The Role of Dlx5 in Murine Cranial Suture Fusion

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in
Murine Cranial Suture Fusion

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ABSTRACT

Objective: The genetic etiology for the majority of sutural growth disturbances, in humans, is unknown. Therefore, the development of our knowledge of the genes involved in suture fusion is essential for progress in the treatment of individuals with cranial suture fusion disorders. In this study, we have examined Distal-less-5 (Dlx5), a homeobox gene, which is required for Bone Morphogenic Protein (BMP)-mediated osteoblast differentiation and is thought to play an important role in the development of mineralized tissues. The significance of Dlx5 in these biologic processes has developed interest in the role of this gene in cranial suture fusion. Methods: We examined the expression of Dlx5 in the fusing and non-fusing cranial sutures of wild-type (wt) mice by RT-PCR and immunohistochemistry. In order to determine the role of Dlx5 in the cranial sutures, the Sagittal (S) and posterior frontal suture (PFS) were analyzed from Dlx5 +/- and wild-type mice at postnatal day 10. To determine if Dlx5 +/- mice had a decrease in osteoblast differentiation in the cranial sutures, Dlx5 +/- and wt mice were bred with mice transgenic for the 2.3 kb fragment of the collagen Type I promoter fused to a Green Fluorescent Protein (2.3 GFP). The 2.3 GFP promoter has been shown to be an accurate marker of differentiated osteoblastic cells (Kalajzic et al., 2002). Results: Dlx5 was abundantly expressed in the S and PFS during sutural maturation (postnatal day (10-45) and there was an increase in the expression of Dlx5 in the posterior frontal suture (PFS) compared to the sagittal suture (S) at day 35. At day 10, there was an increase in space (p< 0.05) between the parietal bone fronts in the sagittal suture from Dlx5 +/- compared to wt mice. Conclusion: Genes involved, or thought to be involved, in cranial suture fusion are of great interest among those studying both normal and
abnormal calvarial development. Knowledge of the intricate processes and genes responsible for proper growth of the cranium is essential to finding therapies for patients who are affected by disorders involving abnormal sutural fusion, namely craniosynostosis. Evidence that a Dlx5 signaling pathway is involved in suture fusion, may make available a new candidate gene for analysis in patients with these sutural growth disturbances.
INTRODUCTION

Cranial sutures function as the major sites of bone expansion during postnatal craniofacial growth and allow development of the skull to accommodate rapid growth of the brain. For sutures to function as growth sites, they need to remain unossified, and simultaneously allow formation of new bone at the edges of the overlapping bone fronts. Too little or delayed bone growth at these sutures results in wide-open fontanels, whereas too much or accelerated bone growth results in osseous obliteration of the sutures (Opperman 2000).

Despite the obvious differences in human and murine craniofacial characteristics, there is a marked similarity and conservation in the molecular pathways for the assembly and growth of the cranial vault between the two species (Warren, Greenwald et al. 2001). In mice, all the sutures of the cranial vault remain patent for the life of the animal; except for the posterior frontal suture (PFS), which fuses in a predictable manner between 21-45 days postnatal (Opperman 2000). In humans, the equivalent metopic suture fuses within the first three years postnatal (Vu, Panchal et al. 2001; Weinzweig, Kirschner et al. 2003). Therefore, the murine PFS serves as an excellent model of post-natal human sutural development (Warren, Greenwald et al. 2001).

BMP signaling has been linked to murine PFS fusion, and is expressed in developing (Kim, Rice et al. 1998) and in adult murine cranial sutures (Warren, Brunet et al. 2003). In fact, Warren et al. showed that inhibition of the BMP signaling pathway, by adenovirus mis-expression of the BMP antagonist Noggin, blocks murine
PFS fusion in vivo (Warren, Brunet et al. 2003). However, BMP added to murine calvarial organ culture sutures did not cause sutural fusion (Kim, Rice et al. 1998). Taken together, these results suggest that BMP is necessary but not sufficient for murine calvarial suture fusion.

Many extracellular and intracellular proteins, such as Dlx5, regulate the BMP signaling pathway. In an avian calvarial explant culture system, Dlx5 transcription was upregulated by BMP and inhibited by the BMP antagonist Noggin (Holleville, Quilhac et al. 2003) which proved that Dlx5 acts downstream from BMP signaling. In subsequent investigations of osteoblastic cell cultures, it was shown that BMP causes an increase in the expression of Dlx5 (Miyama, Yamada et al. 1999; Takagi, Kamiya et al. 2004), and that regulation of osteoblast differentiation by BMP is dependent on Dlx5. Others showed, that in osteoblast-like cells, BMP can induce markers of osteoblast differentiation including alkaline phosphatase (Kim, Lee et al. 2004), Runx2 (Lee, Kim et al. 2003), and Osterix (Lee, Kwon et al. 2003) all of which have been shown to be dependent upon Dlx5.

**RATIONALE**

The incidence of craniosynostosis in the United States is about .04-.1%. Although this developmental disorder is rare, it may result in severe malformations of the cranial vault and in the more harshly affected, increased pressure on the brain. The most detrimental consequences described are mental impairment, loss of hearing, and poor vision. The severity of these complications requires that infants, sometimes at a
young age, receive several surgeries to avoid further damage and to correct the deformity.

The genetic etiology for the majority of sutural growth disturbances in humans is unknown. Providing evidence that a Dlx5 signaling pathway is involved in sutural fusion, may make available a new candidate gene for analysis in patients with sutural growth disturbances.

**HYPOTHESIS**

In this study, we plan to examine the role of the transcription factor, Dlx5, in controlling murine cranial suture development and growth. We hypothesize that Dlx5 expression will be increased in the murine PFS during the time of posterior frontal suture fusion and that there will be a decrease in suture fusion in the PFS in heterozygous Dlx5 (+/-) mice.

**SPECIFIC AIMS/OBJECTIVES/PREDICTIONS**

Aim 1. **Examine the expression and localization of Dlx5 in the posterior frontal and the sagittal cranial sutures.**

The posterior frontal suture in mice fuses between days 21 and 45 postnatally. Our working hypothesis is that Dlx5 expression will increase in the PFS during the period of expected suture fusion (days 21-45 postnatal) relative to that in the non-fusing sagittal suture. We plan on testing this hypothesis by examining expression levels of
Dlx5 by RT-PCR and its localization by immunohistochemistry in the posterior frontal and sagittal sutures from CD-1 mice postnatal day 21, 25, 35, and 45.

**Aim 2. Examine the posterior frontal and sagittal sutures of 10 day-old wild-type and heterozygous Dlx5 (+/-) mice.**

Previous studies have shown that mice deficient in both alleles (Dlx5 -/-), die in utero, therefore we plan on examining heterozygous Dlx5 +/- mice. In order for PF sutural fusion to occur, the bone fronts of the frontal bone must come in contact with each other, which occurs prior to PFS sutural fusion (postnatal day 21-45). Therefore, our objective is to determine the extent of bone front spacing in the posterior frontal and sagittal sutures of 10 day-old wild-type and heterozygous Dlx5 (+/-) mice. Our working hypothesis is that there will be an increase in the space between bone fronts in the PFS in heterozygous Dlx5 (+/-) mice.

**BACKGROUND**

**A. Cranial Sutures**

The skull vault is composed of paired frontal, parietal, squamosal portions of the temporal bones, and the anterior portion of the occipital bone. (Couly *et al.*, 1993; Jiang *et al.*, 2002; Noden, 1978, 1988). These bones form the calvaria and develop through intramembranous ossification; whereas, the bones that form the base of the skull develop through endochondral ossification (Morriss-Kay and Wilkie 2005).
In vertebrates, the skull develops from either neural crest, or mesodermal tissues. Chai et al. and Jiang et al., incorporated markers in mice that enabled visualization of the migration of cranial neural crest cells during development (Chai et al., 2000 and Jiang et al. 2000, 2002). These studies showed that neural crest cells emigrate as three separate populations: the trigeminal crest, the hyoid crest, and the vagal crest. The trigeminal crest migrates to the frontonasal and first branchial arch regions and provides the neural crest component of the trigeminal ganglion; this is the only population of neural crest cells that contributes to the skull.

Cranial sutures separate the bones of the calvariae and form as the bony edges meet. Growth at these sutures occurs perpendicular to the orientation of the suture; normally, it coincides with the expansion of the brain (Morriss-Kay and Wilkie 2005). At birth, the cranial sutures are patent and the flexibility that results allows the bones of the cranium to overlap as the baby passes through the birth canal. Another significant task of the cranial sutures is their role as the primary sites of bone growth during craniofacial development. This is most notable during the period when the brain is rapidly expanding (Baer, 1954).

The human metopic suture undergoes fusion during the first three years and all of the other cranial sutures remain patent well into adulthood (Weinzweig et al., 2003). In the mouse, the PFS, equivalent to the metopic or interfrontal suture in humans, fuses in the first 45 days of life. However, all of the other sutures, including the sagittal and coronal, remain patent (Warren et al. 2001). Similarities between mice and humans in craniofacial development, the specific molecules involved, as well as the structure of those molecules make the murine posterior frontal suture an excellent model for the
study of human suture development and pathology (Richtsmeier et al., 2000; Schneider et al., 1999; Waterston et al., 2002; Wilkie and Morriss-Kay, 2001).

B. Dura

The presence of the dura mater has been found to be essential for normal development and maintenance of cranial vault sutures (Opperman et al., 2005). In fact, the ability of neonatal dura mater to regenerate bone has been observed clinically and demonstrated in several animal models (Lenton et al., 2005). For example, it has been shown that the presence of intact dura is required for regeneration of the skull vault after removal of a small area of the calvaria in infants and young animals (Babler et al., 1982; Mabbutt and Kokich, 1979; Mabbutt et al., 1979; Mossaz and Kokich, 1981). These findings and the intimate association between the dura mater and cranial sutures, initiated investigations by others, to determine a role for dura mater in suture development and fate (Lenton et al., 2005).

Opperman et al. transplanted embryonic and postnatal coronal sutures with and without dura mater to the midparietal bones of syngeneic adult rats and showed that both embryonic and postnatal suture explants fused in the absence of their original, suture-associated dura mater. On the other hand, explants of both sutures transplanted with their dura mater intact remained patent (Opperman et al., 1993). Similar results were obtained when the embryonic sutures were placed in an in vitro organ culture system, with intact sutures remaining patent and those lacking their dura mater fusing (Opperman et al., 1995). These results suggested that factors and/or physical signals from the dura mater associated with the coronal suture are responsible in some way for
maintaining suture patency in the late embryonic and early postnatal stages of development.

In subsequent studies, Opperman and colleagues added conditioned media from dura mater cultures to coronal suture explants placed in organ culture without their suture-associated dura mater. They showed that this conditioned media was capable of preventing suture fusion. When the media was divided into heparin and non-heparin binding fractions, only the heparin-binding was capable of maintaining coronal suture patency (Opperman et al., 1996). These results suggested that heparin-binding growth factors, such as FGFs and Transforming Growth Factor-β (TGF-β) superfamily members, were responsible for differences in coronal suture fate (Opperman, et al., 1996).

The role of the dura mater in maintenance of suture patency has also been demonstrated in the sagittal suture (Kim et al., 1998). Embryonic (E16) murine sagittal suture explants that were cultured without their dura mater, fused within three days; this contrasted with control explants, cultured with their dura intact, that remained patent. However, no difference was demonstrated in the patency of sagittal suture explants from postnatal (P1) mice cultured with and without their associated dura mater (Kim et al., 1998). This prompted investigators such as Kim et al. to study the expression of genes known to act in conserved signaling pathways throughout development. Different patterns of expression of these genes were found at the end of embryonic development in the osteogenic fronts of the sagittal suture. This suggested that different molecular mechanisms are responsible for suture morphogenesis during the embryonic and postnatal stages of development and lead to the hypothesis that in the murine sagittal
suture, the dura mater is responsible for maintaining suture patency prior to birth, while molecular signals from the osteogenic bone fronts are the principal regulators of suture patency during the neonatal stages of development (Kim et al., 1998).

Other studies have focused on the effects of removal or disruption of the dura on PFS fusion. Separating the dura mater from the overlying PFS either temporarily or by placement of an impermeable silicone membrane, resulted in a significant delay in fusion of the PFS and suggested that there are interactions between the PFS and the dura during the process of PFS fusion (Roth et al., 1996).

Verification that molecules originating in the dura mater were responsible for coronal and sagittal suture patency, as well as PFS fusion, led to the hypothesis that the dura mater has a specific “make-up”, based upon it’s location, and is therefore capable of coordinating the differing fate of each cranial suture (Lenton et al., 2005). Levine et al. (1998) tested this hypothesis by removing a portion of the calvariae, in rats, containing both the posterior frontal and sagittal sutures without the dura mater and rotated it 180°. This placed the dura that was previously associated with the PFS in contact with the sagittal suture and vice versa. The new position resulted in obliteration of the sagittal suture which normally remains patent and sustained patency of the normally fused PFS in vivo.

The results of these experiments clearly indicate a role of the dura mater in determining cranial suture fate.

C. Disturbances in Suture Fusion

For sutures to function as growth sites, they need to remain patent, and allow new
bone to be formed at the edges of the overlapping bone fronts. Too much or accelerated bone growth results in osseous obliteration of the sutures; whereas too little or delayed bone growth results in wide-open fontanels (Opperman 2000).

1. Craniosynostosis, or premature fusion of the cranial sutures, results in an abnormal skull shape, and may cause severe deformities such as blindness and mental retardation (McCarthy et al., 1989). Although the majority of craniosynostoses are nonsyndromic, more than 100 different syndromes have been identified that include craniosynostosis as one of their many characteristics (Wilkie, 1997). Because craniosynostosis is such a common finding among syndromes and may result in severe deformities, it is important to study the mechanisms and roles of the molecules involved in cranial suture fusion in an effort to develop therapies to treat the disorder.

In the last ten years, some of the more common syndromes of these have been linked to dominant mutations in genes encoding Fibroblast Growth Factor Receptors (FGFR1, FGFR2, and FGFR3), and the transcription factors TWIST and Msx-2 (Bellus et al., 1996; El Ghouzzi et al., 1997; Howard et al., 1997; Jabs et al., 1994, 1993; Oldridge et al., 1995; Reardon and Winter, 1994). Mutations of FGFRs and Msx-2 genes are usually described as gain-of-function mutations whereas TWIST mutations are known as loss-of-function mutations. (Opperman et al., 2000). Mutations in these genes and transcription factors account for about 25% of all cases of craniosynostosis (Twigg et al., 2004).

Msx-2 (muscle segment homeobox 2) is a highly conserved homeobox gene that is involved in the regulation of inductive tissue interactions during embryogenesis (Jabs et al., 1993). A mutation of the Msx-2 gene was the first to be identified among gene
mutations associated with craniosynostosis (Jabs et al., 1993). Boston-type craniosynostosis is the only syndrome with craniosynostosis that is associated with mutations in the gene for Msx-2 (Jabs et al., 1993). The specific mutation associated with Boston-type craniosynostosis is a cysteine to adenosine transversion on the gene for Msx-2 that leads to an amino acid substitution. (Jabs et al., 1993), this results in an increase in DNA-binding of the Msx-2 protein and augmentation of the normal function of Msx-2 (Ma et al., 1996).

**TWIST** is a transcription factor that was originally identified as a principal stimulator of mesoderm formation in *Drosophila* (Lenton et al., 2005). Mutations in the gene encoding TWIST are also associated with craniosynostosis among many other abnormalities. Heterozygous TWIST-1 mutations have been identified in 80% of patients with Saethre-Chotzen syndrome (Chun et al., 2002; Johnson et al., 1998). Some of the many abnormalities found in patients diagnosed with this disorder are, craniosynostosis involving one or both coronal sutures, midface hypoplasia, and facial asymmetry (El Ghouzzi et al., 1997; Johnson et al., 1998; Reardon and Winter, 1994). In contrast to the Msx-2 gain-of-function or overexpression mutation, the majority of TWIST-1 mutations resulting in craniosynostosis, produce haploinsufficiency and result in a lack of functional protein production (Paznekas et al., 1998; El Ghouzzi et al., 1997).

Heterozygous **FGFR** mutations are the most common genetic abnormality associated with syndromes including craniosynostosis in their phenotype (Lenton et al., 2005). The most frequently occurring mutations are missense, or small in-frame insertions or deletions that affect the ligand-binding domain (Wilkie, 1997). Other
mutations involve the tyrosine kinase domain of the isoform FGFR2 and are thought to have an impact on downstream signaling pathways (Kan et al., 2002). However, most mutations of this gene are dominant and are believed to result in a gain of function by either increasing ligand binding affinity, decreasing the specificity of receptor-ligand interactions, or increased receptor dimerization and stabilization (Lenton et al., 2005).

Most of the FGFR mutations that cause craniosynostotic syndromes are mutations of the FGFR2 isoform. In fact, two mutations that result in an increase in function account for almost all of the cases of Apert syndrome (Lenton et al., 2005). In addition, other mutations of the FGFR2 gene have been associated with Pfeiffer and Crouzon syndromes. An analogous mutation, to one found in the gene encoding FGFR2 that causes Apert syndrome, has been identified in the FGFR1 gene, and is responsible for some cases of Pfeiffer syndrome (Robin et al., 1994; Wilkie et al., 1995). Two mutations in the FGFR3 gene have been linked to Muenke syndrome and type I thanatophoric dysplasia (Bellus et al., 1996; Muenke et al., 1997, 1994; Tavormina et al., 1995).

2. Enlarged Parietal Foramina

Too little sutural growth results in a disease known as Enlarged Parietal Foramina. Loss-of-function mutations in the transcription factors Alx-4 and Msx-2 have been associated with this disorder among many others in development.

Alx-4 and Msx-2 encode homeodomain transcription factors known to have regulatory roles in the development of various parts of the body in vertebrates (Alappat et al., 1999; Meijlink et al., 1999). Studies of Alx-4 and Msx-2 knock out mice have displayed the importance of their roles in skeletal patterning, differentiation, and
growth. These mice had craniofacial, axial, and appendicular abnormalities among their many defects (Antonopoulou et al., 2004; Qu et al., 1997; Satokata et al., 2000).

In humans, heterozygous loss-of-function mutations of either of these genes may result in defects of the skull vault in the form of enlarged parietal foramina (PFM) (Mavrogiannis et al., 2006). Typical PFM are bilateral oval or round openings in the parietal bones on either side of the posterior frontal suture (O’Rhailly et al., 1952). They are easily distinguished from the normally present minute foramina that blood vessels run through (Tubbs et al., 2003), and are a result of delayed ossification during rapid calvarial expansion (Antonopoulou et al., 2004; Satokata et al., 2000). Frequently, PFM can be traced back to a defect in infancy that extends between the anterior and posterior fontanelles; when this persists, it is termed cranium bifidum (CB) (Mavrogiannis et al., 2006). A bridge of bone usually develops along the midline, that creates two separate foramina, which usually decrease in size with age through circumferential bone growth. Eventually, complete closure may occur, leading to clinical nonpenetrance (Mavrogiannis et al., 2006).

D. Members of the TGF-beta superfamily and the regulation of cranial sutural fusion

The TGF-β superfamily includes several molecules that are involved in signaling and have vital roles in various processes throughout development, such as, embryogenesis, tissue formation, and wound repair (Gold et al., 1997; Kingsley, 1994). Some members of this family that have been found to be involved in cranial suture
development are the TGF-β isoforms (1, 2 and 3), and Bone Morphogenetic Proteins (BMPs).

1. BMP

Previous studies have shown, that BMP4 is expressed in the sagittal suture mesenchyme and dura mater of embryonic mice (Kim et al., 1998). Further study of postnatal sutures showed that BMP4 protein is localized to the suture mesenchyme and osteogenic fronts in fusing posterior frontal and patent sagittal and coronal sutures (Warren et al., 2003).

Since BMPs have been found in patent and fusing cranial sutures, Warren et al. screened for BMP antagonist mRNAs before, during, and after the period of expected suture fusion. They discovered the presence of Noggin in the patent sutures in all three of these periods and almost no Noggin expression in the fusing PFS as early as the time period prior to suture fusion (15d) (Warren et al., 2003).

Takao et al., was the first to demonstrate the function of Noggin as a BMP antagonist in cultured murine bone marrow stromal cells (Takao et al., 1996). Noggin protein was found to bind BMP4 with high affinity and inhibit BMP4 activity by blocking its ability to bind to cognate cell-surface receptors (Zimmerman et al., 1996).

Noggin was originally described in the Xenopus and was found to play a role in normal dorsal development (Smith and Harland, 1992). Further studies by Brunet et al., found that Noggin induces lateral mesodermal tissues to form more dorsal elements including muscle, heart, and the pronephros. In addition to these roles, they also found that Noggin was able to rescue dorsal patterning in ventralized embryos (Brunet et al., 1998).
Many of the roles identified for Noggin, oppose those known for BMPs, for example, the role of BMP to rival ventralizing potential in a developing embryo. As a result of these studies, recent work has looked further into the roles of Noggin and suggested that it might play an important part in maintaining suture patency (Lenton et al., 2005).

Recently, the BMP antagonist Noggin has been linked with FGF2 activity and determination of suture fate (Warren et al., 2003). Only the dura of the PFS expresses FGF2 mRNA and protein *in vivo* (Greenwald et al., 2000) and produces high levels of FGF2 in culture. FGF2 disrupts Noggin induction in a dose-dependent fashion, suggesting that environments with a high FGF2 concentration, such as the PFS, reduce Noggin expression and enable suture fusion (Warren et al., 2003).

Further evidence which suggests that Noggin may be responsible in determining suture fate is the 3 day old CD-1 mice injected with a noggin adenovirus that had widely patent posterior frontal sutures after 50 days (Warren et al., 2003).

**E. Transcription factors associated with BMP-Signaling**

**1. Dlx Genes**

Dlx homeobox containing genes are mammalian homologs of the *Drosophila Distal-less (Dll)* gene. They are a group of six genes that are organized into pairs, specifically, Dlx 1/2, Dlx 5/6, and Dlx 3/7 (Robledo et al., 2002). Mammalian Dlx genes are expressed in the primitive craniofacial region, the developing brain, and limbs, as well as, the apical ectodermal ridge (Robledo et al., 2002). Dlx gene products have a conserved 60 amino acid homeodomain and are members of a large family of
DNA-binding transcription factors. They are believed to control the transcription of genes that affect organogenesis for skeletal formation (Miyama et al. 1999).

Dlx5 has been coined by some to be a new candidate responsible for skull formation (Depew et al., 1999; Acampora et al., 1999). Compared with their other family members, Dlx5 and Dlx6 are uniquely localized to all skeletal tissues (Holleville et al., 2003). In fact, Acampora et al., found Dlx5 and Dlx6 in immature chondrocytes and developing skull bones of embryonic mice and rats and in the periosteum of more mature bones (Acampora et al., 1999).

Results from several in vivo studies have provided evidence that indicates the involvement of the Dlx family of homeodomain proteins in the regulation of osteoblast differentiation and bone tissue-specific gene expression (Miyama et al., 1999). Ryoo et al., showed that among the Dlx genes, Dlx5 is a bone-inducing transcription factor that is expressed in fetal rat calvarial cells in the later stages of osteoblast differentiation (Ryoo et al., 1997). In addition, it has been shown, that forced expression of Dlx5 in cultured cells leads to osteocalcin expression and a fully mineralized matrix (Lee et al., 2003, Miyama et al., 1999, and Tadic et al., 2002). These results strongly suggest that Dlx5 plays an important role in the development of mineralized tissues.

In an investigation of potential functional roles for Dlx5, Miyama et al., isolated Dlx5 as a BMP-inducible gene by differential display technique. Subsequently, they demonstrated that mDlx5-transfected cells show a more mature osteoblast phenotype when compared to parental MC3T3-E1 cells, suggesting that mDlx5 plays a role in osteogenesis (Miyama et al., 1999). In the study, mDlx5-transfected cells showed higher alkaline phosphatase activities, osteocalcin production, and mineralization of
extracellular matrix, suggesting that mDlx5 is a positive regulator of osteoblast differentiation.

In addition, when the time course of mDlx5 mRNA induction of MC3T3-E1 cells was examined, transcripts of mDlx5 continuously occurred 48h past the time of BMP treatment. It was proposed from these findings, that the mDlx5 gene product plays an important role in the later stage of the differentiated osteoblast since osteoblast fate has been clearly determined at this time (Miyama et al., 1999).

Recent investigations, showed that Runx2, a transcription factor that plays an essential role in osteoblast differentiation and bone mineralization (Lee et al., 2005), as well as alkaline phosphatase (ALP) expression, was induced, in the absence of BMP-2 with forced expression of Dlx5 (Kim et al., 2004). Another finding was that blocking of Dlx5 completely inhibited BMP2-induced stimulation of Runx2 and ALP expression. These results proved, that Dlx5, is required as an upstream regulator of the expression of Runx2 that is generated by BMP signaling and suggested that Dlx5 enhances ALP expression by increasing Runx2 expression (Kim et al., 2004, and Holleville et al., 2007).

Results of these studies have shown that Dlx5 is essential for BMP-mediated osteoblast differentiation and have suggested that Dlx5 plays a role in the mature stages of mineralization in skeletal tissue. These, and previous findings linking BMP signaling to murine PFS fusion, in addition to its expression in developing (Kim et al., 1998) and adult cranial sutures (Warren et al., 2003), has lead us to believe that Dlx5 may play an important role in cranial suture fusion.
Chapter 2

Wadhwa S, Bi Y, Ortiz A, Embree M, Kilts T, Iozzo R, Opperman L, Young M.

*Impaired posterior frontal suture fusion in the biglycan/decorin double deficient mice.*

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Impaired posterior frontal sutural fusion in the biglycan/decorin double deficient mice

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Abstract

Biglycan (Bgn) and decorin (Den) are highly expressed in numerous tissues in the craniofacial complex. However, their expression and function in the cranial sutures are unknown. In order to study this, we first examined the expression of biglycan and decorin in the posterior frontal suture (PFS), which predictably fuses between 21 and 45 days post-natal and in the non-fusing sagittal (S) suture from wild-type (WT) mice. Our data showed that Bgn and Den were expressed in both cranial sutures. We then characterized the cranial suture phenotype in Bgn deficient, Den deficient, Bgn/Den double deficient, and WT mice. At embryonic day 18.5, alizarin red/salcian blue staining showed that the Bgn/Den double deficient mice had hypomineralization of the frontal and parietal craniofacial bones. Histological analysis of adult mice (45–60 days post-natal) showed that the Bgn or Den deficient mice had no cranial suture abnormalities and immunohistochemistry staining showed increased production of Den in the PFS from Bgn deficient mice. To test possible compensation of Den in the Bgn deficient suture, we examined the Bgn/Den double deficient mice and found that Bgn deficiency led to increased expression of Bmp-4 and Dlx-5 in the PFS compared to their non-fusing suture in WT mice and decreased expression of Dlx-5 in both PF and S sutures in the Bgn/Den double deficient mice compared to the WT mice. Failure of PFS fusion and hypomineralization of the calvaria in the Bgn/Den double deficient mice demonstrates that these extracellular matrix proteoglycans could have a role in controlling the formation and growth of the cranial vault.

Keywords: Small proteoglycans; Biglycan; Decorin; Cranial suture; Mouse

Introduction

Biglycan (Bgn) and decorin (Den) are members of the small leucine repeat proteoglycan family (SLRP). Members of this family are characterized by a small protein core, which consists predominantly of leucine-rich repeats. There are 13 known members of this family that are divided into three classes depending on their genomic organization and the similarity of their amino acid sequences. Bgn and Den belong to the class I type SLRP and are highly expressed in skeletal and connective tissues [1,12,14,15,31]. The exact function of both Bgn and Den is unknown; however, both can bind and modulate members of the TGF-beta superfamily [11].

Bgn deficient mice have impaired post-natal bone formation which may be due to the ability of Bgn to modulate the actions of Bone morphogenic proteins-2/4 (Bmp-2/4) in osteoblastic cells, potentially contributing to the failure to achieve peak bone mass and early onset of osteoporosis [4,36]. Den deficient mice do not have obvious skeletal defects, but instead, have skin fragility which is hypothesized to be due to the ability of Den to modulate collagen fibril structure and integrity [7]. Because of their similarity in structure and their overlapping expression in skeletal and connective tissues, it is probable that Bgn and Den...
function redundantly. Support of this concept comes from the analysis of Bgn/Dcn double deficient mice that have a more severe phenotype in both long bone and skin [2, 5] compared to Wt or singly deficient SLRP mice.

The purpose of this study was to examine the role of Bgn and Dcn in the craniofacial complex. In the cranial region, Bgn and Dcn are present in teeth [22, 28], periodontal tissues [21], nasal cartilage [29], eye [8], temporomandibular joint [18], the developing mandible [16, 35], and the developing calvaria [35]. Despite their prevalence in numerous tissues of the craniofacial complex, little is known about their function in these tissues. In order to examine this further and to eliminate potential compensation of Dcn with Bgn, we examined whether a craniofacial phenotype could be unmasked by creating Bgn/ Dcn double deficient mice and comparing them to the single Bgn deficient, single Dcn deficient, and control Wt mice.

There is similarity and conservation in the molecular pathways that control the assembly and growth of the cranial vault between humans and mice [33]. In mice, all the cranial vault sutures remain open throughout the life span of the animal, except for the posterior frontal suture (PFS), which fuses in a predictable fashion between 21 and 45 days post-natal [25]. In humans, the equivalent metopic suture fuses within the first 3 years post-natal [30, 34]. Therefore, the mouse PFS serves as a model of post-natal human suture growth [33]. Bmp-2/4 signaling has been linked to murine PFS fusion by Warren et al., who showed that inhibitors of the Bmp-2/4 signaling pathway by adenovirus mis-expression of the Bmp antagonist Noggin blocks murine PFS fusion in vivo [32]. Since both biglycan and decorin can modulate Bmp-2/4 signaling, we hypothesized that, in their absence, there would be defective Bmp-2/4 signaling and impaired PF sutural fusion.

In this study, we found that Bgn and Dcn were expressed in the fusing PFS and in the non-fusing Suture in Wt mice. The PFS in both Dcn deficient and Bgn deficient mice revealed no defects. However, further examination of the PFS in the Bgn/ Dcn double deficient mice showed a lack of PFS fusion in a mechanism that is potentially related to a decreased expression of Dlk-5.

Experimental procedures

Generation of Bgn and Dcn single and double deficient mice

All experiments were performed under an institutionally approved protocol for the use of animals in research (NIH-DOCR-IRP-98-058 and 01-151). Mice deficient in Bgn and Dcn were generated by gene targeting in embryonic stem cells as described previously [7, 36]. Heterozygous Bgn/Dcn deficient mice were produced by breeding a homozygous Bgn deficient female (Bgn<sup>−/−</sup>/Dcn<sup>−/−</sup>) female with a Dcn heterozygous deficient male (Bgn<sup>+</sup>/Dcn<sup>+/−</sup>). Bgn<sup>−/−</sup> deficient males are designed as Bgn<sup>−/−</sup> since the Bgn gene is located on the X chromosome and absent from the Y chromosome. F2 Bgn/Dcn double deficient mice were obtained by interbreeding F1 heterozygous Bgn/Dcn deficient animals. In order to maximize the number of double deficient mice obtained, the following breeding scheme was used: Bgn<sup>−/−</sup>/Dcn<sup>−/−</sup> males were bred with Bgn<sup>+/−</sup>/Dcn<sup>+/−</sup> females. The cranial vault of the mice was analyzed at embryonic day 18.5, and post-natal days 35, 42, and 60. We chose to examine 42 days and 60 days post-natal (the posterior frontal suture normally fuses between 21 and 45 days post-natal) because they represent time points during and well past normal PFS closure which allowed us to evaluate if there was a delay or failure of PFS fusion in the transgenic mice. We selected post-natal day 35 for our RT-PCR analysis because we wanted to evaluate whether there would be differences in gene expression during the time of normal posterior frontal sutural fusion.

Genotyping

All mice were genotyped for Bgn and Dcn alleles by PCR analysis as described [35]. PCR products were resolved by electrophoresis through 1.8% agarose gels, yielding bands of 212 bp for the WI Bgn allele, 310 bp for the disrupted Bgn allele, 161 bp for the WI Dcn allele, and 238 bp for the disrupted Dcn allele.

Alizarin red and alcin blue staining of mice litters

In our experimental approach, we used a breeding scheme that would maximize the number of Bgn/Dcn double deficient mice, and for this reason, we were unable to obtain a homozygous Wt mouse and Bgn/Dcn double deficient mouse in the same litter. Therefore, in this first part of the study, we used (Bgn<sup>+/−</sup>/Dcn<sup>+/−</sup>) mice as our controls. 18.5 d.p.c. litters obtained from 5 litters were euthanized, and their skin, muscle, and fat were removed. The animals were fixed in 100% ethanol for 4 days and then placed in alcohol for 3 days. Mice were then stained with alizarin red (0.09%) and alcin blue (0.05%) in a solution containing ethanol, glacial acetic acid, and water (67:5:28) for 3 days. After staining, mouse samples were transferred to 1% KOH until their soft tissues were dissolved and then preserved in a 100% glycerol [23].

Faxitron

Sixty-day-old mice were divided into four groups. WI, Bgn deficient, Dcn deficient, and Bgn/Dcn double deficient mice were sacrificed and the soft tissues on their heads were removed. The calvaria were radiographed using a Faxitron MX-20 Radiography System (Faxitron X-ray Corp., Wheeling, IL, USA) at an energy of 90 kV for 20 s. The images were captured with Eastman Kodak Co. X-Omat 11T (Eastman Kodak Co., Rochester, NY, USA).

In order to determine the optimal length of a rectangular box that could be used to quantitate the amount of radio-opacity in the PFS, the length of the PFS from 60-day-old WI, Bgn deficient, Dcn deficient, and Bgn/Dcn double deficient mice was first measured. The mean length ± the standard error of the PFS was 11.0±0.6 mm for WI, 10.3±0.3 mm from Bgn deficient, 10.7±1.2 mm from Dcn deficient, and 11.7±0.3 mm from Bgn/Dcn double deficient mice. Evaluation of differences among the means by analysis of variance (ANOVA) revealed that there were no significant differences in the length of the PFS between the groups. Therefore, a 10 mm by 3 mm rectangular box was used to measure the amount of radio-opacity in the PFS from the different groups. The amount of radio-opacity in the PFS was quantified by using Image J NIH IMAGE.

Histology and immunohistochemistry

WI, Dcn deficient, Bgn deficient, and Bgn/Dcn double deficient mice were sacrificed by CO<sub>2</sub> inhalation at 35–45 days after birth. Calvaria were removed and fixed for 2 weeks at room temperature in 10% formalin. After being washed in water for 5 min, they were decalcified in formic acid bone decalcifier solution (Immunocal from Dural Corporation, Tallman, NY, USA) for 4 weeks. Specimens were then washed in water for 5 min and fixed for 3 days in buffered zinc formalin (Z-fix from Anatech Ltd., Battle Creek, MI, USA) before being classically processed for histology and sectioned coronally. Sections were stained with H&E.

Tissue sections were deparaffinized with xylene. Following rehydration with decreasing concentrations of ethanol, the sections were treated with 3% peroxide in methanol for 20 min to block endogenous peroxidase activity. To reenergize the antigen, sections were pretreated in 1% hydrogen peroxide (Endogen, Woburn, MA) for 30 min at room temperature. Tissue sections were incubated overnight at 4°C with primary antibody at 1:200 dilution. Dcn and Bgn antibodies (respectively LF 113 and LF 100) were kind gifts from Larry Fisher (NIH, NIH). Biocytin and rabbit anti-goat secondary antibody

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was used and visualized by a streptavidin-peroxidase solution in the presence of DAB chromagen. The negative control consisted of the above mentioned procedures except for the substitution of the primary antibody with rabbit IgG.

The data presented in this paper were reproduced in at least 6 different sections from two separate animals for each group. Serial sections through the whole suture were obtained for each animal and observations were confirmed in different interspaced serial sections chosen to cover the whole suture. In this way, it was ensured that the reported observations were genuine and not local random abnormalities. In each case, data from a single representative experiment are shown.

Isolation of the suture complex for mRNA extraction

Posterior frontal (PF) and sagittal (S) suture complexes (including the associated dura mater, suture mesenchyme, and osteogenic fronts) were harvested from 35 day-old male Wt (n = 4) and Bgn/Dcn (n = 2) double deficient mice. Mice were euthanized with carbon dioxide, and the posterior frontal and sagittal suture complexes were isolated under a dissecting microscope as previously described [24].

Semi-quantitative RT-PCR

RNA was extracted from the cranial suture material using Trizol Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer’s instructions. One microgram total RNA from the sample preparation was reverse transcribed with 50 units of SuperScript II RT using random hexamer primers (Invitrogen Life Technology, Carlsbad, CA) following the manufacturer’s instructions. The primers used for amplification were designed with Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). The primers used for RT-PCR were for Gapdh 5'TCTGGAGCTTATAGATG3' (forward) and 5'CGGGACTTTATGAGG3' (reverse), for Bgn-4 5'GGAGGCTTTTCCTCC3' (forward) and 5'GGGACGCTTTATGAGG3' (reverse), for Dcn 5'CAGGGATCGTGCA3' (forward) and 5'GGCACTGTAGGCTCG3' (reverse), for biglycan 5'ACGTTCCATGCGG3' (forward) and 5'GCCGAGCTTTATGAGG3' (reverse), and for decorin 5'CCGACTAATGCTACT3' (forward) and 5'GECGGCTTTATGAGG3' (reverse), and for 18S 5'AGGATGAGGCAAG3' (forward) and 5'CATTATGAGGCAAG3' (reverse). RT-PCR was performed using a hot start (with Taq Gold™, Applied Biosystems) at 95°C for 10 min followed by 45 cycles (biglycan, decorin, 18S, and GAPDH) or 30 cycles (Dox-5 and Bgn-4) of 1 min at 94°C, 20 s at 57°C and 30 s at 72°C followed by a 7 min extension at 70°C. The reaction was chilled to 4°C afterwards until it was analyzed. Sixteen microliters of the PCR product and a 100-bp DNA ladder (Gibco BRL) were run in a 10% acrylamide gel (TBE) buffer at 100 V. The products separated by electrophoresis were visualized after ethidium bromide staining under a UV light and photographed in a single field of view with a digital camera. Individual bands on digital micrographs of RT-PCR products were analyzed by densitometer using Image J NIH IMAGE. Intensity for Dox-5, Bgn, Dcn, and Bgn-4 was calculated as a percentage of densitometry values for control bands of Gapdh or 18S. Each primer set was tested previously to assure that amplification was in the linear range by analyzing amplification at several different cycle numbers.

Statistical analysis

Statistical significance of differences among means was determined by analysis of variance (ANOVA) with post hoc comparison of more than two means by the Bonferroni method using GraphPad Prism (San Diego, CA).

Results

Alizarin red–alcan blue staining of E 18.5 littermates

Alizarin red–alcan blue staining of E 18.5 control (Bgn+/– / Dcn+/–) was used as the control due to the inability to generate a Wt and a Bgn/Dcn double deficient in the same litter) and Bgn/Dcn double deficient littermates revealed that the Bgn/

Dcn double deficient embryos had severe hypomineralization of the frontal and parietal bones (Figs. 1A–D). Alizarin red and alcan blue staining were also performed on two additional Bgn/Dcn double deficient embryos along with two littermate controls revealing similar results (data not shown).

Expression of Bgn and Dcn in the PFS and sagittal suture from Wt mice

A time course for the expression of Bgn and Dcn was determined in the PF and the S suture in Wt mice at day 21, day 25, and day 35 post-natally by semi-quantitative RT-PCR. The data indicated that mRNA encoding both biglycan and decorin is abundantly expressed in both the posterior frontal and sagittal suture during maturation (Fig. 2A). Since there appeared to be increased expression of Bgn mRNA in the PF compared to the S suture during the late phases of PFS fusion, we repeated the experiments two more times at day 35 post-natal. In the three independent experiments from 35 day-old Wt mice, we found that the ratio of Bgn mRNA expression in the PFS/S suture was 1.1±0.3.

In order to determine the precise localization of Bgn and Dcn protein expression in the PFS and to evaluate possible compensation at the protein level, immunohistochemistry for Bgn and Dcn was performed in 42 day-old Wt and Bgn deficient mice (Figs. 2B–E). In the PFS from the Wt and Bgn deficient mice, Dcn was found in the underlying dura (Figs. 2C, E). In the Wt mice, Dcn appeared to have a more restricted
Fig. 2. Expression of Bgn and Den in cranial sutures. (A) Semi-quantitative RT-PCR analysis performed for Den and Bgn on RNA extracted from the sagittal (S) suture and the posterior frontal suture (PFS) from 21, 25, and 35 day-old wild-type (Wt) mice. (B-E) Immunohistochemistry (brown) for biglycan (B) and decorin (C, E) on coronal sections of the posterior frontal suture from 42 day-old Wt (B, C) and Bgn deficient mice (D, E). Sections were counterstained with hematoxylin (blue).

expression pattern in the dura compared to Bgn deficient mice where it was found at a higher level and throughout the structure. In contrast, the PFS from the Den deficient mice had no change in the localization of Bgn in the underlying dura compared to Wt mice (data not shown). Negative controls consisting of the substitution of the primary antibody with rabbit IgE revealed no visible staining (data not shown).

Analysis of the PFS from Bgn deficient, Den deficient, Bgn/Den Double deficient and Wt mice

In order to determine what role Bgn and Den deficiency had on later developmental stages, 60 day-old Wt and Bgn/Den double deficient mice were examined. Faxitron analysis showed that the Bgn/Den double deficient mice had a clear absence of radio-opacity in the PFS. On the other hand, calvaria obtained from 60 day-old Wt, Bgn deficient, and Den deficient mice, all had PFS that was radio-opaque (data not shown). Quantification of the amount of radio-opacity in the PFS by X-ray scanning and NIH-image analysis revealed that it was significantly decreased in Bgn/Den double deficient mice compared to the other groups (p<0.001) (Fig. 3A). Besides a decrease in mineralization of the PFS, the decrease in radio-opacity in the PFS from the Bgn/Den double deficient could also be due to a difference in the structure and curvature of the calvaria. To test this possibility, coronal histological sections of the PFS from 45 day-old Bgn deficient (Fig. 3C), Den deficient (Fig. 3D), Bgn/Den double deficient (Fig. 3E), and Wt mice (Fig. 3B) were examined. This analysis revealed PFS patency only in the Bgn/Den double deficient samples compared to the other groups.

Fig. 3. Analysis of radio-opacity and histology of the PFS from Wt, Bgn deficient, Den deficient, and Bgn/Den double deficient mice. (A) Quantification of intensity of radio-opacity in the PFS from faxitron images of the calvaria from 60 day-old Wt, Bgn deficient, Den deficient, and Bgn/Den double deficient mice. Each point is the mean and SEM for n=3 for each group. *Significant decrease in the intensity of radio-opacity in the PFS from the Bgn/Den double deficient mice compared to the other groups, p<0.001. (B–E) H&E staining of coronal sections of the posterior frontal suture from 45 day-old Wt (B), Bgn deficient (C), Den deficient (D), and Bgn/Den double deficient (E) mice.
Expression of Bmp-4 and Dlx-5 mRNA in PFS and sagittal suture from 35 day-old Wt and Bgn/Dcn double deficient mice

Because Bmp-2/4 signaling has been previously implicated in controlling PFS fusion [32], we examined the relative expression levels of Bmp-4 mRNA, and the Bmp-4 target gene, Dlx-5 mRNA [10], in our mouse model. Messenger RNA was extracted from the fusing PFS and non-fusing S sutures of 35 day-old Wt and in Bgn/Dcn double deficient and analyzed by semi-quantitative RT-PCR. These analyses showed that Bmp-4 mRNA levels were higher in the (fusing) PFS compared to S suture in both genotypes. Interestingly, when Dlx-5 was measured in the same samples, it was significantly higher in the Wt PFS compared to the S suture but absent in both S and PF sutures in Bgn/Dcn deficient mice (Fig. 4). Similar results were obtained in a second independently repeated experiment (data not shown).

Discussion

This report shows that both biglycan (Bgn) and decorin (Dcn) are abundantly expressed in the cranial sutures of mice, with overlapping yet distinct patterns of expression in the fusing suture and the dura mater. We found that Bgn/Dcn double deficient mice have open sutures and severe hypomineralization of both frontal and parietal bones as early as E 18.5, while mice singly deficient in Bgn or Dcn have no suture formation defects.

Murine PFS fusion is controlled by a complex interaction between many different types of cells, tissue types, and growth factors. For example, Warren et al. [32] has proposed that Fgf-2 secreted by the underlying dura causes a downregulation of Noggin within the posterior frontal suture cells that allows Bmp-2/4 to interact with the bone fronts to cause fusion of the PFS. For such orchestration of events to occur, there must be control of the localization and activity of the secreted growth factors. Proteoglycans that could modulate the activity of growth factors within the extracellular matrix of the posterior frontal suture may provide this control [26].

Several lines of evidence link both Bgn and Dcn to Bmp-2/4 activity. In a recent study, we found that Bgn deficient calvarial osteoblast-like cells had less cell surface Bmpr4 binding, which led to reduced Bmp-4 mediated osteoblast differentiation in Bgn deficient calvarial osteoblast-like cells compared to Wt controls [4]. Similar results have been noted in MC3T3-E1 cell-derived clones expressing higher or lower levels of biglycan [27]. Dcn has been shown to modulate the actions of Bmp-2/4. Specifically Bmp-2 induction of alkaline phosphatase was diminished in Dcn deficient myoblast cells compared to Wt controls [9].

Bgn and Dcn are similar in structure and function and it is possible that they function redundantly. Immunohistochemistry revealed that, in the absence of Bgn, Dcn is upregulated throughout the PFS. Dcn expression was restricted to the inferior portion of the dura in Wt mice but more evenly distributed throughout the underlying dura of the PFS in Bgn deficient mice. We have also previously reported increased expression of Dcn in Bgn deficient osteoblastic cell cultures [4]. Furthermore we found that the ability of Bmp-2 to induce mineralization, as measured by alizarin red accumulation, was decreased 52% in osteoblast-like cells from Bgn/Dcn double deficient mice [2] compared to 30% in Bgn only deficient osteoblast-like cells [4].

We hypothesize that the impaired PFS in the Bgn/Dcn double deficient mice is due to altered Bmp-4 signaling since we found equivalent mRNA expression of Bmp-4 but markedly reduced expression of Dlx-5 in the Bgn/Dcn double deficient cranial sutures. Many extracellular and intracellular proteins, such as Dlx-5, regulate the Bmp-2/4 signaling pathway. Specifically, Bmp-2/4 has been shown to increase Dlx-5 expression in avian calvarial explant cultures [13] and in osteoblastic cell cultures [10]. In addition, the Bmp-2/4 regulation of the osteoblast differentiation markers, Runx-2, OsFetix, and Alkaline phosphatase, appears to be dependent on Dlx-5 [17,19,20]. Impaired PFS fusion in the Bgn/Dcn double deficient mice may be due to failure of appropriate expression of signaling molecules involved in promoting bone formation and mineralization, which is consistent with previous findings of severe osteopenia [2,5] and decreased expression of Dlx-5 in the cranial sutures. However, as has been suggested by others, it is important to consider the possibility that additional factors besides increased or decreased bone formation could control abnormal cranial suture fusion [3,6].

Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>E 18.5 calvaria</th>
<th>Post-natal PF fusion</th>
<th>Suture gene/protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>Normal</td>
<td>Normal</td>
<td>Dlx-5 and Bmp-4 mRNA higher in PF vs. S</td>
</tr>
<tr>
<td>Bgn deficient</td>
<td>ND</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Dcn deficient</td>
<td>ND</td>
<td>Normal</td>
<td>Den protein higher in Bgn deficient</td>
</tr>
<tr>
<td>Bgn/Dcn deficient</td>
<td>ND</td>
<td>Hyperminalized</td>
<td>Bmp-4 protein unaffected Dlx-5 mRNA absent in PF and S sutures</td>
</tr>
</tbody>
</table>

Normal Wt mice and mice deficient in Bgn, Dcn, or both (genotype, column 1) were analyzed at embryonic day 18.5 (E 18.5) by alizarin red/salician blue staining (column 2) or post-natally (60 days after birth) by fastinon X-ray or histology (column 3). Specific mRNA or protein expression in the normal and mutant mice was examined in PF (posterior frontal) or sagittal (S) sutures 35-45 days after birth (column 4). ND = not determined.
In conclusion, we show that the alteration in the extracellular matrix of the posterior frontal suture causes impaired fusion (Table 1). These new data provide the foundation for the identification of novel candidate genes for patients who have natural growth disturbances.

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References

Chapter 3

CRANIAL SUTURAL GROWTH IN HETEROZYGOUS Dlx5 +/- MICE

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Introduction

There are two main types of cranial suture growth disturbances. Too little or delayed bone growth at these sutures results in wide-open fontanels and a human disease known as enlarged parietal formania, whereas too much or accelerated bone growth results in osseous obliteration of the sutures, and the human disease craniosynostosis (Opperman 2000).

We have previously reported that in mice deficient in both biglycan and decorin, there is lack of PFS fusion at 60 days. Analysis of gene expression in the PFS from 35 day-old wt and double deficient biglycan/decorin mice, revealed equivalent expression of BMP-4, but a marked decrease in the expression of Dlx5 in the double deficient mice. Therefore, we hypothesized that Dlx5 plays a role in PFS fusion. In order to test this hypothesis, we examined the expression of Dlx5 in the cranial sutures from CD-1 mice and examined if a cranial suture phenotype was present in Dlx5 +/- mice.

In this study, we found that Dlx5 is abundantly expressed in both the sagittal and PFS between 10 and 35 days postnatal. There was an increase in the expression of Dlx5 in the PFS compared to the sagittal suture at day 35. Examination of the cranial sutures from Dlx5 +/- mice revealed no defects in PFS fusion, but instead an increase in the distance between the parietal bone fronts in the sagittal suture. Taken together, these results suggest that Dlx5 plays a role in postnatal growth of the sagittal suture in CD-1 mice.
Materials and Methods

Mice

To develop the mouse model for mice used in our experiments, Dlx5 transgenic mice (Obtained from Dr Alex Lichler, UCHC, Farmington, CT) were bred with mice transgenic for the 2.3 kb fragment of the collagen Type I promoter fused to a Green Fluorescent Protein (2.3 GFP) (Obtained from Dr. David Rowe, UCHC, Farmington, CT). The specific breeding pairs were Col I 2.3 GFP (+/+) Dlx5 (+/+) with heterozygous Dlx5 (+/-), and Col I 2.3 (+/+) heterozygous Dlx5 (+/-) with Col I 2.3 GFP (+/+) Dlx5 (+/+). Heterozygous Dlx5 (+/-) 2.3 GFP (+/) and homozygous Dlx5 (+/+) 2.3 GFP (+/) littermates were analyzed.

Genotyping

All mice were genotyped for Dlx5 alleles by polymerase chain reaction (PCR) analysis as described previously (Depew et al. 1999). PCR products were resolved by electrophoresis through 1% agarose gels, yielding a 368 bp band for the wt Dlx5 allele and a 459 bp band for the disrupted Dlx5 allele. PCR products were visualized by ethidium bromide staining under an ultraviolet light
Histology and Immunohistochemistry

Frozen Embedding

Wild-type and heterozygous Dlx5 (+/-) mice were sacrificed by CO₂ inhalation at 10 days after birth. Calvaria were removed and fixed for 2 weeks at room temperature in 10% formalin. Next, they were separated into posterior frontal and sagittal regions and placed in separate cassettes. After a 5 minute water wash, they were decalcified in formic acid bone decalcifier solution (Immunocal from Decal Corporation, Tallman, NY, USA) for 4 weeks. Specimens were then washed in water for 5 minutes and fixed for 3 days in buffered zinc formalin (Z-fix from Anatech Ltd, Battle Creek, MI, USA).

Prior to embedding, a 200ml beaker containing 2-methylbutane was pre-chilled over dry ice under a hood. Disposable base molds (Thermo Shandon) were filled with frozen embedding medium (Thermo Shandon), care was taken to avoid the introduction of bubbles. Posterior frontal and sagittal suture regions of the calvariae were immersed in individual molds containing the embedding medium. The embedding media was flash frozen by holding the mold with forceps in a solution of 2-methylbutane keeping the embedding mold on a horizontal level. Once the medium was frozen, the mold was allowed to sink to the bottom of the beaker until it was completely frozen. The molds were removed from the methyl butane solution and wrapped in a square of aluminum foil, placed in a plastic container, and stored at -20°C.
CryoJane Frozen Section

Cryosectioning was performed on a Leica CM1900 Cryostat (D-69226; Leica, Inc., Nussloch, Germany) equipped with CryoJane Frozen Sectioning Kit (Instrumedics Inc.; Hackensack, NJ). The CryoJane system is designed to capture a frozen cryostat section on special cold adhesive tape to assist transferring the section to a cold glass microscope slide coated with an ultraviolet light-curable, pressure-sensitive adhesive. The section is permanently bonded to the adhesive on the slide with a flash of ultraviolet light.

The block containing either the decalcified posterior frontal or sagittal suture was oriented in the block holder to obtain a 5-μm coronal section. After capturing the frozen section on a piece of cold tape, the section was transferred to the cold slide, and the transfer tape was removed, leaving the frozen section behind on the microscope slide. The slides were air dried in the dark and kept in a slide box at 4°C before processing.

Fluorescent Imaging

Slides holding the frozen sections were soaked in 1X PBS for 20 minutes then mounted with cover slips using 50% glycerol in PBS. Col I 2.3 GFP was examined with a microscope under ultraviolet light and pictures were taken with a color digital camera at 10X magnification.
**Immunohistochemistry**

Tissue sections were deparaffinized with xylene. Following rehydration with graded ethanol, sections were treated in 3 % peroxide in methanol for 20 minutes to block endogenous peroxidase activity. Next, sections were incubated in 10% goat serum for 30 minutes to reduce non-specific binding and incubated overnight at 4° C with primary antibody at a 1: 200 dilution. Dlx5 antibody was purchased from Chemicon (Temecula, CA) cat #AB5728 and Biotinylated rabbit anti-goat was used as a secondary antibody. Immunostaining was visualized by a streptavidin-peroxidase solution in the presence of DAB chromagen. The negative control consisted of the above mentioned procedures except for the substitution of the primary antibody with rabbit IgE.

**Semiquantitative RT-PCR**

Posterior frontal and sagittal suture complexes, including the associated dura mater, suture mesenchyme, and osteogenic fronts, were harvested from male wt mice. Mice were euthanized with carbon dioxide, and the posterior frontal and sagittal suture complexes will be isolated under a dissecting microscope as described by (Nacamuli, Song et al. 2004). RNA was extracted from the calvarial sutures using Trizol-Reagent (Molecular Research Center, Cincinnati, OH) following the manufacture’s instructions. One microgram total RNA from the sample preparation was reverse transcribed with 50 units of SuperScript II RT using random hexamer primers (Invitrogen Life Technology, Carlsbad, CA) following the manufacturer’s instructions. The primers used for amplification were designed with Primer 3 software (http://www-
The primers used for RT-PCR were 5’ gagaggccctatcccaact3’ (forward) 5’gtggtgtgcagcgaacttat3’ (reverse) for 18S, 5’catgtggtgtgaggaaa3’ (forward) and 5’gccagagactcatttctt3’ (reverse) for BMP-4, and 5’ccgggacgctttattagatg3’ (forward) and 5’tggacactatcaatggtgcc3’ (reverse) for Dlx5. The reaction was chilled to 4°C until it was analyzed. Sixteen microlitres of the PCR product and a 100-bp DNA ladder (Gibco BRL) were run in a 10% acrylamide gel (TBE) buffer at 100 V. The products were separated by electrophoresis and visualized after ethidium bromide staining under a UV light then photographed with a digital camera. The RT-PCR procedure was repeated three times. Individual bands on digital micrographs of RT-PCR products were analyzed by densitometer using Image J NIH IMAGE. Intensity for Dlx5 and Bmp-4 was calculated as a percentage of densitometry values for control bands of 18S.

Measuring the Width of the Sagittal and Posterior Frontal Sutures

H & E stained coronal sections of the posterior frontal and sagittal sutures from 10 day-old wt and heterozygous Dlx5 (+/-) mice were captured with a digital camera. In Adobe Photoshop the distance between the bone fronts was measured in arbitrary units. A micro-ruler was captured using the same digital camera, at the same magnification, and arbitrary units were converted to μm by measurement against a known distance.

Statistical Analysis

Statistical significance of differences among means was determined by analysis of variance with post-hoc comparison of more than two means by the Bonferroni
method or the Mann-Whitney rank sum test for nonparametric populations using SigmaStat (Jandel Scientific, San Rafael, CA).

**Results**

**Dlx5 expression in cranial sutures**

Immunohistochemistry for Dlx5 was performed in 10 day-old CD-1 mice. In the PFS from 10 day-old mice, Dlx5 expression was in the underlying dura and overlying periosteum (Figure 1A). A similar expression of Dlx5 was noted in the S compared to the PFS (Figure 1B).

A time course for the expression of Dlx5 and BMP-4 was determined in the posterior frontal and sagittal sutures from CD-1 mice at day 21, day 25, day 35, and day 45 postnatally by semi-quantitative RT-PCR. The data indicated that mRNA encoding Dlx5 and BMP-4 were abundantly expressed in both the posterior frontal and sagittal sutures during maturation (Figure 2). Since there appeared to be an increase in expression of Dlx5 and BMP-4 mRNA in the PF compared to the S suture during the late phases of PFS fusion, we repeated the experiments two more times at day 35 postnatal. In three independent experiments from 35 day-old wt mice, the mean decrease and standard error of Bmp-4 mRNA expression in the sagittal suture compared to the PFS was 83 +/- 15%. Additionally, in three independent experiments from 35 day-old wt mice, the mean decrease and standard error of Dlx5 mRNA expression in the sagittal compared to the PFS was 59 +/- 14 % (Figure 2 b).
Analysis of the Posterior frontal and Sagittal sutures from 10 day-old heterozygous Dlx5+/- and wt mice

Mice deficient in both Dlx5 alleles die shortly after birth and have almost a complete absence of their calvariae. In contrast, Dlx5 +/- heterozygous mice are viable. In order for PF sutural fusion to occur, the bone fronts of the frontal bone must come in contact with each other, which is expected to occur between 21 and 45 days postnatal. However, we have observed this process of suture fusion beginning at a time point prior to 21 days postnatal. Therefore, the cranial sutures from 10 day-old wt and Dlx5 +/- were examined. H & E coronal sections of the Posterior frontal (Figure 3) and Sagittal sutures (Figure 5) revealed that there appeared to be greater separation of the bony fronts in the sagittal suture of Dlx5 +/- mice compared to wt mice. Measurement of the distance between the bone fronts of the Posterior frontal and Sagittal sutures from 10 day-old wt (n=3) and Dlx5 +/- mice (n=3), revealed a significant increase (p<.05) in the distance between the parietal bone fronts in the sagittal suture from Dlx5 +/- compared to wild-type controls (Figure 6). No significant differences were noted in the PFS suture (Figure 4),

Osteoblast differentiation in the cranial sutures from 10 day-old heterozygous Dlx5 +/- and wt mice

Dlx5 has been show to mediate osteoblast differentiation (Ryoo et al., 1997), therefore we hypothesized that there would be a decrease in osteoblast differentiation in the cranial sutures from Dlx5 +/- mice compared to wt mice. In order to examine this, we bred Dlx5 +/- mice with mice transgenic for the 2.3 kb fragment of the collagen
Type I promoter fused to a Green Fluorescent Protein (2.3 GFP). Previous investigators have shown that the 2.3 GFP promoter is an accurate marker of differentiated osteoblastic cells (Kalajz et al., 2002). We have initial histological sections of the PFS (Figure 7) and S suture (Figure 8) from 10 day-old Dlx5 +/+ 2.3 GFP and Dlx5 +/- 2.3 GFP mice. However, we have not analyzed the samples.

Discussion

Our experiments show that Dlx5 is expressed both in the fusing posterior frontal and non-fusing sagittal sutures of mice during the time period of expected suture fusion. Holleville et al. found, in chick calvariae, that Dlx5 is highly expressed at the osteogenic fronts and at the edges of the suture mesenchyme, but not in the suture itself (Holleville et al., 2003). Data from Acampora et al., who studied mice, also indicated that the expression of Dlx5 was more pronounced in periosteal bone and was also observed in endosteal cells (Acampora et al., 1999). These findings are similar although some were found in chick embryos and others were found in mice. Our results also showed that Dlx5 was expressed in the osteogenic fronts as well as the periosteum and underlying dura but not in the suture space.

In this study, we chose to use heterozygous Dlx5 (+/-) mice to investigate the role of Dlx5 in cranial suture fusion due to previous findings that Dlx5 (-/-) mice are affected by a severe phenotype. Depew et al. found that Dlx5 (-/-) mutants die shortly after birth and approximately 25% of them are born encephalic. The mutant mice born without encephaly have hypomineralized parietales and interparietals and all of
the mutants had the phenotype of regional defects in their nasal and otic capsules (Depew et al., 2005). Our results lacked a distinct phenotype in the PFS of Dlx5 (+/-) mice. Previous reports from Depew et al. also recorded that no morphologic changes were noted with the loss of a single Dlx5 allele. Therefore, it is very possible that the phenotype of our heterozygous Dlx5 (+/-) mice was too mild for changes in fusion of the PFS to be visible. In addition, several studies have shown evidence which might suggest that other Dlx genes compensate when their family member is deficient. Dlx homeobox genes are organized into pairs as follows: Dlx1/2, Dlx5/6, and Dlx 3/7 (Robledo et al., 2002). Compared with their other family members, Dlx5 and Dlx6 are uniquely localized to all skeletal tissues (Holleville et al., 2003). In fact, Acampora et al., found Dlx5 and Dlx6 in immature chondrocytes and developing skull bones of embryonic mice and rats and in the periosteum of more mature bones (Acampora et al., 1999). Robledo et al., 2002 showed that there are redundant roles for Dlx5 and Dlx6 during osteogenesis in his study of Dlx5/6 (-/-) mice whose phenotype included a complete lack of calvarial bones.

Developmental defects found in Dlx5 mutants that support our findings have been described by Depew et al. in 1999. Scanning electron microscope analysis of Dlx5 homozygous mutant embryos revealed that a neural tube closure defect is centered in the midbrain but also includes the forebrain and hindbrain in the more severe cases. Examination of the calvarial roofs of non-exencephalic Dlx5 mutants revealed small, hypomineralized parietal and interparietals (Depew et al., 1999). This developmental phenotype, both in the specific areas of the brain, and in the parietal and interparietals
of the Dlx5 mutants supports our findings in the sagittal sutures of Dlx5 heterozygous (+/-) mice.

Another possible reason for the lack of phenotype that was seen in the PFS but was seen in the sagittal suture from heterozygous Dlx5 (+/-) mice could be that PFS fusion is dependent in part on sox-9 expression and a cartilage template (Sahar et al., 2005). The role of Dlx5 in chondrocyte differentiation is different than that in bone, and this is why we propose that an obvious phenotype was not seen in the PFS. However, we did appreciate a phenotype in the sagittal suture of Dlx5 heterozygous (+/-) mice; this may be attributed to the lack of the necessity for a cartilage template in the sagittal suture and the role of Dlx5 in regulating osteoblast differentiation (Sahar et al., 2005 & Ryoo et al., 1997).

We hypothesize that the phenotype observed in the sagittal sutures of Dlx5 (+/-) mice is due to a decrease in osteoblast differentiation compared to wt controls. In order to test this hypothesis, we bred Dlx5 (+/-) mice with mice transgenic for the 2.3 kb fragment of the collagen Type I promoter fused to a Green Fluorescent Protein (2.3 GFP). The 2.3 GFP promoter has been shown to be an accurate marker of differentiated osteoblastic cells (Kalajzc et al., 2002). However, we have not analyzed the samples.

Although craniosynostosis is a rare finding, it may cause severe malformations and developmental disorders in those that are affected. Since the genetic etiology is unknown in the majority of human patients with sutural growth disturbances, evidence that a Dlx5 signaling pathway is involved in cranial suture fusion, may provide a new candidate gene for analysis in patients with sutural growth disturbances.
The development of our knowledge of the genes involved in suture fusion is essential for progress in the medical and genetic treatment of individuals with cranial suture fusion disorders.
Chapter 4

**Discussion**

Previously, our lab studied PFS fusion in biglycan/decorin double deficient mice. In the study, we found that biglycan and decorin are highly expressed in fusing cranial sutures as well as the dura in overlapping but distinct patterns. The double deficient mice that were studied had patent sutures and severe hypomineralization of frontal and parietal bones. However, mice deficient with either biglycan or decorin separately were absent of suture formation defects.

Similar to Dlx5, both Biglycan and Decorin have been linked to BMP2/4 activity. Recently, it was found that Biglycan deficient calvarial osteoblast-like cells have less cell surface BMP4 binding. This led to a decrease in BMP4 mediated osteoblast differentiation in the Biglycan deficient calvarial osteoblast-like cells compared to the wild-type controls (Chen *et al.*, 2004). Similarly, Parisuthiman *et al.* found that MC3T3-E1 clonal cells expressing higher or lower levels of biglycan show less BMP4 mediated osteoblast differentiation (Parisuthiman *et al.*, 2005). Decorin has also been shown to alter BMP2/4 activity. For example, Gutierrez et al showed a decrease in BMP2 induction of alkaline phosphatase in decorin deficient myoblast cells compared to wild-type controls (Gutierrez *et al.*, 2006).

In this previous study, we hypothesized that the deficiency in PFS fusion that was observed in biglycan/decorin double knock out mice was due to altered BMP4 signaling because we found equal amounts of mRNA expression of BMP4 but markedly reduced expression of Dlx5 in the cranial sutures of biglycan/decorin double deficient
mice. This finding drove our interest in studying the role of Dlx5 in cranial suture fusion.

Our experiments show that Dlx5 is expressed both in the fusing posterior frontal and non-fusing sagittal sutures of mice during the time period of expected suture fusion. Holleville et al. found that Dlx5 is highly expressed at the osteogenic fronts and at the edges of the suture mesenchyme, but not in the suture itself in chick calvariae (Holleville et al., 2003). Although we studied murine sutures, we also found that Dlx5 was expressed in the osteogenic fronts as well as the periosteum and underlying dura but not in the suture space. We also found that 10 day-old heterozygous Dlx5 (+/-) mice had increased space between their bony fronts in the sagittal suture compared to wt controls, and that there was no apparent phenotype in the PFS from the Dlx5 (+/-) heterozygous mice.

Different models have been proposed in regards to the factors responsible for Dlx5 expression in cranial sutures (Figure 9). Choi et al. found that the addition of FGF2 to developing bone fronts in murine sutures stimulated BMP2 expression but that the addition of BMP2 could not induce FGF2 expression. Another finding, was that disruption of Runx2 completely eliminated expression of BMP2 and its downstream genes Dlx5 and Msx2. These results suggested that Runx2 is required in addition to FGF2 to work upstream of BMP2 for the expression of Dlx5 (Choi et al., 2005).

Alternatively, Holleville et al. used a dominant-negative construct (DlxHD-EnR) that interfered with chick Dlx5 transactivating activity in E6 calvaria cells transfected with a constitutively active type I BMP receptor (AcBMPR). Results of their studies showed that DlxHD-EnR prevents the induction of Runx2 activation by BMP signals. They
concluded that Dlx5 or related family members such as Dlx6 are necessary for the initiation of Runx2 in immature chick calvaria mesenchyme triggered by BMP signaling (Holleville et al., 2007). These results contrast with the findings of Choi et al. who found that Runx2 was involved in this cascade prior to activation of BMP2/4. Differences in the results obtained by these authors may be explained by their use of animals from different species (Figure 9).

The increase in spacing between parietal bone fronts in the sagittal sutures from Dlx5 (+/-) mice could be due to a decrease in osteoblast differentiation, changes in apoptosis, and/or changes in proliferation in the sutural complex. Holleville et al. recently showed that Dlx5 does not effect proliferation in immature calvarial cells (Holleville et al., 2007). In addition, Acampora et al. (1999) showed no difference in the apoptic cells in a variety of tissues from the Dlx5 (-/-) compared to wt controls. Therefore taken together, we hypothesize that increased spacing in the sagittal suture seen in the Dlx5 (+/-) mice is due to a defect in osteoblast differentiation. In our study, we started to examine differentiation by the use of mice transgenic for Col I 2.3 GFP, however we have not finished analyzing the samples.

A. Future studies

Future studies may be useful in furthering our knowledge of Dlx5 and its role in cranial suture fusion. Since a phenotype was observed only in the sagittal suture of mutant mice, these studies may involve examining the proliferation, differentiation and apoptosis of cells in the sagittal suture of Dlx5 mutant and wild-type mice.
Additional studies that may enhance our knowledge of Dlx5 may be to knock out the gene postnatally. Because the homozygous mutant has such a severe phenotype, resulting sometimes in death at birth, we propose that the gene be knocked out conditionally by flox Dlx5 and giving a tamoxifen cre postnatally. This would avoid the problem of the lethal phenotype and still allow examination of the role of Dlx5 during suture fusion.

B. Significance

Although craniosynostosis is a rare finding, it may cause severe malformations and developmental disorders in those that are affected. Since the genetic etiology is unknown in the vast majority of human patients with sutural growth disturbances, evidence that a Dlx5 signaling pathway is involved in cranial suture fusion, may provide a new candidate gene for analysis in patients with sutural growth disturbances.

The development of our knowledge of the genes involved in suture fusion is essential for progress in the medical and genetic treatment of individuals with cranial suture fusion disorders.
References


Iseki, S., A. O. Wilkie, et al. (1997). "Fgfr2 and osteopontin domains in the developing skull vault are mutually exclusive and can be altered by locally applied FGF2." Development 124(17): 3375-84.


Figure Legends

Figure 1. Expression of Dlx5 in the cranial sutures. Immunohistochemistry (brown) for Dlx5 in the Posterior frontal suture (A) and Sagittal suture (B) from 10 day-old CD-1 wild-type mice. Sections were counterstained with Hemotoxylin (Blue).

Figure 2. Expression of BMP4 and Dlx5 in the cranial sutures from 21 to 45 day old wild-type mice. Semiquantitative RT-PCR analysis performed for BMP4, Dlx5, and the housekeeping gene 18S on RNA extracted from the posterior frontal and sagittal sutures (A). Relative expression of BMP4 and Dlx5 in the Sagittal/Posterior frontal suture from 35 day-old wild-type mice. RT-PCR products separated by electrophoresis were visualized after ethidium bromide staining under a UV light and photographed in a single field of view with a digital camera. Individual bands on digital micrographs of RT-PCR products were analyzed by densitometer using Image J NIH IMAGE. Intensity for Dlx5 and BMP4 was calculated as a percentage of densitometry values for control bands of 18S. Each primer set was tested previously to assure that amplification was in the linear range by analyzing amplification at several different cycle numbers (B).

Figure 3. H&E stained coronal sections of the posterior frontal suture from 10 day-old wild-type (A) and heterozygous Dlx5 (+/-) (B) mice.

Figure 4. The mean distance in μm and standard error between osteogenic fronts of the posterior frontal suture in 10 day-old Dlx5 (+/+) (mean=36.5; SE=21; n=3) and Dlx5 (+/-) (mean=78.1; SE=27.4; n=3) mice.

Figure 5. H&E stained coronal sections of the sagittal suture from 10 day-old wild-type (A) and heterozygous Dlx5 (+/-) (B) mice.

Figure 6. The mean distance in μm and standard error between parietal bone fronts of the sagittal suture in 10 day-old Dlx5 (+/+) (mean=-45.9; SE=15.5; n=3) and Dlx5 (+/-) (mean=69; SE=27.9; n=3) mice.

Figure 7. Transgenic Dlx5 (+/-) mice were bred with mice transgenic for the 2.3kb fragment of the Collagen Type I alpha 1 promoter fused to a Green Fluorescent Protein (2.3 GFP). GFP expression in the posterior frontal suture of 10 day-old Dlx5 (+/+)/2.3 GFP (+/-) (A) and Dlx5 (+/-)/2.3 GFP (+/-) (B) mice.

Figure 8. Transgenic Dlx5 (+/-) mice were bred with mice transgenic for the 2.3kb fragment of the Collagen Type I alpha 1 promoter fused to a Green Fluorescent Protein (2.3 GFP). GFP expression in the sagittal suture of 10 day-old Dlx5 (+/+)/2.3 GFP (+/-) (A) and Dlx5 (+/-)/2.3 GFP (+/-) (B) mice.

Figure 9. Proposed model of Dlx5 expression in cranial sutures
Figure 1
Expression of Genes in Sag/PF

BMP4

Dlx5

18S

A

Figure 2
Figure 3

**Dlx5 +/- PFS**

![Image A]

**Dlx5 (+/-) PFS**

![Image B]

100 μm
Distance between Osteogenic Bone Fronts in the PFS of 10 day-old Dlx5 (+/+ ) and Dlx5 (+/- ) Heterozygous Mice

Figure 4
**Dlx5 +/+ Sagittal suture**

A

**Dlx5 +/- Sagittal Suture**

B

**Figure 5**
Distance Between Parietal Bone Fronts in 10 day-old Dlx5 (+/+ and Dlx5 (+/-) Heterozygous Mice

Figure 6
2.3 GFP Dlx5 +/+ PFS

Figure 7

2.3 GFP Dlx5 +/- PFS

Figure 7
2.3 GFP Dlx5 +/- Sagittal suture

Figure 8
Models of Factors Involved in the expression of Dlx5 in the Cranial Sutures

- Choi et al.
- Holleville et al.

FGF2 Added to Bone Fronts
- Runx2
  - BMP2
    - Dlx5 and Msx2

BMP
- Dlx5
  - Runx2

Figure 9