasmid gene therapy for bone fracture repair in elderly human subjects who suffer from osteoporosis. It is a lyophile that consists of plasmid DNA code for human parathyroid hormone 1-34 peptide in a biodegradable bovine type I collagen sponge. After delivery Mat-100 lyophiles should remain localized at the site of implantation until biodegradation is complete.

Collagen Gel

Collagen gels are primarily used for injectable, controlled delivery systems and have the advantage that, they can be introduced into the patient in a minimally invasive manner. Studies by others and those documented in chapter 3 clearly demonstrate the ability of collagen gel to deliver therapeutic proteins in vitro and in vivo (Premaraj et al., 2006). Collagen gel could also be used to deliver plasmids, viruses and transduced cells in ex-vivo gene therapy (Adachi et al., 2002).
**Collagen-Glycosaminoglycan (GAG) Matrix**

The collagen-GAG matrix has the potential to immobilize the DNA to effectively increase the local concentration of DNA (Doukas *et al.*, 2001). It is possible to control the release of DNA from the matrix by regulating the degradation of the matrix by appropriate choice of cross linking (Weadock *et al.*, 1996) or by altering the degree to which the plasmid is integrated into the collagen–GAG network (i.e., by swelling the collagen fibers at the acidic or basic pH during the addition of the plasmid). Collagen-GAG matrices have the potential to increase the transfection efficiency due to cell-matrix interactions. Release of plasmid DNA from collagen-Glycosaminoglycan(GSCG) matrices showed a biphasic pattern consisting of a faster early release rate over the initial 8 hour leaching followed by a slower, late release rate that was relatively constant over the subsequent 28 days of leaching (Samuel *et al.*, 2002).

An important issue for gene delivery from polymers is what polymer formulations will give an efficient incorporation and sustained local delivery in a reproducible manner. Collagen has shown promise for these applications; however its limitations include requirements for its isolation from an animal or other biological sources and limited control over its degradation rate.

**1.8.2. DNA Encapsulated in Polymeric Nanoparticle**

Encapsulation techniques can be used to protect plasmid DNA from degradation. PLG polymers protect the plasmid DNA from digestion by DNAase I in vitro. Cohen and coworkers evaluated the gene expression mediated by the nanoparticles in vivo and in...
vitro in comparison with cationic-liposome delivery (Cohen et al., 2000). Nano size range (600 nm) plasmid DNA loaded in PLG polymer particles exhibited sustained release of plasmid DNA of over a month. The entrapped plasmid maintained its structural and functional integrity. In vitro transfection by plasmid DNA-nanoparticles resulted in significantly higher expression levels in comparison to naked plasmid DNA.

1.8.3. Poly (lactide-co-glycolide) (PLG)

Polyesters of naturally occurring α-hydroxy acids, including PGA, PLA and PLGA are widely used as matrices for gene delivery in tissue engineering. The ester bonds in these polymers are hydrolytically labile and these polymers degrade by non-enzymatic hydrolysis. Since these polymers are thermoplastic, they can be easily formed into three dimensional scaffolds with a desired microstructure. PLG polymers can be readily designed to exhibit varying degradation rates. Additionally, by manipulating the PLG matrix properties (fiber diameter, porosity, pore size, etc.) and method of DNA incorporation into the matrix it is possible to accurately control the release of DNA from the scaffold (Luu et al., 2003). Shea and coworkers investigated the feasibility and potency of PLG polymer matrix as biodegradable vehicle for sustained plasmid gene delivery to rat skin dermis (Shea et al., 1999). Incorporated DNA was released over times ranging from days to a month in vitro. In vivo delivery of a plasmid encoding platelet derived growth factor B led to a three-to four fold enhancement of granulation tissue at the implantation site. This was contrasted with direct injection of the platelet derived growth factor B-plasmid, which did not significantly enhance local tissue formation, a result that emphasizes the utility of the matrix carrier. Klugherz and coworkers used a
PLGA coating on to an expandable stent to achieve sustained DNA release for 10 days and yield positive gene expression in the coronary artery wall (Klugherz et al., 2000). These findings demonstrate the potential advantage of sustained plasmid delivery.

1.8.4. Alginate Hydrogel

Alginate is a polysaccharide made of $\alpha$-L-guluronic and $\beta$-D-mannuronic acids, isolated from sea weed. Alginate is relatively biocompatible and is approved by the FDA for human use as wound dressing material. It has been demonstrated that sustained plasmid delivery can be achieved from these biodegradable hydrogels. They can be injected via a syringe and therefore introduced into the patient in a minimally invasive manner (Madsen and Mooney, 2000). These hydrogels can also be designed to degrade over widely varying time frames (Lee et al., 1999) and thus could be used to promote expression of a desired gene within a specifically defined time frame.

1.8.5. Fibrin

Fibrin is the natural matrix for migrating cells in wounds. It is thought that this matrix may bind the plasmid DNA and allow the cells to take up the gene by endocytosis during migration. Andree and coworkers showed a reproducible transfection method for human keratinocytes resuspended in fibrin glue containing the hEGF plasmid with protein expression increased up to 120-fold compared to controls (Andree et al., 2001). Therefore, this fibrin-matrix with plasmids of interest could be used for gene delivery in vivo without any suspended cells.
1.8.6. Poly (2-aminoethyl propylene phosphate) (PPE-EA)

PPE-EA is a water soluble and biodegradable polyphosphoester with positively charged side chains. It is synthesized with relatively high molecular weight to ensure the necessary hydrolytic stability to protect plasmid in physiological conditions. Wang et al., (2002) studied the sustained release of plasmid DNA from PPE-EA complexes in mouse muscle, when delivered as intramuscular injection. Sustained release of plasmid was achieved from PPE-EA/DNA complexes as a result of PPE-EA degradation (Wang et al., 2002). The DNA release profiles are mainly controlled by carrier degradation and the release rate could be adjusted by varying the charge ratio of PPE-EA to DNA.

Other injectable polymer systems that can provide protective, interactive delivery system include polyvinyl and N-methyl-2 pyrrolidones, polyvinyl alcohol and propylene glycol. Recent work demonstrates that the delivery of plasmid encoding for β-galactosidase in a polyvinyl pyrrolidone delivery system has an eight-fold higher muscle fiber content than the traditional plasmid saline formation (Mumper et al., 1998).

In conclusion, the premise that incorporation of DNA into a polymer can offer protection from degrading enzymes and provide an enhanced biological effect owing to localized and sustained release. The release of DNA from polymer scaffolds can be controlled through microsphere properties and the processing conditions. The integration of recent research on liposomes, cationic lipids and condensing agents for DNA into this approach might, in the future, lead to increased transfection efficiency of non-viral plasmids.
1.9. Research Goals

Experiments documented in this dissertation were aimed at understanding the molecular mediators associated with cranial suture fusion and developing novel cytokine- and gene-based therapies to prevent or rescue cranial suture fusion. These studies were performed in a rat model of cranial suture fusion. The rat is an excellent animal model to study cranial suture development and physiological suture fusion. The rat has previously been used to study cranial suture development (Kokich, 1986). In rats, the posterior frontal suture begins to unite on the endocranial aspect 12 days postnatally and complete closure occurs by the age of 22 days (Moss, 1958a). The remaining cranial sutures (coronal and sagittal) never fuse during its life time.

This dissertation is organized into five chapters. Chapter 1 is a general introduction and a review of literature pertinent to the body of research presented in this dissertation. Characterizing the molecular mechanisms of physiological cranial suture fusion is essential to understand the pathological premature fusion of sutures. The present study aimed at understanding some of these molecular mechanisms in an effective way. Chapter 2 examines the expression profile of some of the growth factor and receptor genes in a fusing and nonfusing cranial suture complex in rats. Use of cytokines as localized therapeutic agents is limited by the lack of a satisfactory delivery system and there is a strong need to identify and develop simple and easy to use, resorbable vehicles for sustained delivery of cytokines to cranial suture sites. In order to fully exploit the potential of a dense collagen gel as a vehicle for cytokine and plasmid delivery, it is necessary to study its release properties and cell and tissue compatibility.
Chapters 3 and 4 investigate the release characteristics of a dense collagen gel and examine the suitability of this dense collagen gel vehicle to deliver cytokines and non-viral plasmids as therapeutic agents to inhibit or delay cranial suture fusion. These studies also analyzed the effects of Tgf-β3 protein or Tgf- β3 non-viral plasmid on the fusion of posterior frontal sutures in rats. Further initial studies were conducted to characterize the possible mechanisms by which increased production of Tgf-β3 interferes with suture fusion.
2.1. INTRODUCTION

Cranial suture fusion is regulated through multiple pathways (Warren et al., 2001). Recent developments in molecular biological techniques have provided new insights into the etiopathogenesis of craniosynostosis. Although these studies have identified chromosomal abnormalities and implicated several genes as a cause for craniosynostotic syndromes (Cohen, 2005; Jabs et al., 1993; Wilkie, 1997), the mechanisms of molecular regulation of cranial suture fusion has yet to be understood in detail. A precise understanding of quantitative differences in the temporal and spatial pattern of growth factor and cognate receptor gene expression between normally fusing and patent cranial sutures is essential to develop new therapies to prevent premature cranial suture fusion.

Several studies have examined the expression pattern of several genes implicated in the cranial suture morphogenesis, patency and fusion (Law et al., 2005; Lin et al., 1997;
Opperman et al., 1997; Poisson et al., 2004; Roth et al., 1997a; Spector et al., 2000).

These studies implicated two growth factor families as closely associated with cranial suture biology: transforming growth factor-betas (Tgf-βs) and fibroblast growth factors (Fgfs). Most of these studies analyzed the expression of the above factors in rats, mice, rabbits and humans using a variety of techniques such as immunohistochemistry, in situ hybridization, Northern blot, Southern blot and semi-quantitative RT-PCR analysis (Gosain et al., 2004; Law et al., 2005; Lin et al., 1997; Opperman et al., 1997; Poisson et al., 2004; Roth et al., 1997a; Spector et al., 2000). Most of these studies are qualitative or semi-quantitative in nature.

The biologic activity of the Tgf-β isoforms is mediated by two transmembrane, serine-threonine kinase receptors, Tβr-I and Tβr-II. In rats, the expression of both receptors is increased in the actively fusing posterior frontal suture compared with patent sagittal suture (Mehrara et al., 1999). Tgf-βs also bind with lower affinity to Tβr-III receptor (betaglycan). It is thought that Tβr-III receptors concentrate Tgf-β ligand before presentation to the Tβr-I and Tβr-II (Cohen, 2000b). Relative temporal and spatial expression of receptors for Tgf-β growth factors remain to be quantitatively characterized. The osteogenic effects of Fgf-2 are mediated by three high affinity tyrosine kinase receptors and gain-of-function mutations in these receptors are associated with syndromic craniosynostoses (Cohen, 1997; Malcolm and Reardon, 1996).

Fusion of cranial sutures is thought to be an active process involving increase in osteogenesis. The role of growth factors known to regulate osteogenesis such as noggin
and Bmps has only recently been studied in programmed cranial suture fusion. Bmp4 is abundant in both fusing and patent cranial sutures (Warren et al., 2003). Immunohistochemistry localized Bmp4 protein to the suture mesenchyme and osteogenic fronts of the posterior frontal suture as well as to the patent sutures (Warren et al., 2003). Noggin, which is a Bmp antagonist, is expressed postnatally in the suture mesenchyme of patent cranial sutures. Noggin expression is suppressed by Fgf-2 and syndromic Fgfr signalling (Warren et al., 2003). In rats, expression of Bmp3, a bone morphogenetic protein antagonist decreased over time in posterior frontal sutures whereas it increased in sagittal sutures (Nacamuli et al., 2005). Fgf-2 suppressed the expression of Bmp3 in osteoblasts (Nacamuli et al., 2005). In a semi-quantitative gene expression study profiling rat cranial sutures, Law and coworkers reported a bimodal expression of Bmp3 in posterior frontal and sagittal sutures (Law et al., 2005).

The role of apoptosis in cranial suture biology remains poorly understood and controversial (Agresti and Gosain, 2005; Chen et al., 2003; Fong et al., 2004; Furtwangler et al., 1985; Rice et al., 1999). There are studies in the literature to support either an increase or decrease in apoptosis with premature suture fusion (Bourez et al., 1997; Dry et al., 2001; Lemonnier et al., 2001; Mansukhani et al., 2000; Mathijssen et al., 2000; Mathijssen et al., 2001; Opperman et al., 2000).

Recently investigators have used real-time RT-PCR to quantitate gene expression in mouse cranial sutures (Mathy et al., 2003; Nacamuli et al., 2004). Although the underlying biology regulating posterior frontal suture in mice and rats is thought to be
similar, quantitative analysis of gene expression during suture fusion in rat has yet to be
described. We used quantitative real-time reverse transcriptase polymerase chain reaction
( realtime RT-PCR) to characterize the gene expression patterns of eight genes which are
implicated in apoptosis and osteogenesis. Studies described in this chapter were
performed to analyze the gene expression pattern within the rat posterior frontal and
sagittal suture mesenchyme before and at the time of closure of the posterior frontal
sutures. We also compared the pattern of gene expression between posterior and sagittal
sutures during this period. A better understanding of the gene expression associated with
normal suture fusion will help to develop targeted strategies to address premature fusion.

2.2. MATERIALS AND METHODS

Animals

All animal research was approved by the Institutional Laboratory Animal Care and Use
Committee (ILACUC) at The Ohio State University. Sprague-Dawley rats were
purchased from Harlan, Indianapolis, IN. Animals were housed in a light-, temperature-
and humidity controlled environment until time of death. Food and water were provided
ad libitum.

Isolation of Posterior Frontal and Sagittal Sutures

Rats were euthanized with carbon dioxide and the posterior frontal and sagittal sutures
were meticulously dissected. The dimension of a dissected suture specimen was about 5
mm long and 3 mm wide. Posterior frontal and sagittal sutures were harvested from 10,
12, 14, 16 and 22 day old rats. (n=6 per suture per time point). These time points were
selected to analyze the posterior frontal and sagittal sutures during the period of pre-fusion and fusion. Underlying dura mater and vascular tissue were removed before the sutures were snap-frozen in liquid nitrogen.

RNA Isolation Using Trizol

Tissue samples were homogenized in 1 ml of TRIzol (Invitrogen, Carlsbad, CA) for 1 minute. Chloroform (200 µl) was added to the homogenates and mixed by vortexing for 10 seconds. Mixture was incubated for 3 minutes at room temperature and centrifuged at 12000 rpm at 4°C for 15 min for phase separation. Aqueous phase was transferred to a new tube and 200 µl of isopropanol was added. Mix was allowed to precipitate at room temperature for 10 minutes and RNA was precipitated by centrifuging at 12 000 rpm at 4°C for 10 minutes. Supernatants were discarded, pellets were washed with 75% ethanol and centrifuged at 12 000 rpm at 4°C for 5 minutes. Supernatants were discarded, pellet was air-dried for 5 to10 minutes and RNA was re-dissolved in 50% formamide. RNA quality was examined by 1% agarose gel.

Preparation of cDNA - Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Complimentary DNA (cDNA) was synthesized by RT-PCR using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA). Following RT-PCR conditions were used; 65°C for 5 minutes, 37°C for 50 minutes , and 70°C for 15 minutes.
Primer Design for Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Primers designed by Law and coworkers and Sun and coworkers (Law et al., 2005; Sun et al., 2002) were used for real time RT-PCR. Sequences of each primer were confirmed to be specific for the target gene alone using BLAST (National Institute of Health, Bethesda, MD). Primers were synthesized by Invitrogen, Carlsbad, CA. The sequences of primer sets for each mRNA transcript are shown in Table 2.1.

SYBR Green Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Real time RT-PCR was performed in a 25 µl final reaction volume using AmpliTaq Gold polymerase (Applied Biosystems, Branchburg, NJ). Conditions for real time PCR were 95°C for 10 min, 40 cycles of 94°C at 15 seconds, 58°C for 30 seconds, 72°C for 40 seconds and 82°C for 16 seconds. All assays were performed with Mx 3000p Real-Time PCR system (Stratagene, La Jolla, CA). Cyclophilin mRNA levels from each sample were used as internal controls to normalize the mRNA levels. Gene expression was calculated using ∆Ct method and presented as ratio to cyclophilin.

Statistical Analysis

Differences in gene expression between time points within posterior frontal or sagittal suture were assessed for statistical significance using analysis of variance (ANOVA). Unpaired t test was performed to identify growth factors or receptors with statistically significant difference between posterior frontal or sagittal suture at each time point. A p
value of less than 0.05 was considered to be statistically significant. Data were analyzed by SAS 9.1 for Windows (SAS Institute, Inc., Cary, NC).

2.3. RESULTS

Expression of Bmp4 in Posterior Frontal (PF) and Sagittal (SAG) Sutures
Levels of Bmp4 transcripts followed similar patterns of expression in PF and SAG sutures (Fig. 2.1). Expression of mRNA for Bmp4 was greater in the PF suture than SAG at all time points except day 22. These differences were statistically significant (p<0.05) on day 12 and day 16 (Fig 2.2). Highest gene expression was measured on day 14 on both sutures.

Expression of Bmp3 in PF and SAG Sutures
Differences were measured in the pattern of Bmp3 expression between posterior frontal and sagittal sutures before and during the programmed posterior frontal suture fusion. In the posterior frontal suture, Bmp3 transcripts decreased between 10 and 22 days of life (Fig. 2.3). In contrast Bmp3 expression in the patent sagittal suture increased between days 10 and 22 (Fig.2.3). There were no statistically significant differences between posterior frontal and sagittal suture mesenchyme expression profiles of Bmp3 (Fig.2.4).

Expression of Bax in PF and SAG Sutures
Expression of pro-apoptotic Bax mRNA followed an almost similar pattern in both suture mesenchyme and osteogenic fronts (Fig. 2.5). Transcript levels of Bax were higher in the
sagittal sutures at all the time points examined. These differences were statistically significant (p<0.05) on day 12 and day 14 (Fig.2.6).

**Expression of Bcl-2 in PF and SAG Sutures**

The anti-apoptotic Bcl-2 molecule had very low and similar expression patterns in PF and SAG sutures in most of the time points examined (Fig. 2.7). However levels of Bcl-2 expression increased markedly in PF sutures by day 22 and this difference was statistically significant (p<0.005) (Fig.2.8).

**Expression of Fgfr-1 in PF and SAG Sutures**

Transcript levels of Fgfr-1 were significantly higher in the mesenchyme and osteogenic fronts of PF sutures than SAG sutures at all time points (Fig. 2.9). These differences were statistically significant at all time points except day 14 (Fig. 2.10). Gene expression for Fgfr-1 in both PF and SAG sutures were higher at earlier time points and then decreased subsequently.

**Expression of Tβr-I in PF and SAG Sutures**

Differences were seen in the pattern of Tβr-I expression between posterior frontal and sagittal sutures during the programmed posterior frontal suture fusion (Fig.2.11). In the posterior frontal suture, Tβr-I transcripts increased between 10 and 14 days of life and then gradually decreased by the time suture fusion is completed (Fig. 2.12). In contrast Tβr-I expression in the patent sagittal suture remained low until day 16 and then
increased by day 22. These differences between sutures were significant on day 12, 14 and 16.

**Expression of Tβr-II in PF and SAG Sutures**

Expression of Tβr-II mRNA followed an almost similar pattern in both suture mesenchyme (Fig. 2.13). Gene expression for Tβr-II in both PF and SAG sutures were higher at earlier time points and then decreased subsequently (Fig. 2.14). This decrease in expression was more in posterior frontal sutures by day 22.

**Expression of Tβr-III in PF and SAG Sutures**

Transcript levels of Tβr-III followed a similar pattern in both suture mesenchyme in most of the time points (Fig. 2.15). Expression showed a peak in both mesenchyme on day 14, decreasing subsequently (Fig. 2.16). Difference in expression was significantly different on day 22.

**2.4. DISCUSSION**

An improved understanding of the molecular events leading to premature fusion of cranial sutures is essential to develop treatment strategies for this developmental disorder. Techniques such as positional cloning and candidate gene approach have identified several mutations associated with syndromic craniosynostosis and these advances have lead to improved diagnosis of these conditions (Warren et al., 2001). Qualitative and quantitative characterization of gene expression in patent and fusing sutures in animal models will help us to understand and compare normal and craniosynostotic sutures.
Although there is a large body of literature describing the qualitative differences in gene expression in sutures which are destined to fuse or remain patent in animal models and humans, very few studies have quantitatively compared the difference in gene expression between these two sutures of opposing fates.

In the current study we have used real-time RT-PCR methodology to profile the expression levels various families of growth factors and cognate receptors in the suture mesenchyme and osteogenic fronts of fusing and patent sutures in rats. This is one of the first gene profiling studies of cranial sutures in rats using quantitative real time RT-PCR technique. Real time RT-PCR is rapidly becoming the state of the art method for quantifying gene expression in studies of cranial suture biology and pathology (Mathy et al., 2003; Nacamuli et al., 2004; Nacamuli et al., 2005; Singh et al., 2006). Quantitative real-time RT-PCR allows quantification of levels of transcript with much greater precision than Northern blot analysis or semi-quantitative reverse transcriptase polymerase chain reaction (Bustin, 2000). Real-time RT-PCR requires much less RNA and yields results that are more sensitive and reproducible than conventional assays (Bustin, 2000; Mathy et al., 2003). Ten femtogram of RNA could be reliably detected by real-time method, in comparison 500 ng is needed for the detection using a conventional PCR method (Singh et al., 2006). In addition this technique allows for analysis of statistical significance among results. Data generated from the real-time method were less variable (low coefficient of variation) than conventional RT-PCR data in examining gene expression (Singh et al., 2006).
The rat is an appropriate model to study the molecular events associated with cranial suture development and fusion. This animal model provides an opportunity to utilize the sagittal suture as a model of normal patent suture and the posterior frontal suture as a model for suture fusion. Using this model one can compare the expression of cytokines and cognate receptors which regulate programmed suture fusion with those involved with suture patency. In our study, we examined the gene expression pattern in the osteogenic fronts and suture mesenchyme, free of dura mater or periosteum.

The Bmp family of ligands was included in this study because of their role in osteogenesis. It is thought that premature suture fusion occurs because of disrupted suture mineralization. In mouse, Bmp4 protein was localized to suture mesenchyme and osteogenic fronts of the posterior frontal sutures 1 week before the onset of fusion as well as to patent sutures (Warren et al., 2003). Our results in rats indicate that expression of Bmp4 was higher in PF sutures than SAG mesenchyme. However, both mesenchyme showed a peak in expression on day 14. A similar finding was reported by Law and co-workers in a semi-quantitative study in rats (Law et al., 2005). Presence of Bmp4 in fusing and patent sutures suggests that there may be a suture-specific regulation of Bmp activity by secreted Bmp antagonists (Warren et al., 2003). Bmp3 has an anti-osteogenic effect and can inhibit the activity of Bmp4 (Daluiuki et al., 2001). In our study, little difference was seen in the expression of Bmp3, between PF and SAG sutures. Levels of Bmp3 were elevated in the posterior frontal suture before the onset of fusion and decreased during the period of fusion. Similar finding were reported by Nacamuli and coworkers in a study in rats using real time RT-PCR and microarray (Nacamuli et al.,
Taken together, this data suggest that there may be other inhibitors of Bmp4 involved in the regulation.

To determine the role of apoptosis in the maintenance of suture patency and programmed cranial suture fusion, we examined the expression of Bax and Bcl-2 in sagittal and posterior frontal sutures. Apoptosis is governed in part by Bax and Bcl-2 which are pro-apoptotic and pro-survival proteins, respectively. Overexpression of Bcl-2 increases the viability of cells under various adverse circumstances including cytokine withdrawal, loss of cell adhesion as well as γ−irradiation (Sorenson, 2004). Our studies demonstrated higher levels of Bax in patent sagittal sutures than fusing PF sutures. In contrast expression of Bcl-2 was very high in PF sutures during the completion of suture fusion. These data validate the previous finding that maintenance of suture patency is associated with apoptosis (Agresti and Gosain, 2005; Opperman et al., 2000). In mice, posterior frontal sutures did not show any difference in the number of TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) and Bcl-10 positive cells at day 25 and day 45, in sagittal sutures a very pronounced apoptotic activity was observed on day 45 (Agresti and Gosain, 2005).

We found that expression of Fgfr-1 was significantly higher in the PF suture than in SAG sutures at most of the time points examined. At day 10 and 12, expression was twofold higher in the PF than in the SAG suture. Law and coworkers reported similar findings in a semi-quantitative gene profiling study in rats (Law et al., 2005). A similar pattern of expression was reported in a real time RT-PCR study in mice (Nacamuli et al., 2004). However, in immunohistochemical characterization studies of rats, Fgfr-1 protein was
expressed in greater degree in the patent SAG suture than in PF sutures (Mehrara et al., 1998). This difference in expression may be due to the qualitative nature of the immunohistochemical studies.

Previous studies show that expression patterns of Tgf-β receptors are complex and are not well characterized. In our studies, at early time points, there was about a four-fold difference in the expression of Tβr-I between PF and SAG sutures. This difference in expression decreased subsequently and there was more Tβr-I expression in SAG than in PF around 20 to 22 days. Both SAG and PF sutures demonstrated high levels of Tβr-II transcripts at early time points, which subsequently reduced over time. Near constant level of Tβr-III expression in both sutures in all the time points studied indicates its minimal involvement is the signal transduction in Tgf mediated suture fusion. Similar distribution of Tβr-I and Tβr-II was reported in studies by Law and coworkers (Law et al., 2005). An altered pattern of Tgf-β receptor expression was seen in the osteogenic fronts and underlying dura of fetal mice subjected to intra-uterine constraint (Hunenko et al., 2001). Immunostaining for Tβr-I and Tβr-II was markedly increased in the dura mater of the posterior frontal suture during programmed suture fusion in rats compared with dura mater underlying the patent sagittal suture (Mehrara et al., 1999). However, there was no difference in Tβr-I and Tβr-II expression pattern in the sutural tissues of posterior frontal and sagittal sutures before or during the active posterior frontal suture fusion.
Several reports implicate the regional dura mater in the regulation of osseous activity within the overlying suture. In our study we have not examined the gene expression pattern within regional dura mater. Therefore, findings from our study could possibly illustrate the final common gene expression pathways leading to suture patency or suture fusion. Our study has provided baseline information to establish a time line for the expression of various growth factors and receptors which may be used to compare normal and craniosynostotic sutures. This data is useful to develop intervention strategies to manage pathological suture fusion.
<table>
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<th>3’ primer</th>
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Table 2.1: Primer design for real time reverse transcriptase polymerase chain reaction (RT-PCR).
Figure 2.1: Quantitative real-time reverse transcriptase polymerase chain reaction analysis of Bmp4 expression in rat sagittal (A) and posterior frontal (B) sutures.

Error bars denote SEM within the sample. Asterisk indicates significant (p<0.05) difference in Bmp4 expression between different time points.
Figure 2.2: Comparison of Bmp4 mRNA expression in posterior frontal suture (PF) versus sagittal suture (SAG). Normalized Bmp4 cytokine mRNA levels were derived from rat cranial suture tissues from postnatal days 10 to 22. Shown are mean values (n=5 to 6) for each data set with standard errors of mean indicated by vertical brackets. Asterisk indicates significant difference (p< 0.05) between posterior frontal and sagittal suture from same time point.
Figure 2.3: Quantitative real-time reverse transcriptase polymerase chain reaction analysis of Bmp3 expression in rat sagittal (A) and posterior frontal (B) sutures. Error bars denote SEM within the sample.
Figure 2.4: Comparison of Bmp3 mRNA expression in posterior frontal suture (PF) versus sagittal suture (SAG). Normalized Bmp3 cytokine mRNA levels were derived from rat cranial suture tissues from postnatal days 10 to 22. Shown are mean values (n=5 to 6) for each data set with standard errors of mean indicated by vertical brackets.
Figure 2.5: Quantitative real-time reverse transcriptase polymerase chain reaction analysis of Bax expression in rat sagittal (A) and posterior frontal (B) sutures. Error bars denote SEM within the sample. Asterisk indicates significant (p<0.05) difference in Bax expression between different time points.
Figure 2.6: Comparison of Bax mRNA expression in posterior frontal suture (PF) versus sagittal suture (SAG). Normalized Bax mRNA levels were derived from rat cranial suture tissues from postnatal days 10 to 22. Shown are mean values (n=5 to 6) for each data set with standard errors of mean indicated by vertical brackets. Asterisk indicates significant difference (p< 0.05) between posterior frontal and sagittal suture mesenchyme from same time point.
Figure 2.7: Quantitative real-time reverse transcriptase polymerase chain reaction analysis of Bcl-2 expression in rat sagittal (A) and posterior frontal (B) sutures. Error bars denote SEM within the sample. Asterisk indicates significant (p<0.05) difference in Bcl-2 expression between day 22 and other time points, in PF suture.
Figure 2.8: Comparison of Bcl-2 mRNA expression in posterior frontal suture (PF) versus sagittal suture (SAG). Normalized Bcl-2 mRNA levels were derived from rat cranial suture tissues from postnatal days 10 to 22. Shown are mean values (n=5 to 6) for each data set with standard errors of mean indicated by vertical brackets. Asterisk indicates significant difference (p< 0.05) between posterior frontal and sagittal suture on day 22.
Figure 2.9: Quantitative real-time reverse transcriptase polymerase chain reaction analysis of Fgfr-1 expression in rat sagittal (A) and posterior frontal (B) sutures.

Data are presented as percentile mean values (n=5 to 6). Error bars denote SEM within the sample.
Figure 2.10: Comparison of Fgfr-1 mRNA expression in posterior frontal suture (PF) versus sagittal suture (SAG). Normalized Fgfr-1 mRNA levels were derived from rat cranial suture tissues from postnatal days 10 to 22. Shown are mean values (n=5 to 6) for each data set with standard errors of mean indicated by vertical brackets. Asterisk indicates significant difference (p<0.05) between posterior frontal and sagittal suture mesenchyme from same time point.
Figure 2.11: Quantitative real-time reverse transcriptase polymerase chain reaction analysis of Tβr-I expression in rat sagittal (A) and posterior frontal (B) sutures. Data are presented as percentile mean values (n=5 to 6). Error bars denote SEM within the sample. Asterisk indicates significant (p<0.05) difference in Tβr-I expression between different time points.
Figure 2.12: Comparison of Tβr-I mRNA expression in posterior frontal suture (PF) versus sagittal suture (SAG). Normalized Tβr-I mRNA levels were derived from rat cranial suture tissues from postnatal days 10 to 22. Shown are mean values (n=5 to 6) for each data set with standard errors of mean indicated by vertical brackets. Asterisk indicates significant difference (p< 0.05) between posterior frontal and sagittal suture mesenchyme from same time point.
Figure 2.13: Quantitative real-time reverse transcriptase polymerase chain reaction analysis of Tβr-II expression in rat sagittal (A) and posterior frontal (B) sutures.

Data are presented as percentile mean values (n=5 to 6). Error bars denote SEM within the sample. Asterisk indicates significant (p<0.05) difference in Tβr-II expression between different time points.
Figure 2.14: Comparison of Tβr-II mRNA expression in posterior frontal suture (PF) versus sagittal suture (SAG). Normalized Tβr-II mRNA levels were derived from rat cranial suture tissues from postnatal days 10 to 22. Shown are mean values (n=5 to 6) for each data set with standard errors of mean indicated by vertical brackets. Asterisk indicates significant difference (p< 0.05) between posterior frontal and sagittal suture mesenchyme from same time point.
Figure 2.15: Quantitative real-time reverse transcriptase polymerase chain reaction analysis of Tβr-III expression in rat sagittal (A) and posterior frontal (B) sutures. Data are presented as percentile mean values (n=5 to 6). Error bars denote SEM within the sample. Asterisk indicates significant difference (p< 0.05) between time points.
Figure 2.16: Comparison of Tβr-III mRNA expression in posterior frontal suture (PF) mesenchyme versus sagittal suture (SAG) mesenchyme. Normalized Tβr-III mRNA levels were derived from rat cranial suture tissues from postnatal days 10 to 22. Shown are mean values (n=5 to 6) for each data set with standard errors of mean indicated by vertical brackets. Asterisk indicates significant difference (p< 0.05) between posterior frontal and sagittal suture mesenchyme from same time point.
CHAPTER 3

SUSTAINED DELIVERY OF BIOACTIVE CYTOKINE USING A DENSE COLLAGEN GEL VEHICLE

3.1. INTRODUCTION

Craniosynostosis is a condition characterized by premature fusion of cranial sutures. Treatment of prematurely fused sutures typically involves a series of invasive surgical procedures beginning in infancy (Jane and Persing, 2000). In many cases, surgical sites show rapid resynostosis. This re-fusion requires additional surgical procedures which increase the patient’s risk of complications. Therefore, an adjunct therapy to surgery that could safely prevent resynostosis would greatly improve treatment outcomes for children with craniosynostosis.

The use of cytokines as localized therapeutic agents is limited by the lack of a satisfactory delivery system. Local delivery of these cytokines has been hindered by their short half-lives, poor tissue penetration, large molecular size and potential toxicity at high doses (Mooney et al., 2004). Incorporation of a cytokine into an appropriate vehicle can overcome some of these obstacles and facilitate localized release over an extended period of time while maintaining the biological activity of the cytokine. Several
elaborate delivery systems have been studied using polymers or hydrogels as vehicles to deliver cytokines for therapeutic and tissue engineering purposes (Babensee et al., 2000; Friess, 1998; Parker et al., 2002; Saltzman and Olbricht, 2002). These vehicles differ in their chemical and structural properties and in the method by which the cytokine is incorporated. Many of these vehicles may require very sophisticated techniques and equipment for preparation (Holland et al., 2003; Maire et al., 2005a; Maire et al., 2005b).

In this study we used a simple FDA-approved collagen gel as a vehicle rather than extensively processed hydrogels or complex polymers. Also, we delivered the cytokine in an un-manipulated manner, without cross-linking or other treatments.

In selecting a material for this study our goal was to identify a resorbable vehicle which could eventually be used to localize delivery of cytokines to cranial suture sites over a prolonged period. The suitability of collagen as a biomaterial is due largely to its ubiquitous presence in tissues and its low immunogenecity. Collagen is also attractive as a vehicle because it is highly resorbable, and can be processed into a number of forms such as sheets, tubes, sponges, injectable gels and dispersions (Friess, 1998; Lee et al., 2001). The collagen gel used in this study (NeuColl Inc., Campbell, CA) had several properties that made it an attractive candidate for use as a vehicle. At 65 mg/ml it had a density much greater than conventional hydrogels. This highly purified, bovine Type-I collagen gel has had the antigenic N- and C-terminal domains removed from the collagen molecule by pepsin digestion thereby, greatly reducing the likelihood of inducing an immune response. Although highly purified, this formulation retains the native fibrillar conformation of the collagen molecule as opposed to other commercially available
degraded collagen gels. In addition, the injectable material is semi-viscous at room temperature and becomes a firm gel at 37°C, making it ideal for placement into surgical sites. These attributes make this gel vehicle easy to manipulate while still providing excellent biocompatibility. This collagen gel has been most widely used clinically as an FDA-approved, intra-dermal injection for soft tissue augmentation where it will resorb in approximately six months to a year (Pachence, 1996).

Preliminary studies by Moursi and coworkers (Moursi et al., 2003) suggested that, unlike most collagen substrates, this very dense formulation of collagen gel did not promote osteoblast proliferation, migration or mineralization. Based on these results and the attractive properties noted above, we conducted initial studies to examine the ability of the collagen gel to deliver cytokines, or anti-cytokine neutralizing antibodies, to sites of cranial suture fusion in vitro (Morgan et al., 2004; Moursi et al., 2000; Moursi et al., 2003) The successful morphological results obtained from these initial studies prompted the need for a thorough characterization of the collagen gel vehicle prior to further studies. This characterization allowed us to optimize the delivery of protein reagents by determining the minimal effective dose and establishing consistent delivery over the desired time period. These data also increased efficiency, and reduce the likelihood of toxicity, in later animal studies.

The aim of the studies described in this chapter was to determine the release kinetics and bioactivity of a cytokine/collagen gel system designed to achieve sustained, local delivery of cytokines in a bioactive form. For this study we chose Tgf-β3 as our test cytokine due
to its critical role in the regulation of cranial suture patency and fusion. We also investigated the ability of Tgf-β3 protein delivered in a collagen gel to rescue or delay the posterior frontal suture in rat calvarial organ culture. Several studies have shown that Tgf-β3 is highly expressed in patent sutures and poorly expressed in fusing sutures (Lin et al., 1997; Opperman et al., 1997; Poisson et al., 2004; Roth et al., 1997a; Roth et al., 1997b). Recently, mutations in the genes encoding Tgf-β receptors I and II have been associated with a human cohort diagnosed with craniosynostosis (Loeys et al., 2005). In addition, we considered its potential future use as a potent therapeutic delivered locally to craniotomy sites in the surgical management of craniosynostosis.

3.2. MATERIALS AND METHODS

Cytokine Release from Collagen Gel

Recombinant human Tgf-β3 (100 or 500 ng, R&D Systems, Minneapolis, MN) was mixed with a collagen gel (65 mg/ml, NeuColl Inc.) at ratio of 1:1, for a total volume of 200 µl. The mixture was placed in twelve-well culture plates and allowed to gel at 37°C. Serum-free culture media (Advanced DMEM, Gibco, Grand Island, NY) was added to submerge the collagen gel. Conditioned media was collected from wells containing the following conditions after 1, 3, 5, 7, 9, 12 and 14 days:

- Media Only (Untreated Control)
- Collagen Gel in Media
- Collagen Gel + Tgf-β3 in Media
The amount of Tgf-β3 in the conditioned media was measured by Tgf-β3 specific enzyme linked immunosorbent assay (ELISA) (DuoSet ELISA kit #DY243, R&D Systems) per manufacturer’s instructions. Tgf-β3 concentrations in the ELISA samples were measured in an ELISA plate reader (Multiskan plus, Labsystems, Helsinki, Finland). All samples were assayed in duplicate and at least three independent experiments were conducted.

Bioactivity Assay

Mink Lung Epithelial Cell Culture

The bioactivity of Tgf-β3 released into the conditioned media was first assessed by mink lung epithelial cell assay. This method is based on the ability of activated Tgf-β3 to inhibit the proliferation of mink lung epithelial cells (Cheifetz et al., 1987; Graycar et al., 1989; Jen et al., 2002; Jennings et al., 1988; Moses et al., 1990). The method as described by Parker et al. (2002) was followed with a few modifications. Briefly, mink lung epithelial cells (CCL-64 cells, ATCC, Manassas, VA) were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum and collected before they reached confluency to ensure exponential growth. Cells were then seeded at a density 10^4 cells/well in a 96-well plate, and incubated at 37°C for five hours. The media was then replaced with conditioned media collected from the cytokine release assays (method and experimental conditions described above). As a positive control Tgf-β3 in advanced DMEM was added directly to the mink lung epithelial cell cultures. In addition, there was an untreated media control, with mink lung epithelial cells cultured in untreated
advanced DMEM media. All samples were assayed in duplicate and at least three independent experiments were conducted.

**Alamar Blue Dye Assay**

After incubation at 37°C for 48 hrs, epithelial cell proliferation was determined by AlamarBlue dye assay (Trek Diagnostic Systems, Inc., Westlake, OH) (Ahmed et al., 1994; Fields and Lancaster, 1993; Johnson et al., 1998). Briefly, fresh media containing 10% AlamarBlue dye was added to the mink lung epithelial cells. After a 3-hour incubation, a small sample of the medium was collected and the cell number was determined by measuring the fluorescence intensity of the dye in a fluorescent spectrophotometer (Fluorskan II, Labsystems). The excitation wavelength was 544 nm and the emission wavelength 590 nm. Results are reported in arbitrary absorption units. The fluorescence intensity of the sampled media is directly related to cell proliferation. This has been previously verified with this particular cell line to establish the correlation between cell number and absorbance.

**Osteoblast Cell Culture**

Osteoblasts were isolated from day 19 fetal rat calvariae as described by Bellows and co-workers (Bellows et al., 1986) with several modifications (Moursi et al., 1996). Briefly, calvariae were finely minced and treated for 10 minutes at 37°C with 570 U/ml Type II collagenase (Worthington Biochemical Corp., Freehold, NJ). The mixed population of cells released during this first digestion was discarded. Minced calvariae were subjected to two 10-minute then two 20-minute sequential collagenase digestions; osteoblasts
released during these digestions were pooled. The pooled cells were then grown overnight at 25,000 cells/cm\(^2\) on plastic dishes (Corning, Corning, NY). The cells were then detached; using 0.05% trypsin and 0.53 mM EDTA in Hank’s buffered salt solution (Gibco). Cells were then plated at a density of 36,000 cells/cm\(^2\) and grown in alpha-Minimum Essential Medium supplemented with 10% heat-inactivated fetal bovine serum, 0.5% Fungizone, 1% L-glutamine, 0.1% gentamicin, and 0.5% non-essential amino acids (Gibco). After 3 days (cell confluence) the medium was further supplemented with freshly prepared ascorbic acid (100µg/ml) and beta-glycerophosphate (3 mM, Sigma, St. Louis, MO). In all conditions containing collagen gel, cells were loaded directly onto the gel surface. Pilot studies were conducted using bovine serum albumin (BSA, Sigma) mixed into the collagen gel as a control for non-specific protein response then compared to the osteoblast response with collagen gel alone. The results were similar therefore; collagen alone was used subsequently as a control condition. Osteoblasts were cultured in 8-well chamber slides on the following substrates for differentiation, proliferation and cell morphology assays:

- Tissue culture plastic
- Collagen gel alone
- Collagen gel + Tgf-β3 (500 ng/ml, 5ng/well).

All samples were assayed in duplicate and at least three independent experiments were conducted.
Differentiation Assay

As an additional method of measuring Tgf-β3 bioactivity, the alkaline phosphatase activity of osteoblasts was examined. Osteoblasts collected as described above were cultured in the conditions listed above for 3, 5, 7, 9, 11, 13 and 15 days and media was changed every 48 hrs. Alkaline phosphatase activity, a measure of osteoblast differentiation, was assessed by measuring the release of p-nitrophenol at 37 °C in 1 h when p-nitrophenylphosphate was used as a substrate (Sigma Diagnostics). The enzymatic reaction was stopped with 50 mM NaOH and absorbance measured at A₄₀₅ using an ELISA plate reader (Multiskan plus, Labsystems). The results are expressed as nM/PNP/min/no. of cells.

Proliferation Assay

Cells collected as described above were cultured in the conditions listed above for 3, 5, 7, 10 and 15 days and media was changed every 48 hrs. At the designated times osteoblasts were gently washed with phosphate buffered saline to eliminate any unattached cells. Cell number was determined by use of the AlamarBlue dye assay as described above.

Cell Morphology

Osteoblasts were collected and cultured as described above. Cells were fixed with cold methanol for 7 min., air dried, stained with 0.5% toluidine-blue for 5 min., rinsed with distilled water and then cover-slipped with Gel/Mount (Biomedia Corp. Foster City, CA). Morphology was qualitatively evaluated by visualizing fixed cells under light microscopy. Cell attachment, cell spreading and fusiform morphology was assessed.
**Calvarial Organ Culture**

Sprague-Dawley rats were sacrificed by CO₂ narcosis on post-natal day 15. Calvariae were dissected with dura mater intact as previously described (Moursi *et al.*, 2003) and placed in organ culture in serum-free DMEM media and supplemented with 0.5% fungizone, 0.1% gentamicin, 1mM L-Proline, 1% insulin, transferrin, and selenium (ITS+, Collaborative Research, Bedford, PA), freshly prepared ascorbic acid (100 µg/ml) and beta-glycerophosphate (3mM). The protein-collagen admixtures were prepared under sterile conditions by mixing the viscous collagen (65 mg/ml) at room temperature with Tgf-β3 protein (500 ng/ml) (R&D Systems, Minneapolis, MN) or bovine serum albumin (BSA) (500 ng/ml).

Calvariae were randomly divided into three groups, each containing three calvariae per time point as follows, untreated, collagen plus BSA and collagen plus Tgf-β3 protein. The collagen gel was implanted on the posterior frontal suture by injection under the periosteum (Fig.3.1). Approximately 20 µl of collagen was applied to each treated suture. Tissues were collected after 7, 10 and 15 days in culture. Media were changed every 48 hours.

**Tissue Processing and Sectioning**

Collected tissue was fixed in formalin for 24 hrs, thoroughly washed in deionized water and decalcified in formic acid and sodium citrate overnight. Control and experimental tissues were mounted in the same paraffin blocks with similar orientation. The coronal suture was placed at the base of the paraffin form ensuring consistent orientation in all
tissue samples. This allowed for side by side comparison of multiple specimens. Tissue samples were then sectioned. Six \( \mu \text{m} \) sections were taken at 100 \( \mu \text{m} \) intervals beginning at the coronal suture. The sections were stained with hematoxylin and eosin stains utilizing standard protocols.

**Cranial Suture Measurements**

All sections were imaged with a Diagnostic Instruments, Inc. digital camera attached to an Olympus BX60 microscope at 200X. The digital images were analyzed using Image J 1.36 b (National Institute of Health, Bethesda, MD). All samples were analyzed in an endocranial-ectocranial dimension to determine the extent of cranial suture bridging. The coronal suture was used as a landmark and all measurements were taken from the coronal suture anteriorly. This allowed for the comparison of similar locations along the posterior frontal suture on different specimens at 100 \( \mu \text{m} \) increments. All measurements are reported as a distance from this coronal suture landmark. It is important to note that the posterior frontal suture fuses from anterior to posterior. Therefore, sites of earlier fusion will be found at a greater distance (longer \( \mu \text{m} \) measurement) from the coronal suture. Data were collected beginning posteriorly at the coronal suture and continued to a point 2000 \( \mu \text{m} \) anteriorly. This is the approximate length of the actively fusing portion of the posterior frontal suture in the 15 day old rat. In order to determine the amount of sutural bridging in the endocranial-ectocranial dimension, measurements were taken of the entire suture height and the amount of bony bridging as described previously (Moursi *et al.*, 2003). This measuring technique is illustrated in Fig.3.2. The most inferior point of the suture ridge in the sutural gap (SG) was labeled point A. A line was then drawn
from point A to a point where it intersects a line connecting the most superior points of
the bony fronts. This intersection was labeled point B. The distance from point A to
point B was defined as the sutural height (SH). From point A, a second line was drawn to
the most superior point of bony bridging, labeled point C. The distance from point A to
point C was defined as the bridging height (BH). The percent bridging was calculated for
each section by dividing the bridging height by the sutural height. Approximately 20
sections per specimen were obtained along the entire posterior frontal suture.

**Statistical Analysis**

Standard Student’s t-tests were used to determine the significance between a control and
experimental condition. Results with a statistical significance of $P < 0.05$ are denoted
with an asterisk on the graph. In addition the standard deviations between the repeats of
each assay are represented as error bars on each individual experimental condition.

In organ culture studies, each condition and time point was duplicated, in at least three
independent experiments. A random sample of histological sections was measured twice.
Intra-examiner reliability was determined to be $r = 0.998$ for suture height and $r = 0.999$
for bridge height using the intraclass correlation coefficient. Percent bridging was
analyzed using factorial analysis of variance with control versus treated (two levels) and
days 7, 10 and 15 (four levels).
3.3. RESULTS

Kinetics of Tgf-β3 Released from Collagen Gel

To measure the kinetics of Tgf-β3 released from the collagen gel, an *in vitro* release assay was performed in cell culture medium. Culture medium was chosen for these studies so that conditions could be optimized for future organ culture experiments where medium will be necessary to maintain viable tissue. For gels loaded with 100 and 500 ng we found a sustained release of Tgf-β3 over 14 days with a pattern of initial large release followed by a gradual reduction in the amount released over time. The reduced release over time was correlated to the amount initially loaded. In the gels loaded with 100 ng (Fig. 3.3) after 1 day, 12.1% of the loaded cytokine was released from the gel into the medium. During the entire incubation period, 14 days, a cumulative total of 57.7% of the Tgf-β3 loaded into the gel was released into the medium. The amount released on day 14 was 38.0% of that released on day 1.

In the gels loaded with 500 ng (Fig. 3.4) there was less of a reduction in the release over time. Retention was also greater, after 1 day, 3.2% of the loaded cytokine had been released from the gel into the medium. During the entire incubation period, 14 days, a cumulative total of 21.0% of the Tgf-β3 loaded into the gel was released into the medium. The difference between day 1 and day 14 was less pronounced than in the gels loaded with 100 ng. The amount released on day 14 was 84.6% of that released on day 1.

These results indicated that at both loading doses the collagen gel could retain and release cytokine over a prolonged period of time. Since the 100 ng dose was sufficient to
maintain a potentially functional release similar to concentrations used by others (Opperman et al., 2000; Opperman et al., 2002a; Opperman et al., 2002b; Parker et al., 2002), even at day 14, all subsequent experiments were conducted with this dose.

**Bioactivity of Tgf-β3 Released from Collagen Gel**

Biological activity of the released Tgf-β3 was measured using the mink lung epithelial cell assay (Fig. 3.5). The greater the amount of activated Tgf-β3 present the stronger the inhibition of proliferation of mink lung epithelial cells and thus, the lower the cell number. Conditioned media containing cytokine released from the collagen gel inhibited epithelial cell proliferation, demonstrating Tgf-β3 bioactivity at all time points. Conditioned media collected on day 1, containing Tgf-β3 at 20.1 ng/ml, produced a 50.4% inhibition compared to conditioned media collected from untreated control wells. Although slightly reduced, Tgf-β3 bioactivity was maintained at day 14. Conditioned media collected on day 14, containing Tgf-β3 at 7.7 ng/ml, produced a 62.6% inhibition compared to conditioned media collected from untreated control wells.

As another method of measuring Tgf-β3 bioactivity, osteoblast differentiation was determined by measuring alkaline phosphatase activity. The activity of alkaline phosphatase is considered a marker of osteoblast differentiation and function. Figure 3.6 shows the alkaline phosphatase activity of the osteoblasts cultured on the plastic and collagen with or without Tgf-β3. All the control groups showed the expected increase in alkaline phosphatase activity during the incubation period. However, we
found that alkaline phosphatase activity of osteoblasts cultured on collagen gel with Tgf-β3 was reduced 43-83% compared to cells cultured on collagen alone between days 9 and 13.

**Osteoblast Response**

The proliferation and cell morphology of primary calvarial osteoblasts cultured on collagen gel, with and without cytokine, was evaluated in order to characterize cell response to this cytokine/collagen gel delivery system. As expected, collagen gel alone showed a significant reduction in cell number compared to plastic at all time points (Fig. 3.7). An additional reduction in cell number was not detected when Tgf-β3 was added to the gel. These results indicated that the collagen gel alone demonstrated a strong inherent inhibitory effect on osteoblast proliferation.

The morphology of osteoblasts cultured on collagen gel with Tgf-β3 was also assessed. A qualitative analysis of the toluidine blue staining demonstrated that there were no observable differences in cell morphology between osteoblasts cultured on collagen alone, those cultured on collagen with Tgf-β3, and those cultured on plastic alone (Fig. 3.8). All conditions exhibited cell attachment, cell spreading, and the fusiform morphology associated with an osteoblastic phenotype.

**Posterior Frontal Suture Fusion in Endocranial-Ectocranial Dimension**

In all calvaria fusion began on the endocranial side and extended ectocranially. To determine whether an effect of the Tgf-β3 protein could be demonstrated along the entire
suture, mean percent bridging was examined in three selected regions. The anterior (1800 to 2000 µm), middle (900 to 1100 µm), and the posterior (100 to 300 µm) regions of the suture were defined by their distance from the coronal suture. The overall percent bridging increased slightly in both control and Tgf-β3-treated samples as suture fusion progressed from anterior to posterior sections. However, in the anterior portion of calvariae treated with collagen gel loaded with Tgf-beta 3, a statistically significant reduction in mean percent bridging, compared to controls, was measured at all time points. (Fig.3.9). In this region the Tgf-beta 3 treated calvaria were found to have 65% to 89 % less bony bridging than control groups. The percent bridging data represent mean values of all the sections in a given region of the calvaria for up to three independent experiments. However, in contrast to these mean values, many of the sections in the Tgf-beta 3 treated group were completely patent with absolutely no fusion of sutures. The effect of Tgf-beta 3 protein on the fusion of posterior frontal sutures of the rat is illustrated in the representative histological sections in Fig. 3.10

3.4. DISCUSSION

Recent advances in the understanding of bone biology pathways and molecular events occurring during normal and premature suture fusion may allow us to design targeted and complementary cytokine therapies to decrease complications inherent in the high-risk surgical procedures used to correct synostotic sutures (Mooney et al., 2004).

The three major isoforms of Tgf-β (1, 2 and 3) are present in the dura and in the osteoblasts lining the dura mater and periosteal surfaces of the cranial bones, throughout suture morphogenesis and after the suture is fully formed. Distribution of Tgf-βs differs
during the various stages of suture formation and suture obliteration. While Tgf-β1 and 2 are highly expressed during suture fusion it has been shown that Tgf-β3 is absent from the bony fronts of fusing sutures (Lin et al., 1997; Opperman et al., 1997; Poisson et al., 2004; Roth et al., 1997b). Based on these findings it has been hypothesized that local delivery of exogenous Tgf-β3 protein could prevent cranial suture fusion.

Few studies have utilized collagen gel to deliver cytokines to cranial suture sites (Chong et al., 2003; Opperman et al., 2002b). Although the results of these studies showed promise for the collagen gel as a vehicle, the studies were limited by a lack of information regarding the collagen gel. The results of this study allowed subsequent studies particularly those conducted in vivo, to be conducted with more accurate dosing and time point selection. This reduced the risk of potentially toxic doses and made for more efficient use of time and animals.

This study was planned to further develop an appropriate vehicle to deliver cytokines to cranial sutures in a biologically active form. We examined the kinetics and bioactivity of our test cytokine, Tgf-β3, during release from a collagen gel. Release data showed that when Tgf-β3 was mixed with collagen gel it was not immediately released as is found with many of the less dense collagen vehicles (Friess, 1998; Lee et al., 2001; Uludag et al., 1999). Instead, the rate of release was gradual over a period of time. The decrease was found to be directly related to the amount of cytokine initially loaded. At the end of the incubation period we found with the 100 ng dose that 42.3% of the protein was retained while with the 500 ng dose 79.0% was retained. This retention was
demonstrated by the collagen gel despite being completely submerged for 14 days in a relatively large volume of media which was completed changed every 48 hours. At the 500 ng dose we did not exceed the carrying capacity of the vehicle as demonstrated by the similar release kinetics on day 1 as compared to the 100 ng dose. Had we overloaded the gel the 500 ng dose would have produced a much higher release on day 1 as compared to the 100 ng dose. Although the 100 ng dose showed a substantial reduction in release between days 1 and 14, the release concentration at day 14 was still within the physiological range, equivalent to that found in approximately 140 mg of human bone (Hering et al., 2001). This concentration was also within the range used by others for in vitro and in vivo studies (Opperman et al., 2000; Opperman et al., 2002a; Opperman et al., 2002b; Parker et al., 2002).

We used the mink lung epithelial cell assay to examine whether Tgf-β3 released from the collagen gels remained biologically active. The results indicated that Tgf-β3 released from the collagen gel retains its bioactivity following incorporation into the collagen gel and release into the media. This bioactivity was evident even after 14 days of incubation when a concentration of 7.7 ng/ml produced a 62.6% inhibition of epithelial cell proliferation. The results from this late time point indicate that, even after a 38% reduction in release from day 1, sufficient amount of cytokine was released to generate a functional response. Also, the reduced cytokine release on day 14 translated into only a modest drop in bioactivity. Together, these results suggest that low amounts of cytokine release are sufficient to induce a cellular response and that even smaller loading doses may be sufficient to achieve desired effects in future studies.
In addition to the mink lung epithelial cell assay, the bioactivity of released Tgf-β3 was further illustrated by the decreased alkaline phosphatase activity measured in osteoblasts cultured on the gels loaded with Tgf-β3. A large decrease in alkaline phosphatase activity was measured at the latest time point, when the release concentration was at its lowest. This further demonstrates the ability of the collagen gel to deliver bioactive cytokines over a prolonged period. This result concurs with other studies showing a Tgf-β3-induced decrease in alkaline phosphatase activity in osteoblasts (ten Dijke et al., 1990).

To further characterize the osteoblast response, we examined osteoblast proliferation and morphology. Cell number was significantly reduced for cells cultured on the collagen gel compared with those grown on tissue culture plastic. This confirmed our previous studies indicating that the collagen gel alone demonstrated an inherent inhibitory effect on cell proliferation (Moursi et al., 2003). These results also correlate well with other studies showing reduced osteoblastic proliferation when cultured on a collagen substrate (Ichimura, 1993; Lynch et al., 1995; Rattner et al., 2000; Talley-Ronsholdt et al., 1995). This is a beneficial property for the intended use of this vehicle to prevent bone formation at cranial suture sites.

The overall reduced proliferation observed on the collagen gel was not augmented by the presence of Tgf-β3. Tgf-β3 has been shown to induce decreased cell proliferation in calvarial tissue (Opperman et al., 2000). However, this effect on proliferation is known to be dependent on the concentration used and the duration and conditions of the experiment.
(Opperman et al., 2000; ten Dijke et al., 1990). We were aware of the strong collagen-mediated inhibition of proliferation therefore, we did not expect an additional Tgf-β3-mediated reduction in cell number. It is likely that a Tgf-β3-mediated reduction of proliferation was masked by the strong inherent inhibition of the collagen gel vehicle. Results from both the mink lung epithelial cell and the alkaline phosphatase activity assays demonstrated the bioactivity of the cytokine. The combined synergistic effect of a bioactive cytokine and an anti-proliferative collagen gel could provide an effective cytokine delivery system where inhibition of bone formation is desired.

Obliteration of the fibrous gap between suture bone margins by ossification is viewed as the hallmark of calvarial suture fusion (Bradley et al., 1996b; Opperman et al., 1993). Tgf-β3 protein delivered in a dense collagen gel maintained the patency of the naturally fusing posterior frontal sutures. These results were similar to those reported in vitro and in vivo studies in rats (Opperman et al., 2000; Opperman et al., 2002b). Together, these studies indicate that an increase in exposure to Tgf-β3 protein by exogenous application can inhibit programmed cranial suture fusion in rats. It is important to understand the mechanisms by which Tgf-β3 maintains the suture patency before it can be utilized as an agent to intervene pathological cranial suture fusion. Inhibition of osteoblast differentiation, demonstrated by the reduction of alkaline phosphatase described above, could be one of the mechanisms responsible for the reduced suture bridging.

Results from the studies presented here provide further evidence for the role of Tgf-β3 in cranial suture fusion. In addition, these studies indicate that localized sustained delivery
of Tgf-β cytokines in a biocompatible matrix such as collagen gel may provide a strategy to intervene in cranial suture fusion in vivo.
Figure 3.1: Collagen gel implant in calvarial organ culture. Box indicates site of collagen gel implantation over posterior frontal suture.
Figure 3.2: Micrograph illustrating technique for measurement of bony bridging.

A = most inferior (endocranial) point of the suture ridge; B = intersection of line drawn from point A to line connecting the most superior (ectocranial) points of the bony fronts; C = most superior point of bony bridging; SH = suture height, distance from point A to point B; BH = bridging height, distance from point A to point B; BH = Bridging height, distance from point A to point C; SG = suture gap. Scale bar = 40 µm.
Figure 3.3: Tgf-β3 (100 ng) release from collagen gel as measured by ELISA.

Error bar represents standard deviation.
Figure 3.4: Tgf-β3 (500 ng) release from collagen gel as measured by ELISA.

Error bar represents standard deviation.
Figure 3.5: Tgf-β3 bioactivity as measured by mink lung epithelial cell proliferation assay. Data presented as percent of untreated control. Error bars represent standard deviation. Asterisk denotes statistically significant difference ($p<.05$) between the collagen + Tgf-B3 (Coll/Beta3) and collagen control (Coll) conditions.
Figure 3.6: Osteoblast alkaline phosphatase activity. Error bars represent standard deviation. Asterisk denotes statistically significant difference ($p<.05$) between the collagen + Tgf-B3 and collagen control conditions.
Figure 3.7: Osteoblast proliferation as measured by AlamarBlue assay. Error bars represent standard deviation. Asterisk denotes statistically significant difference ($P<.05$) between the collagen control and plastic only conditions.
Figure 3.8: Osteoblasts after 48 h in culture stained with toluidine blue: (A) Tgf-β3 in collagen gel, (B) Collagen gel alone and (C) plastic. No observable differences in cell morphology between osteoblasts cultured on collagen alone, those cultured on collagen with Tgf-β3 and those cultured on plastic alone (original magnification: 200 X).
Figure 3.9: Mean percent bony bridging of sutures in three suture regions after 7(A), 10 (B), and 15 (C) days in culture. Error bars represent standard deviation. Asterisks denote statistically significant (p<0.05) difference between collagen plus bovine serum albumin (BSA) and collagen plus Tgf-β3 protein.
Figure 3.10: Micrograph showing calvaria treated with collagen plus BSA (A) and collagen plus Tgf-β3 protein (B) after 10 days in culture. Both sections are from the midpoint of the posterior frontal suture. Original magnification: X 20. Sutures in the Tgf-β3 treated group were completely patent. Arrows indicate bridging height (A) and suture patency (B).
4.1. INTRODUCTION

A significant body of literature supports a role for transforming growth factors in the regulation of suture fusion. Differential distribution of Tgf-β isoforms has been observed in the peri-sutural tissues of human infants and rodent models as well as in the rabbit model of human craniosynostosis. In these tissues Tgf-β3 was down-regulated in fusing sutures and abundant in patent sutures (Lin et al., 1997; Opperman et al., 1997; Poisson et al., 2004; Roth et al., 1997a; Roth et al., 1997b).

The functionality of this differential expression of Tgf-β3 has been studied by several researchers. Studies in rodents have shown that delivery of exogenous Tgf-β3 protein prevented cranial suture fusion both in vitro (Opperman et al., 1999; Opperman et al., 2000; Opperman et al., 2002a) and in vivo (Opperman et al., 2002b). In a rabbit model of delayed-onset craniosynostosis, Chong and co-workers delivered Tgf-β3 protein in a
collagen gel which rescued coronal sutures destined to synostose (Chong et al., 2003).

These studies indicate the potential of Tgf-β3 as a therapeutic agent in the management of craniosynostosis.

Although Tgf-β3 protein therapy has been successful in animal models, very high concentrations and a longer treatment time would be necessary for clinical applications. Since growth factors have very short biological half lives and are difficult to retain at sites of local application, large doses would be required to inhibit suture fusion for long periods. Also, under these conditions, the release of growth factors is not uniform over time. Initially there is a rapid efflux which saturates the surrounding tissue with supra-physiological concentrations of growth factor which could lead to systemic exposure. Subsequent release, although slower, provides suboptimal concentrations (Uludag et al., 1999). Another disadvantage of using recombinant cytokines is their high cost.

Gene transfer or therapy can provide an opportunity to overcome such problems. By delivering cDNAs that encode growth factors and cytokines, rather than delivering them as proteins themselves; it is possible to achieve a sustained, local presence of cytokine at optimal concentrations with minimal exposure to non-targeted sites (Evans et al., 2004). In addition, a cytokine synthesized in situ as a result of gene transfer undergoes authentic posttranslational modifications and is presented to the surrounding tissues in a natural, cell based manner (Betz et al., 2006). Endogenously expressed cytokines are therapeutically more effective than systemically applied recombinant proteins (Makarov et al., 1996). Gene transfer of growth factors or cognate receptors into sutural tissues to
prevent or disrupt cranial suture fusion is an attractive concept to circumvent the
difficulties associated with growth factor delivery. cDNAs can be transferred to sutural
tissues by viral or non-viral methods. Previous studies have demonstrated the principle
and utility of viral-mediated gene transfer in preventing programmed suture fusion in
mouse (Mehrara et al., 2002; Song et al., 2004; Warren et al., 2003).

Clinical use of viruses as gene therapy vectors is associated with safety concerns which
are related to the immunogenicity of both the virus itself and the cells transduced by the
virus (Chuah et al., 2003; Debyser, 2003). Non-viral vectors are simpler and less
expensive. Non-viral vectors that contain only naked DNA and some type of carrier to
facilitate cell uptake have low immunogenicity and low likelihood of being inserted into
the host cell genome (Oligino et al., 2000). However, non-viral vectors are less efficient
than viral vectors. For greater transfection efficiency, plasmid DNA can be complexed
with liposomes and various polymers. Also, gene delivery from biomaterials allows for
localized and sustained delivery of plasmids. Collagen gels can be used in injectable,
controlled delivery systems and has the advantage that it can be introduced in a
minimally invasive manner. Studies directed towards using different forms of collagen as
a carrier for naked plasmid DNA have shown the potential of these carrier matrices in
therapeutic gene delivery and tissue engineering (Fang et al., 1996; Sano et al., 2003;
Scherer et al., 2002). Although previous studies have shown the ability of the collagen
gel to deliver therapeutic proteins in vitro and in vivo (Chong et al., 2003; Moursi et al.,
2003; Premaraj et al., 2006), little is known about gene delivery using dense collagen gel.
Studies described in this chapter were designed to test the following hypothesis an increase in non-viral plasmid-encoded Tgf-β3 production, localized to posterior frontal suture, can prevent programmed fusion of posterior frontal sutures in rats. These studies made use of a non-viral plasmid, delivered in a collagen gel vehicle, to produce a localized and sustained, overproduction of Tgf-β3 recombinant protein in cell culture and in the posterior frontal sutures of wild-type rats.

4.2. MATERIALS AND METHODS

4.2.1. DNA Release Assays

Transforming growth factor beta 3 (Tgf-β3) – pCMV6-xl5 plasmid DNA (Fig.4.1), 5 µg (Origene Technologies, Rockville, MD, USA) was mixed 1:1 volume with collagen gel (32 mg/ml, NeuColl Inc., Campbell, CA, USA) and placed in six-well culture plates (Corning life science, Corning, NY). Dulbecco’s Modified Eagle’s Medium (Gibco) was added to immerse the gel mixture and media samples were collected at various time points. The PicoGreen double stranded DNA Quantitation kit (Molecular Probes, Eugene, OR, USA) was used to detect DNA released into the media from collagen. Picogreen is an ultra sensitive fluorescent nucleic acid stain that is highly specific for the detection of super coiled and double stranded DNA, but not for single stranded DNA and oligonucleotides (Rengarajan et al., 2002). The samples were measured using a fluorescent spectrophotometer (Fluorskan II; Labsytems, Helsinki, Finland). Solutions were excited at 485 nm and emission was measured at 530 nm. Integrity of the released DNA was examined by 0.8% agarose gel electrophoresis and visualized by ethidium bromide staining. Collagen gel loaded with plasmids was digested
with collagenase (Worthington Biochemical Corp., Freehold, NJ) and collected DNA was analyzed by 0.8% agarose gel electrophoresis for changes in the structural integrity.

4.2.2. In Vitro Transfection of Calvarial Osteoblasts Using Collagen Gels Loaded with pEGFP-C2 and pDsRed2

Fetal rat osteoblasts obtained as described in Section 3.2 were grown at 37°C in an atmosphere of 5% CO₂ in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics. On the day prior to addition of gels loaded with plasmids, cells were seeded at a density of 20,000 cells/well into 8-well chamber slides (BD Biosciences, San Jose, CA) giving rise to an almost confluent cell layer on the day of transfection. Plasmids encoding green fluorescent protein (pEGFP-C2) and DsRed (pDsRed2) (Clontech, Palo Alto, CA, USA) (1 µg/well) combined with Geneporter (Gene therapy systems, Inc.) were mixed with collagen gel. On the day of transfection, collagen alone (control) or collagen with plasmids was placed into the chambers. Plasmid release from the gel and subsequent transfection of osteoblasts were examined by visualization of fluorescent proteins by fluorescent microscopy (Olympus BX60, Melville, NY) after 48 and 72 hours of incubation.

4.2.3. In Vitro Transfection of Calvarial Organ Culture Using Collagen Gels Loaded with pEGFP-C2 and pDsRed2

Calvariae were dissected from 15-day old rats as described in Section 3.2. pEGFP-C2 or pDsRed2 was mixed with collagen and GenePorter transfection reagent. The transfection mix was placed at 4°C for one hour to form gel mixture. EGFP/collagen or
DsRed/collagen mixture was loaded into a syringe and implanted superficial to the posterior frontal suture by injection under the periosteum. The calvarial tissues were cultured for 10 days in 12-well cell culture microplates (Corning life science, Corning, NY), with media changes occurring every other day. Calvarial tissues were fixed with 4% paraformaldehyde overnight and decalcified with formic acid and sodium citrate. The tissue was then mounted on a slide and fluorescent images were visualized by Zeiss 510 META laser scanning confocal microscope (Carl Zeiss Advanced Imaging Microscopy, Jena, Germany).

4.2.4. In Vitro Transfection of Calvarial Osteoblasts Using Collagen Gel Loaded Non-Viral Plasmid DNA Encoding Tgf-β3

Fetal rat osteoblasts were grown at 37°C in an atmosphere of 5% CO₂ in DMEM supplemented with 10% fetal calf serum and antibiotics. On the day prior to addition of gels loaded with plasmids, cells were seeded at a density of 50 000 cells/well into 48-well plates (Corning life science, Corning, NY) giving rise to an almost confluent cell layer on the day of transfection. Plasmid DNA encoding Tgf-β3 (Origene Technologies, Rockville, MD, USA) (3 µg/well) combined with Geneporter was mixed with collagen gel. On the day of transfection, collagen alone (control) or collagen with DNA was added to the wells then supernatants were collected at various time-points up to 14 days. DNA release from the gel and subsequent transfection was quantified by measurement of Tgf-β3 production using ELISA.
**Tgf-β3 Bioactivity Assay**

The bioactivity of Tgf-β3 produced in the above experiment was assessed by mink lung epithelial assay as described in Section 3.2.

**4.2.5. Collagen Gel Delivery of Non-Viral Transforming Growth Factor Beta3 (Tgf-β3) Plasmid in Rat Calvarial Organ Culture**

The plasmid-collagen admixtures were prepared by mixing the viscous collagen (65 mg/ml) at room temperature in a 1:1 volume (final concentration, 32.5 mg/ml) with the Tgf-β3 or control plasmid (Tgf-β3–pCMV-XL5 and pCMV-XL5 Origene Technologies, Rockville, MD) and Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). Plasmids were admixed with the collagen gel at concentrations of 3 µg/calvaria.

Plasmid/collagen mixture was loaded into a syringe and placed at 4°C for one hour to form gel mixture. Sprague-Dawley rats were sacrificed by CO₂ narcosis on post-natal day 15. Calvariae were dissected with dura mater intact and placed in one of the following control or experimental groups:

- **Group 1:** Untreated Control- No treatment
- **Group 2:** Collagen and control plasmid (3 µg/calvaria)
- **Group 3:** Collagen and Tgf-β3 plasmid (3µg/calvaria)

Three animals were used in each of the 3 conditions and experiments were repeated three times. Approximately 40 µl of collagen vehicle, with Tgf-β3 or control plasmid was implanted superficial to the posterior frontal suture by injection under the periosteum described previously (Fig. 3.1) (Moursi et al., 2003). Calvaria were then placed in organ
culture in serum-free DMEM media supplemented with 0.5% fungizone, 0.1% gentamicin, L-Proline, and 1% insulin, transferrin, and selenium (ITS+, Collaborative Research, Bedford, PA) and freshly prepared ascorbic acid (100 µg/ml), beta-glycerophosphate (3mM).

**Histological Analysis of Cranial Suture Fusion**

After 15 days in culture, the calvarial tissues were processed, as previously described in Section 3.2 (Moursi et al., 2003). Briefly, tissues were fixed in 4% paraformaldehyde, pH 7.5 at 4°C for 1 hr. then decalcified in formic acid and sodium citrate. Control and experimental tissues were mounted in the same paraffin blocks with similar orientation. The coronal suture was placed at the base of the paraffin form to ensure consistent orientation in all tissue samples. This allowed for side by side comparison of multiple specimens. Tissue samples were then sectioned. Six µm sections were taken at 100 µm intervals beginning at the coronal suture. The sections were stained with hematoxylin and eosin stains utilizing standard protocols. All sections were imaged with a digital camera attached to an Olympus BX60 microscope at 200X. The digital images were analyzed using Image J 1.36 6 software (National Institute of Health, Bethesda, MD). All samples were analyzed in an endocranial-ectocranial dimension to determine the extent of cranial suture bridging. The coronal suture was used as a landmark and all measurements were taken from the coronal suture anteriorly as described previously. This allowed for the comparison of similar locations along the posterior frontal suture on different specimens at 100 µm increments. In order to determine the amount of sutural bridging in the
endocranial-ectocranial dimension, measurements were taken of the entire suture height and the amount of bony bridging as described in Section 3.2.

Immunohistochemistry

Immunohistochemistry was performed on rat calvarial organ cultures to determine the efficiency of transfection and protein production after Tgf-β3 plasmid treatment. Calvarial tissues were fixed with 4% paraformaldehyde for 4 hours. Specimens were blocked with 10% donkey serum in wash buffer for 30 minutes at 37°C. Specimens were incubated with primary antibody (goat anti-chicken Tgf-β3, AF-243-NA, R&D Systems, Minneapolis, MN) at a concentration of 5µg/ml for at least one hour. Specimens were washed then incubated with secondary antibody (fluorescein-conjugated donkey anti-goat IgG, Jackson Immunoresearch, West Grove, PA) at 1:200 for 30 minutes at room temperature. Whole calvaria were mounted in wells containing gel mount for visualization with Zeiss 510 META laser scanning confocal microscope.

4.2.6. Collagen Gel Delivery of Non-Viral Plasmid Encoding Transforming Growth Factor Beta3 (Tgf-β3) in a Collagen Gel Vehicle In Vivo

Animals

All animal studies were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) at The Ohio State University. Sprague-Dawley rats were purchased from Harlan, Indianapolis, IN. Animals were housed in a light-, temperature- and humidity controlled environment until time of death. Food and water were provided ad libitum.
Preparation of Collagen Gels

The plasmid-collagen admixtures were prepared under sterile conditions by mixing the viscous collagen (65 mg/ml) at room temperature in a 1:1 volume (final concentration, 32.5 mg/ml) with the Tgf-β3 or control plasmid and Fugene 6 transfection reagent. Plasmids were admixed with the collagen gel at concentrations of 15μg/animal. Tgf-β3 protein was mixed with collagen gel at concentrations of 30 ng /animal.

Animals and Surgery

Fifteen days old, rats were anesthetized with an intra-peritoneal injection (2 mg/kg) of a solution of 91% Ketsest (Ketamine Hydrochloride, 100 mg/ml, Hospira, Inc., Lake Forest, IL) and 9% Rompun (Xylazine, 20 mg/ml, Phoenix Scientific, Inc., St. Joseph, MO) and their scalps were prepared for injections. Animals were randomly assigned to each of the following 6 conditions:

Group 1: Untreated Control- No treatment

Group 2: Collagen + Control vector plasmid (15μg/animal)

Group 3: Collagen + Tgf-β3 plasmid (15μg/animal)

Group 4: Collagen + Tgf-β3 protein (30 ng/animal)

Group 5: Sham surgical control- PBS

A volume of 100 μl of the mixture was injected sub-periosteally with a 21G needle superficial to the posterior frontal suture. The collagen gel is enough to cover 3-4 mm of the posterior frontal suture anterior to the coronal suture. Animals in the sham control group were injected with 100μl of PBS.
Ten days after injections, animals were euthanized by CO$_2$ narcosis and calvariae were harvested. Tissues were fixed in 4% paraformaldehyde, pH 7.5 at 4$^\circ$ C for 3 hours. Calvarial issues for the histology and immunohistochemistry were processed as described above (see section 4.2.5). Calvariae for the micro CT analysis were stored in 70% ethyl alcohol until the imaging.

**Micro CT**

An attempt was made to validate the histomorphometric analysis of suture fusion by micro-CT analysis of the sutures. Micro-CT scans (Scanco USA, Inc., Pennsylvania) of the posterior frontal suture area were performed and series of cross-sectional images were generated. These images were magnified and mean percent bridging of the control and treated calvariae were quantified using Image J 1.36 b (National Institute of Health, Bethesda, MD). The quantification technique used was similar to that used for histomorphometric analysis of histological sections (see Section 3.2).

**Scoring and Analysis of Tissues**

The results of four separate experiments were combined for data analysis. A random sample of micro CT images was measured twice. Intra-examiner reliability was determined to be $r = .988$ for bridge height and $r = 0.897$ for suture height using intraclass correlation coefficient. Percent bridging data for in vitro and in vivo studies were analyzed by analysis of variance (ANOVA) and Tukey’s posthoc test. $p < 0.05$ was considered as a significant difference. Data were analyzed by SAS 9.1 for Windows (SAS Institute, Inc., Cary, NC).
**Immunohistochemistry**

For immunohistochemical staining, mounted and unstained tissue specimens were deparaffinized. Cell and Tissue staining kits (HRP-DAB system, rabbit kit -CTS005, goat kit-CTS008, R&D system, Minneapolis, MN) were used according to manufacturer’s instructions. Briefly, an indirect immunoperoxidase method was used to detect reactivity of polyclonal antibodies specific for, Tgf-β2 (AF-302, R&D Systems, Minneapolis, MN), Tβr-I, Tβr-II, Bax and Bcl-2 (sc-402, sc-400, sc-526, sc-492; Santa Cruz Biotechnologies, Santa Cruz, CA) using diaminobenzidine (DAB) staining. Rat intestinal sections were used as positive control. Negative control slides were incubated in the absence of primary antibody. All sections were counterstained with Mayer’s hematoxylin, dehydrated through serial alcohols, cleared in xylene, and the coverslips secured with Permount (Sigma, St Louis, MO). All sections were imaged with a digital camera attached to an Olympus BX60 microscope. Immunoreactivity of the perisutural areas (i.e., osteogenic fronts, sutural mesenchyme -when present, dura mater, periosteum and defect site) were subjectively scored on a scale of 0 (no stain) to 3 (intense brown). Results were compared between Tgf-β3 plasmid treatment group and untreated control group to determine up- or down-regulation.

**4.3. Results**

**DNA Release Kinetics**

DNA release studies showed that plasmid release increased at each time-point with the total collected over 14 days was equal to approximately 20% of the total loaded (Fig. 4.2). After 14 days, 80% of the loaded plasmid DNA remained in the gel. Following
characterization of DNA release kinetics, studies were carried out to determine whether the released plasmid was structurally intact. Results from gel electrophoresis studies demonstrated that the released plasmid maintained its structural integrity as evidenced by distinct bands present on the gel (data not shown).

**Transfection in Rat Osteoblast and Calvarial Organ Culture**

In order to monitor the transfection efficiency and patterns of protein production in calvarial tissues, transfections using reporter plasmids, pEGFP-C2 and pDsRed2 were carried out before Tgf-β3 plasmid studies. Bioavailability of the released plasmid from collagen gel was determined via transfection of primary calvarial osteoblasts. Results demonstrated that pEGFP-C2 and pDsRed2 plasmids released from the collagen gel directly incubated with calvarial osteoblasts were successfully transfected and translated into fluorescent proteins by the cells (Fig. 4.3). However, the transfection efficiency was low.

Experiments were carried out to determine pEGFP-C2 and pDsRed2 plasmid’s ability to transfec sutural tissues in an organ culture environment. Transfection studies demonstrated that released pEGFP-C2 and pDsRed2 plasmids were able to transfec cells in calvarial organ culture and express fluorescent proteins up to 14 days (Fig. 4.4).

Similar transfection studies were carried out to determine the ability of Tgf-β3 plasmid delivered in a collagen gel to transfect and produce Tgf-β3 protein in primary osteoblasts and sutural tissues in vitro. In the primary osteoblast culture system, supernatant samples
were collected over several days then assayed by ELISA for recombinant Tgf-β3. Tgf-β3 plasmid was able to transfect the osteoblasts and express Tgf-β3 protein. Tgf-β3 production was considerably higher than endogenous production (control) and increased at each time point up to 14 days (Fig. 4.5). Successful transfection and expression of Tgf-β3 expression was also seen in sutural tissues as demonstrated by the fluorescent immunohistochemistry viewed by confocal laser scanning microscopy (Fig. 4.6).

**Biological Activity of the Plasmid Encoded Tgf-β3 Protein**

Biological activity of the plasmid encoded Tgf-β3 protein was measured by mink lung epithelial (MLE) assay. Supernatants collected from the osteoblasts transfected with Tgf-β3 plasmid inhibited the proliferation of MLE cells. The amount of inhibition was similar to that produced by recombinant Tgf-β3 protein (Fig. 4.7).

**Effect of Tgf-β3 on the Programmed Fusion of Posterior Frontal Sutures**

**Adverse effects**

The implantation of collagen gel loaded with Tgf-β3 plasmid or control plasmid did not produce any obvious systemic adverse effect or weight loss to the rats during the experimental period. Macroscopic examination of the implantation site at the time of tissue collection and microscopic examination of the histological sections did not reveal any signs of infection or adverse immunological or inflammatory reactions.
Histomorphometric Analysis of the Effect of Tgf-β3 Plasmid on Posterior Frontal Suture Fusion In Vitro

All sutures increased in size in organ culture environment and bridging started on the endocranial side and extended ectocranially. In all groups, sutural tissue appeared healthy without any signs of toxic effects from collagen gel or plasmid DNA. The percent bridging data represent mean values of all the sections in the middle region of the posterior frontal sutures. In this region Tgf-β3 plasmid treated group was found to have 77% to 85% less bony bridging than collagen control and untreated groups after 15 days in culture (Fig. 4.8). Mean percent bridging of the Tgf-β3 plasmid treated group was 3.59 ± 3.21 (mean ± S.D). In contrast, to this mean value many of the sections in the Tgf-β3 plasmid treated group had no bony bridging at all as illustrated in the representative histological sections in Fig. 4.9.

Histomorphometric Analysis of the Effect of Tgf-β3 Plasmid on Posterior Frontal Suture Fusion In Vivo

At 25 days of age (10 days post-implantation) posterior frontal sutures were harvested for histomorphometric, immunohistochemical and micro CT analysis. In all calvariae, bridging began on the dural side and extended ectocranially as observed in the in vitro studies. The percent bridging data represent mean values of all the sections in the middle region (700 μm to 1100 μm) of the posterior frontal sutures. In animals treated with Tgf-β3 plasmid or Tgf-β3 protein, there was a significant reduction in suture fusion in the middle region of the posterior frontal sutures when compared to control groups. In this
region Tgf-β3 plasmid treated group was found to have 70% to 75% less bony bridging than control groups (untreated, sham and collagen plus control plasmid) (Fig 4.10). Although the inhibition of suture fusion was more in the Tgf-β3 plasmid treated group than Tgf-β3 protein group, this difference was not statistically significant. Mean percent bridging of the Tgf-β3 plasmid treated group was 20.91 ± 20.90 (mean ± S.D). Even though there was a dramatic reduction in the suture fusion, these rescued sutures did not show any anatomical abnormalities in the sutural arrangement and morphology (Fig. 4.11).

**Micro CT Analysis of the Effect of Tgf-β3 Plasmid on Posterior Frontal Suture Fusion In Vivo**

Micro CT analysis of suture fusion supported the histomorphometric analysis of mean percent bridging (Fig. 4.12). The profiles of suture fusion among control and treatment groups were similar to that derived from histomorphometric analysis (Fig. 4.13). However, statistical analysis of micro CT data was not performed due to inadequate sample size.

**Immunohistochemistry**

Different isoforms of Tgf-β can have distinct effects using the same receptors. Also, Tgf-βs can regulate the expression and activity of each other, either by regulating different signaling pathways or by regulating the expression of Tgf-β receptors. Therefore, the effects of Tgf-β3 on expression of Tβr-I, Tβr-II and Tgf-β2 was investigated. Previous
studies demonstrated a relationship between apoptosis and suture fusion and patency. Therefore we also examined the effects of Tgf-β3 on the expression of pro-apoptotic and anti-apoptotic proteins.

**Effect of Tgf-β3 on Tβr-I immunoreactivity in Posterior Frontal Sutures**

In fusing or fused posterior frontal sutures, fibroblast, osteoblasts lining dural surface and residual sutural tissues showed an intense immunoreactivity for Tβr-I (Fig.4.14) In contrast, fibroblasts in the suture matrix of animals treated with Tgf-β3 plasmid showed a very low or no immunoreactivity for Tβr-I (Fig.4.14). Tissue sections which were not treated with primary antibody were used as controls and did not show any immunoreactivity for Tβr-I in sutural tissues.

**Effect of Tgf-β3 on Tβr-II immunoreactivity in Posterior Frontal Sutures**

Osteoblasts and fibroblasts lining the residual suture matrices showed a strong staining intensity for Tβr-II in both treated and untreated sutures (Fig.4.15).

**Effect of Tgf-β3 on Tgfβ-2 immunoreactivity in Posterior Frontal Sutures**

Suture matrices of fusing and fused posterior frontal sutures showed strong immunoreactivity for Tgfβ-2 (Fig. 4.16 A, B). Similar levels of immunoreactivity were observed within the osteoblasts lining the osteogenic fronts of the sutures. In contrast to the above finding, immunoreactivity of sutural matrix and cells of the posterior frontal sutures treated with Tgf-β3 plasmid was very low (Fig. 4.16 C, D). Similarly, posterior
frontal sutures treated with Tgf-β3 protein showed very low immunoreactivity for Tgfβ-2 (data not shown). Tissue sections which were not treated with primary antibody were used as controls and did not show any immunoreactivity for Tgfβ-2 in sutural tissues.

**Effect of Tgf-β3 on Bax Immunoreactivity in Posterior Frontal Sutures**

In fusing or fused posterior frontal sutures, fibroblast, osteoblasts lining dural surface and residual sutural tissues showed an immunoreactivity for Bax (Fig. 4.17 C, D) In contrast, osteoblast and fibroblasts in the suture matrix of animals treated with Tgf-β3 plasmid showed a very intense immunoreactivity for Bax (Fig.4.17 A, B).

**Effect of Tgf-β3 on Bcl-2 Immunoreactivity in Posterior Frontal Sutures**

Distribution of Bcl2 expression in sutural tissues treated with Tgf-β3 was different from that of tissues from control animals. Suture matrices of fusing and fused posterior frontal sutures (untreated) showed strong immunoreactivity for Bcl-2 (Fig. 4.18 C, D). Similar levels of immunoreactivity were observed within the osteoblasts lining the osteogenic fronts of the sutures. In contrast to the above findings, Bcl-2 immunoreactivity of sutural matrix and cells of the posterior frontal sutures treated with Tgf-β3 plasmid was very low (Fig. 4.18 A, B).

**4.4. Discussion**

It is well established in several studies that Tgf-β1 and Tgf-β3 suppress cranial suture fusion while Tgf-β2 enhance the suture closure (Lee et al., 2006; Moursi et al., 2003;
Opperman et al., 1997; Opperman et al., 2000). This phenomenon of differential activity of Tgf-β isoforms in cranial suture fusion and patency can be manipulated to intervene in cranial suture fusion in health and disease. In Chapter 3, we have shown that in vitro exogenous application of Tgf-β3 protein can rescue the rat cranial sutures which are destined to fuse. In vivo studies with Tgf-β3 protein in wild type rats and craniosynostotic rabbits have produced similar results (Chong et al., 2003; Opperman et al., 2002b). These results suggest a therapeutic potential of Tgf-β3 in the management of non-syndromic craniosynostosis.

The ability to deliver proteins continuously and limit their spread to adjacent tissues is important in the use of cytokines to prevent resynostosis. A major limitation to direct a long term protein delivery approach is degradation of proteins when exposed to the in vivo environment even when it is delivered in a polymer such as a collagen gel. A gene transfer approach can bypass this issue by promoting the production of specific growth factors at a specific tissue site. It has been reported that sustained release of plasmid DNA and subsequent gene expression in vivo was possible by incorporating plasmid DNA in biomaterials such as poly lactic acid-glycolic acid copolymer (Murphy et al., 2000; Shea et al., 1999), alginate (Aggarwal et al., 1999), chitosan (Lee et al., 1998) and gelatin (Truong-Le et al., 1998). The main aim of the experiments documented in this chapter was to identify, characterize and test an easy to use, biodegradable polymer which can be used as a gene activated matrix to deliver therapeutic non-viral plasmids to sutural tissues in vivo. By combining gene expression profiling data from Chapter 2 with the immunohistochemical findings from this chapter, we gain a better
understanding of possible mechanism by which Tgf-β3 regulates the cranial suture fusion.

Collagen has excellent properties when compared with other biomaterials as a delivery vehicle for therapeutic proteins and cytokines. The same properties make collagen a suitable material for non-viral plasmid delivery. At room temperature, collagen is in a semi-liquid state and when it is implanted it becomes fibrillar and gels at body temperature. Because of this property plasmids can be easily mixed with the collagen gel. The gel nature of the collagen after implantation protects the plasmid DNA from enzymatic destruction and other immunological damage. Collagen is a positively charged molecule and when it interacts with a negatively charged nucleic acid, it forms a collagen/DNA complex.

Results from DNA release and in vitro transfection studies indicate that the collagen vehicle was able to deliver the plasmid intact to the cells for uptake, transfection and production of the protein. Tgf-β3 plasmids released from the collagen gel were able to transfect and generate Tgf-β3 protein in calvarial osteoblasts and sutural tissues. Tgf-β3 production continued to increase throughout the duration of the experiment. This result correlated well with DNA release data and suggests that Tgf-β3 production could remain elevated for an extended period.

Several studies have reported that delivery of DNA from polymers associated with damage to the DNA such as nicking and loss of super coiled structure (Hirosue et al.,
2001; Perez et al., 2001; Wang et al., 1993). This damage is associated with the physical and chemical stress created by the loading or manufacturing process of the plasmids into polymers. Plasmid DNA can be degraded by chemical and physical stress. Plasmid DNA that is not degraded is circular in nature and when it is degraded; its shape becomes a linear strand. Efficiency of gene expression is affected by the shape of the plasmid and loss of its supercoiled structure usually associated with reduction in the efficiency (Ochiya et al., 2001). These changes can be easily examined by agarose gel electrophoresis because of the differences in the rate of migration between different shapes. Dense collagen gel used in this study does not need any special manipulations to incorporate plasmid DNA in its matrix. This is an advantage as it will reduce the damage to the plasmid DNA. This has been demonstrated by the migration profile in the gel electrophoresis.

We have demonstrated that a non-viral Tgf-β3 plasmid delivered in a sustained manner is capable of enhanced Tgf-β3 protein production in the sutural tissues. Results from in vitro and in vivo experiments showed that this plasmid-encoded production of Tgf-β3 cytokine resulted in increased biological activity and rescued the posterior frontal futures from its programmed fusion. To test the ability of Tgf-β3 to abrogate the suture fusion in a situation similar to craniosynostosis, we intervened at the age of day 15, when the suture fusion has already started and active. These data demonstrate for the first time that gene transfer of non-viral plasmid DNA to the sutural tissues can prevent programmed cranial suture fusion. Although there is a significant reduction in the suture fusion, there was at least some suture fusion in all the animals treated with Tgf-β3. This
may be due to the fact that some degree of suture fusion had occurred by postnatal day 15 when the collagen gels were implanted. Also, this may be possibly due to compensatory changes in other growth factors implicated in suture fusion.

Opperman and co-workers have shown that cultured calvaria and a rat model treated with recombinant Tgf-β3 protein were rescued from sutural fusion (Opperman et al., 1999; Opperman et al., 2000; Opperman et al., 2002a; Opperman et al., 2002b). In our in vivo study, plasmid encoded Tgf-β3 produced more inhibition of posterior frontal suture fusion than locally delivered exogenous recombinant Tgf-β3. Even though this difference was not statistically significant, it could be hypothesized that endogenously produced cytokines are more biologically effective than recombinant proteins. Makarov and coworkers reported that locally expressed secreted interleukin 1 receptor antagonist (sIL-1ra) was about 4 times therapeutically efficient than recombinant sIL-1ra (Makarov et al., 1996). Similar findings have been reported in another study of intra-articular gene transfer of human interleukin-1 receptor antagonist protein (IRAP) to antagonize the intra-articular actions of interleukin-1 (Hung et al., 1994).

Tgf-β3 regulation of cranial suture fusion may be mediated by a number of mechanisms. Tgf-βs are potent regulators of osteoblast migration, proliferation and differentiation. Despite reports claiming that cell proliferation plays a key role in the pathogenesis of craniosynostosis, the role of programmed cell death and cellular proliferation in premature fusion remain poorly understood and controversial. Reports exist in the literature to support either an increase or decrease in cell proliferation in syndromic
craniosynostosis (De Pollack et al., 1996; Lemonnier et al., 2001; Lomri et al., 1998; Mansukhani et al., 2000; Zhou et al., 2000). Recent studies demonstrate that Tgf-β2 induced suture obliteration in cultured fetal mouse and rat calvaria was accompanied by elevated levels of cell proliferation (Lee et al., 2006; Opperman et al., 2000). This increased cell proliferation and cranial suture closure was mediated by Erk-MAPK pathway (Lee et al., 2006; Opperman et al., 2006). Tgfβ-2 inhibits apoptosis in human osteoblasts and decreases the Bax/Bcl-2 ratio (Hay et al., 2001).

It is believed that Tgf-β3 regulates suture patency by regulating suture cell proliferation and apoptosis directly or indirectly through other Tgf-β isoforms (Opperman et al., 2000). Rescue of sutures from obliteration by addition of Tgf-β3 to cultured calvaria was associated with decreased levels of cell proliferation (Opperman et al., 2000). In our study we examined the effect of Tgf-β3 on the expression of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins. Overexpression of Bax was seen in the sutural tissues rescued by the Tgf-β3. Increased expression of Bcl-2 was seen in the untreated fusing or fused sutures. These results showed increased apoptosis could be associated with delayed suture closure. Similar findings were reported by Opperman and coworkers where they examined the apoptosis by presence of apoptotic cells in the histological sections (Opperman et al., 2000).

Tgf-β2 and Tgf-β3 have antagonistic effects in the regulation of suture morphogenesis and fusion (Opperman et al., 2000). They share the same transmembrane receptors and
downstream signalling pathways (Cohen, 2003). We examined the effect of Tgf-β3 on the expression of Tgf-β2, Tβr-I and Tβr-II in the rescued and fusing sutures. Tgf-β3 plasmid treatment was associated with differential distribution of Tgf-β2 and Tβr-I, but not Tβr-II, in sutural cells. Tgf-β3 exposure reduces the expression of Tgf-β2 in the sutural tissues of posterior frontal sutures when compared to untreated posterior frontal sutures. Tgf-β3 gene transfer decreased the expression of Tβr-I in the sutural tissues of rescued posterior frontal sutures in comparison to untreated posterior frontal sutures. We did not see any difference in the expression of Tβr-II between Tgf-β3 treated and untreated sutures. Similar findings were reported by Opperman and coworkers in an in vivo study in rats (Opperman et al., 2002b). Our results confirm the previous findings that Tgf-β3 regulates the suture fusion by its effects on apoptosis and by the regulation of the activity of other growth factors such as Tgf-β2 and their receptors.

In conclusion, collagen gel can be formulated to provide sustained release of non-viral plasmid DNA which maintains a substantial transfection capacity. This sustained release of plasmid resulted in prolonged osteoblast transfection and elevated Tgf-β3 protein production. Tgf-β3 is an important regulator of suture fusion and an increase in plasmid-encoded Tgf-β3 protein was found to be effective in inhibiting programmed suture fusion in rats. Together, these findings indicate this approach may have therapeutic potential to achieve localized and controlled, non-viral gene delivery in vivo.
Figure 4.1: Full length human Tgf-β 3 cDNA cloned into a mammalian expression vector.
Figure 4.2: Tgf-β3 plasmid release from collagen gel.

Error bars represent standard deviation.
Figure 4.3: DsRed (A) and GFP (B) plasmid delivered via collagen gel transfects calvarial osteoblasts. Visualized by fluorescent microscopy 24 hours after placement of collagen gel (original magnification: 100X).
Figure 4.4: GFP plasmid delivered via collagen gel transfects calvarial tissue.

Visualized by laser scanning confocal microscope 10 days after placement of collagen gel.
Figure 4.5: Plasmid-encoded Tgf-β3 production in osteoblast culture as measured by ELISA. Error bars represent standard deviation.
Figure 4.6: Tgf-β3 plasmid delivered via collagen gel transfects calvarial tissue.

Immunohistochemistry for Tgf-β3, Visualized by Laser scanning confocal microscope 15 days after placement of collagen gel.
Figure 4.7: Plasmid encoded Tgf-β3 protein is biologically active as measured by mink lung epithelial (MLE) assay. Data presented as fluorescence intensity which is directly related to proliferation of MLE cells. Error bars represent standard deviation. Untreated: untreated MLE cells; rhTgf-B3: MLE cells treated with recombinant human Tgf-β3 protein at $10^3$ pg/ml; Control conditioned media: MLE cells treated with media collected from osteoblasts treated with collagen only; B3 conditioned media: MLE cells treated with media collected from osteoblasts transfected with Tgf-β3 plasmid in collagen gel.
Figure 4.8: Non-viral Tgf-β3 plasmid delivered in a collagen gel inhibits posterior frontal suture fusion in vitro

Compiled data from three independent experiments are expressed as mean percent bridging. Bars represent mean ± SEM. Asterisk denotes statistically significant difference between groups (p < .05).
Figure 4.9: Micrograph showing bony bridging of collagen plus control plasmid (A) and collagen plus Tgf-β3 plasmid (B) after 15 days in culture.

Both sections are from the midpoint of the posterior frontal suture. (Original magnification: 200 X).
Figure 4.10: Non-viral Tgf-β3 plasmid delivered in a collagen gel inhibits rat posterior frontal suture fusion in vivo.

Compiled data from four independent experiments are expressed as mean percent bridging. Bars represent mean ± SEM. Asterisk denotes statistically significant difference between groups (p < .05). Note that data is from sections.
Figure 4.11: Micrograph of calvariae 10 days after collagen gel implantation.

All the sections are from the midpoint of the posterior frontal suture. BH = bridging height, SG = suture gap. (Original magnification: 200 X).
Figure 4.12: Micro CT image showing bony bridging of untreated (A) and collagen plus Tgf-β3 plasmid (B) 10 days after implantation.

Arrows indicate suture bridging (A) and suture gap (B).
Figure 4.13: Non-viral Tgf-β3 plasmid delivered in a collagen gel inhibits rat posterior frontal suture fusion in vivo.

Non-viral Tgf-β3 plasmid delivered in a collagen gel inhibits rat posterior frontal suture fusion in vivo. Graph showing mean percent bridging of posterior frontal sutures, as measured in micro CT images. The values are given as the mean and standard deviation (n=2 to 4 rats). Note that measurements are from micro CT.
Figure 4.14: Micrographs of Tβr-I immunoreactivity in posterior frontal sutures.

Low (A) and high (B) – power micrographs of untreated posterior frontal sutures. Low-
(C) and high (D) – power micrographs of posterior frontal sutures treated with Tgf-
β3 plasmid. Osteoblasts in the residual sutural tissues are immunoreactive to Tβr-I (large
arrows). Osteoblasts in the peristeum and treated sutures are non reactive (small
arrows).
Figure 4.15: Micrographs of Tβr-II immunoreactivity in posterior frontal sutures

Low (A) and high (B) – power micrographs of posterior frontal sutures treated with Tgf-β3 plasmid. Low- (C) and high (D) – power micrographs of untreated posterior frontal sutures. Osteoblasts in the residual sutural tissues are immunoreactive to Tβr-II (large arrows).
Figure 4.16: Micrographs of Tgfβ–2 immunoreactivity in posterior frontal sutures

Low (A) and high (B) – power micrographs of untreated posterior frontal sutures. Low-(C) and high (D) – power micrographs of posterior frontal sutures treated with Tgf-β3 plasmid. Osteoblasts in the residual tissues of the untreated sutures are intensely immunoreactive (large arrows). Osteoblasts in the periosteum and sutural fibroblasts in the sutures treated with Tgf-β3 plasmid are non-reactive to Tgf-β2 (small arrows).
Figure 4.17: Micrographs of Bax immunoreactivity in posterior frontal sutures

Low (A) and high (B) - power micrographs of posterior frontal sutures treated with Tgf-β3 plasmid. Low- (C) and High (D) – power micrographs of untreated posterior frontal sutures. Osteoblasts and sutural tissues are intensely immunoreactive to Bax in sutures rescued by Tgf-β3 plasmid (large arrows). Osteoblasts and sutural tissues in the untreated sutures are nonreactive (small arrows).
Figure 4.18: Micrographs of Bcl-2 immunoreactivity in posterior frontal sutures

Low (A) and high (B) – power micrographs of posterior frontal sutures treated with Tgf-β3 plasmid. Low- (C) and high (D) – power micrographs of untreated posterior frontal sutures. Osteoblasts and residual sutural tissues are intensely immunoreactive to Bcl-2 in untreated sutures (large arrows). Sutural tissues and osteoblasts in the sutures rescued by Tgf-β3 show low immunoreactivity to Bcl-2 (small arrows).
CHAPTER 5

CONCLUSIONS

Cranial suture fusion is a multifactorial process regulated by several signaling molecules and environmental factors such as tissue expansile forces produced by the growing brain. This dissertation began with a description of studies examining the temporal expression of several growth factors and cognate receptors in posterior frontal (PF) and sagittal (SAG) sutures in rats. These studies also illustrated the time line of molecular events associated with suture patency and suture fusion.

Gene expression studies of mitochondrial apoptotic pathways indicated that apoptosis is associated with the maintenance of suture patency. There are very few studies in the literature which describe the expression of the apoptosis-regulating molecules Bax and Bcl-2 in cranial sutures. Our results indicate that maintenance of apoptosis is essential to preserve the suture patency as indicated by increased expression of Bax in the SAG sutures compared to PF sutures. Similar findings were reported in an immunohistochemical study of the apoptotic marker Bcl-10 in mouse (Agresti and Gosain, 2005). In this study, an increased number of apoptotic cells were observed in the
leading edges of sagittal sutures compared to posterior frontal sutures. These findings were further confirmed by results from the immunohistochemical studies described in Chapter 4. In these studies, PF sutures which were rescued by the Tgf-β3 treatment showed upregulation of Bax as compared to fusing sutures.

Tgf-β3 has a pro-apoptotic role in several tissues as demonstrated in Tgf-β3 (+/-) heterozygous mice where programmed cell death was significantly reduced (Dunker et al., 2002). Bcl-2 expression was upregulated in Tgf-β3 (+/-) heterozygous mice (Dunker et al., 2002). We also found an upregulation of the anti-apoptotic marker, Bcl-2, in the PF sutures as compared to sagittal sutures. However, immunoreactivity for Bcl-2 was down regulated in the PF sutures treated with Tgf-β3 plasmid.

Recently it has been shown that, Tgf-β2 induced suture fusion was associated with cellular proliferation (Lee et al., 2006). These findings confirm that programmed suture fusion is associated with cell proliferation or down regulation of apoptosis. In contrast to our findings, in a study using both fluorescent and technetium -99m-labelled annexin V (an in vivo marker of apoptosis) to image the apoptotic activity during suture fusion in rats, Fong and coworkers did not find any difference in the apoptotic activity between PF and SAG sutures. However, there was an increased apoptotic activity in both sutures during the period of suture fusion (Fong et al., 2004). They did not find any difference in the expression of Bax between PF and SAG sutures but did find an increase in Bcl-2 expression in PF sutures as measured by microarray analysis. However, the authors acknowledged that these results need to be validated by quantitative real time RT-PCR
studies (Fong et al., 2004). In conclusion, gene expression and immunohistochemical findings suggest a definite role for apoptosis in the maintenance of suture patency.

Bmps are involved in the regulation of balance between the undifferentiated and differentiated states of osteogenic cells (Kim et al., 1998). Our findings indicated that both mesenchyme showed a peak in Bmp4 expression on day 14. However, Bmp4 expression levels were higher in the PF sutures than in SAG sutures. A similar finding was reported by Law and co-workers in a study in rats (Law et al., 2005). Presence of Bmp4 in fusing and patent sutures suggests that there may be a suture-specific regulation of Bmp activity by secreted Bmp antagonists (Warren et al., 2003). Bmp3 has anti-osteogenic effects and can inhibit the activity of Bmp4 (Daluiski et al., 2001). In our study, very little difference was seen in the expression of Bmp3, between PF and SAG sutures. Levels of Bmp3 were elevated in the posterior frontal suture before the onset of fusion and decreased during the period of fusion. Similar finding were reported by Nacamuli and coworkers in a rat study using real time RT-PCR and microarray (Nacamuli et al., 2005). Taken together, this data suggest that there may be other inhibitors of Bmp4 in addition to Bmp3, such as Noggin, involved in the maintenance of suture patency. Similar Bmp profiles in fusing and non-fusing sutures suggest that suture fusion is a default process in all the sutures and other anti-osteogenic factors are necessary to counteract this process and maintain suture patency (Nacamuli et al., 2003).

Fgfr-1 is associated with initiation of osteoblast differentiation (Iseki et al., 1999). Expression of Fgfr-1 was significantly higher in PF sutures as compared to SAG sutures.
in most of the time points examined. Law and coworkers reported a similar finding using semi-quantitative gene profiling in rats (Law et al., 2005). Also, a similar pattern of expression was reported in a real time RT-PCR study in mice (Nacamuli et al., 2004). Upregulation of Fgf-2 derived from dura mater has been associated with programmed PF suture fusion (Mehrara et al., 1998; Most et al., 1998). In addition, Fgf-2 is known to upregulate the expression of Fgfr-1 (Mathy et al., 2003). Therefore, Fgf-2 secreted from the dura mater may be responsible for the upregulation of Fgfr-1 expression in PF sutures at the onset of suture fusion.

Receptors for Tgf-βs are present in suture fibroblasts and osteoblasts. At the beginning of the PF suture fusion; there was an approximately four-fold difference in the expression of Tβr-I between PF and SAG sutures. This difference in expression decreased subsequently and there was more Tβr-I expression in SAG than in PF suture around 20 to 22 days. A different pattern of expression was observed for Tβr-II. Both SAG and PF sutures demonstrated high levels of Tβr-II transcripts at early time points, which subsequently reduced over the time. A similar distribution of Tβr-I and Tβr-II was reported in studies by Law and coworkers (Law et al., 2005). In contrast, Mehrara and coworkers reported an increased immunoreactivity of Tβr-I and Tβr-II in the osteoblasts of the sutural margin of PF sutures during suture fusion as compared with that of SAG sutures (Mehrara et al., 1999). However, there was no difference in Tβr-I and Tβr-II expression patterns in PF and SAG sutures before or during the active PF suture fusion. This difference in the findings between gene expression and immunohistochemical studies
indicate the need to supplement the real time RT-PCR gene expression studies with in-situ hybridization or immunohistochemistry to localize the gene expression pattern. Near constant levels of Tβr-III expression in both sutures in all the time points studied indicates its minimal involvement in the signal transduction of Tgf-β mediated suture fusion.

Real time RT-PCR was used to measure the transcript levels of growth factor ligands and receptors. This methodology measures the mRNA levels only and it is one of the limitations of this technique. Since there is an extensive post-transcriptional and post-translational processing of transcripts, there may be a difference between mRNA levels and secreted cytokines or cell surface receptor levels. To compare this difference in mRNA and protein levels, real time RT-PCR studies need to be supplemented with western blot assays or protein arrays. Since numerous reports indicate a role for regional dura mater in the regulation suture patency, future studies will also need to quantitatively describe the gene expression pattern within dura mater to get a complete sequence of events.

We have identified and characterized a biocompatible biopolymer for the localized sustained delivery of cytokines. This FDA-approved dense collagen gel does not need any special manipulation to incorporate the cytokines in its matrix. This collagen has been in clinical use for soft-tissue augmentation for over 25 years. Cytokines such as Tgf-β3 can be readily incorporated into this dense collagen gel allowing for a sustained and localized release at the implant site. Results from our organ culture study indicated that
the released Tgf-β3 protein was bioactive and capable of rescuing cranial suture fusion in vitro. Results from this study indicated that fusion of cranial suture fusion can be modulated by manipulating these cytokines, as reported previously by others (Chong et al., 2003; Opperman et al., 2002b). This collagen gel/cytokine delivery system can retain and release cytokine over a two week period. However, in the future it will be necessary to study its release pattern over an even longer time period as the duration of cytokine release needed in a human clinical situation is much longer.

Delivery of Tgf-β3 could be accomplished using either the protein directly or a gene transfer approach. The localized delivery of cDNA, which can be administered locally in a mammalian expression vector, is taken up by the sutural fibroblasts and osteoblasts and the transgene product is expressed. We developed and tested a gene delivery system based on a dense collagen gel to deliver non-viral plasmids which, encode for therapeutic proteins. The profile of non-viral plasmid release from this gel during a period of two weeks was characterized by well-established methods. Initial studies demonstrated that a non-viral plasmid encoding fluorescent proteins can be delivered in a localized, sustained manner in an organ culture environment. Released plasmids retained their structural integrity and maintained their biological activity for transfection. These studies were followed by Tgf-β3 gene transfer studies. We demonstrated that a non-viral plasmid encoding Tgf-β3 delivered in a localized, sustained manner can significantly increase Tgf-β3 cytokine activity. This increased Tgf-β3 cytokine activity resulted in the reduction of PF suture fusion in rats, both in vitro and in vivo.
Histological examination of treated PF sutures revealed normal suture morphology and sutural tissue arrangement. Based on this observation, it could be speculated that the Tgf-β3 treatment effect was limited to the osteoblasts, which mainly affected the mineralization of sutures. The in vivo study was conducted during a period of 10 days. Tgf-β3 cDNA used in the present study was spliced to a cytomegalovirus (CMV) promoter for high levels of transgene expression. However, this expression is not well sustained. Duration of transgene expression can be lengthened by utilizing mammalian promoters which are associated with certain housekeeping genes that are constantly expressed in mammalian cells. The duration of expression of transgenes can also be controlled by incorporating inducible promoters into the gene cassette construct. This will allow for stricter regulation of gene expression by exogenously administered substances such as tetracycline.

One of the problems associated with in vivo gene transfer is targeting the gene to specific tissues. Gene expression can be targeted to the sutural fibroblasts and osteoblasts by placing tissue specific promoters into the gene cassette which will limit the gene expression to these tissues only. Therefore, future studies need to be carried out with gene cassettes containing promoters for temporal and spatial regulation of gene expression. This will enhance our ability to limit the Tgf-β3 gene expression to sutural tissues, during a desired window of time. Further studies with a longer duration need to be carried out to evaluate this gene delivery system’s ability to rescue suture fusion and maintain its patency for a long period. It is also important to determine the long term effects of Tgf-
β3 on suture morphology and the effects of supra-physiological doses of Tgf-β3 on other cranial tissues.

The above findings are relevant to therapeutic applications in terms of amounts and persistence of produced gene products. Delivery of other genes, such as Noggin, which are known to be associated with suture patency, can be combined with Tgf-β3 plasmid delivery. This combination of factors theoretically should act synergistically and increase the inhibition of suture fusion, compared with individual factors alone. In the in vivo study, it was assumed that plasmid release and transfection was localized to the suttural tissues and the periosteum only. However, future studies could utilize Tgf-β3 plasmid with a reporter construct (e.g. Tgf-β3 cDNA inserted into the pIRES2-EGFP plasmid) to monitor the transfection efficiency and pattern of protein production in tissues.

Future studies that preserve the integrity of the plasmid DNA incorporated into the collagen gel will further increase the level of gene expression attainable by these gene delivery vehicles. Currently, several biomaterials and polymers are being tested for gene delivery applications. Most of these materials need to be chemically modified to improve its gene transfer ability and these may affect the plasmid integrity, in addition to the safety concerns. Collagen gel used in this study can be used without any modifications such as cross-linkage. This study provided a rational basis for the development of collagen gel-based matrices for the controlled release of therapeutic levels of non-viral plasmid DNA for craniosynostosis.
It is important to establish the mechanism by which Tgf-β3 rescues PF suture fusion before this strategy can be considered for therapeutic applications. Tgf-β3 plasmid treatment was associated with increased apoptosis as demonstrated by immunohistochemistry. There was an upregulation of pro-apoptotic Bax expression and down regulation of anti-apoptotic Bcl-2 expression in sutures treated with Tgf-β3. This increased immunoreactivity of Bax was localized to the osteoblasts lining the PF sutural margin. This suggests that apoptosis of the osteoblasts reduced osteogenesis in the sutures and maintained patency. Plasmid-mediated upregulation of Tgf-β3 protein produced a differential distribution of Tgf-β2 and Tβr-I, but not Tβr-II, in sutural cells. Tgf-β2 is known to induce cranial suture fusion and Tgf-β3-induced down regulation of Tgf-β2 can help to maintain the suture patency. Tgf-β3 treatment down regulated the expression of Tβr-I in rescued sutures. Tgf-β3 is known to down regulate the expression of Tβr-I (Opperman et al., 2002a). Similar immunoreactivity pattern of Tgf-β2, Tβr-I and Tβr-II was reported by Opperman and coworkers in the rat PF sutures treated with Tgf-β3 protein in vivo (Opperman et al., 2002b). Recently it has been shown that repeated exposure to Tgf-βs suppresses the Tβr-I expression by differentiated osteoblasts (Kim et al., 2006). In conclusion, a working hypothesis for the mechanism of Tgf-β3-induced suture maintenance is that Tgf-β3 induces apoptosis through the upregulation pro-apoptotic Bax expression and the reduction of osteogenesis through the down regulation of Tgf-β2 and Tβr-I.
To summarize, identification of growth factors and receptors required for rat cranial suture fusion and elucidation of their temporal patterns of expression help to identify possible targets and time windows by which to effectively modulate suture fusion. These new findings, combined with recent developments in gene therapy and tissue engineering, will enhance the development of adjunct therapies to prevent post-operative resynostosis.


Loeys BL, Chen J, Neptune ER, Judge DP, Podowski M, Holm T, Meyers J, Leitch CC, Katsanis N, Sharifi N, Xu FL, Myers LA, Spevak PJ, Cameron DE, Backer JD,


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