

Elsevier Editorial System(tm) for Bone

Manuscript Draft

Manuscript Number: BONE-D-06-00055R3

Title: Impaired Posterior Frontal Sutural Fusion in the Biglycan/ Decorin Double Deficient Mice

Article Type: Full Length Article

Section/Category:

Keywords: Small Proteoglycans; biglycan; decorin; cranial suture; mouse

Corresponding Author: Dr Sunil Wadhwa, DDS, PhD

Corresponding Author's Institution: University of Connecticut School of Dental Medicine

First Author: Sunil Wadhwa, DDS, PhD

Order of Authors: Sunil Wadhwa, DDS, PhD; Yaming Bi, PhD; Ana T Ortiz, DDS; Mildred C Embree, BA; Tina Kilts; Renato V Iozzo, MD; Lynne A Opperman, PhD; Marian F Young, PhD

Manuscript Region of Origin:

Abstract: Biglycan (Bgn) and decorin (Dcn) are highly expressed in numerous tissues in the craniofacial complex. However, their expression and function in the cranial sutures is 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 21-45 days post-natal and in the non-fusing sagittal (S) suture from wildtype (Wt) mice. Our data showed that Bgn and Dcn were expressed in both cranial sutures. We then characterized the cranial suture phenotype in Bgn deficient, Dcn deficient, Bgn/ Dcn double deficient and Wt mice. At embryonic day 18.5, alizarin red/ alcian blue staining showed that the Bgn/ Dcn 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 Dcn deficient mice had no cranial suture abnormalities and immunohistochemistry staining showed increased production of Dcn in the PFS from Bgn deficient mice. To

test possible compensation of Dcn in the Bgn deficient sutures we examined the Bgn/ Dcn double deficient mice and found they had impaired fusion of the PFS. Semi-quantitative RT-PCR analysis of RNA from 35 day-old mice revealed increased expression of Bmp-4 and Dlx-5 in the PFS compared to their non-fusing S suture in Wt tissues and decreased expression of Dlx-5 in both PF and S sutures in the Bgn/ Dcn double deficient mice compared to the Wt mice. Failure of PFS fusion and hypomineralization of the calvaria in the Bgn/Dcn double deficient mice demonstrate these extracellular matrix proteoglycans could have a role in controlling the formation and growth of the cranial vault.

To Whom it May Concern:

Thank you for kindly considering our paper, “Impaired Posterior Frontal Sutural Fusion in the Biglycan/ Decorin Double Deficient Mice”, for publication into *Bone*. The work has not been published previously and is not under consideration for publication elsewhere. In this report, we show that both biglycan and decorin are abundantly expressed in the cranial sutures with overlapping yet distinct patterns of expression in the cranial sutures and in the dura mater. In addition, we show, for the first time, that mice singly deficient in Biglycan or Decorin have no cranial suture abnormalities and that in the absence of Biglycan, the related Small Leucine Repeat Proteoglycan, Decorin is up regulated throughout the posterior frontal suture. The potential for a compensatory role for Decorin in the absence of Biglycan was uncovered in *Biglycan/ Decorin* double deficient mice, which we have shown, have open sutures and severe hypomineralization of both frontal and parietal bones. This new data provides the foundation for the identification of novel candidate genes for patients who have sutural growth disturbances.

Thank you again,

Dr. Sunil Wadhwa DDS, PhD
Assistant Professor
Dept. of Orthodontics
University of Connecticut School of Dental Medicine
Farmington, CT 06030
860-679-4899
wadhwa@uchc.edu

Concern: Resubmission of manuscript Bone-D-06-00055R2

Dr. Masaki Noda
Editor
Bone

Dear Dr. Noda:

You will find enclosed, for resubmission to Bone, a revised version of our manuscript entitled "Impaired Posterior Frontal Sutural Fusion in the Biglycan/ Decorin Double Deficient Mice" (Bone-D-06-00055R2).

The manuscript has been revised based on the comments of the reviewer. Our answers to each of the comments are point by point and are enclosed. We took into account all the comments and modified our manuscript accordingly.

By making the changes recommended by the reviewers, we hope that our manuscript now fulfills the quality requirements for publication in *Bone*.

Sincerely,

Sunil Wadhwa, DDS PhD

Answers to comments

We would like to thank the anonymous reviewer for the valuable remarks, which were used to improve our manuscript. Following these remarks, modifications have been made to the manuscript.

Answers to reviewer 1's comments:

1) "The rationale for the 3 different age groups should be included in Materials & Methods"

We have now included the rationale for using the three different age groups in the revised materials and method.

2) "The down regulation of Dlx-5 expression observed by RT=PCR should also be visible by ISH".

We, too, thought examining Dlx-5 by in-situ hybridization would be a logical and important experimental approach. Towards this goal we obtained probes for Dlx-5 and used them on sections from Wt and Bgn/ Dcn DKO mice. In three separate experiments we could not detect Dlx-5 mRNA in any of the sections. We presumed that in the material we used the absolute levels of Dlx-5 were low and simply not detectable using this procedure. For this reason we turned to RT-PCR using RNA obtained from sutures dissected from the different sutures described in our paper. We feel confident that our dissections were not contaminated and contained only tissue from the PFS and sagittal sutures. Using RT-PCR we could amplify the low levels of Dlx-5 present and then show significant differences in expression levels between the Wt and DKO suture mRNA.

3) Solving the discussion problems perceived by this reviewer and the feeling of a "disjointed collection of statements" by the other reviewer requires more than rewriting the last paragraph.

We have carefully reviewed and revised the discussion.

We thank the reviewers for their helpful comments and hope our paper is now suitable for publication in *Bone*.

Sincerely,

Sunil Wadhwa, DDS, PhD

Impaired Posterior Frontal Sutural Fusion in the Biglycan/ Decorin Double Deficient Mice

Sunil Wadhwa ^{1,2}, Yanming Bi ², Ana T. Ortiz ¹, Mildred C. Embree ², Tina Kilts ²,
Renato Iozzo ³, Lynne A. Opperman ⁴, Marian F. Young ²

¹ **Division of Orthodontics, School of Dental Medicine, UCHC, Farmington, CT 06030**

² **Molecular Biology of Bones and Teeth Section, Craniofacial and Skeletal Diseases Branch, NIDCR, NIH, DHHS Bethesda, MD 20892**

³ **Departments of Pathology and Anatomy and Cell Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107**

⁴ **Center for Craniofacial Research and Diagnosis, Baylor College of Dentistry, Texas A&M University System Health Science Center, Dallas, TX 75246**

Corresponding Author:
Dr. Sunil Wadhwa
Division of Orthodontics
School of Dental Medicine
UCHC, Farmington CT 06030
860-679-4899
Wadhwa@uchc.edu

Key words- Small Proteoglycans, biglycan, decorin, cranial suture and mouse

ABSTRACT

Biglycan (Bgn) and decorin (Dcn) are highly expressed in numerous tissues in the craniofacial complex. However, their expression and function in the cranial sutures is 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 21-45 days post-natal and in the non-fusing sagittal (S) suture from wildtype (Wt) mice. Our data showed that Bgn and Dcn were expressed in both cranial sutures. We then characterized the cranial suture phenotype in *Bgn* deficient, *Dcn* deficient, *Bgn/ Dcn* double deficient, and Wt mice. At embryonic day 18.5, alizarin red/ alcian blue staining showed that the *Bgn/ Dcn* 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 *Dcn* deficient mice had no cranial suture abnormalities and immunohistochemistry staining showed increased production of Dcn in the PFS from *Bgn* deficient mice. To test possible compensation of Dcn in the *Bgn* deficient sutures we examined the *Bgn/ Dcn* double deficient mice and found they had impaired fusion of the PFS. Semi-quantitative RT-PCR analysis of RNA from 35 day-old mice revealed increased expression of *Bmp-4* and *Dlx-5* in the PFS compared to their non-fusing S suture in Wt tissues and decreased expression of *Dlx-5* in both PF and S sutures in the *Bgn/ Dcn* double deficient mice compared to the Wt mice. Failure of PFS fusion and hypomineralization of the calvaria in the *Bgn/Dcn* double deficient mice demonstrate these extracellular matrix proteoglycans could have a role in controlling the formation

and growth of the cranial vault.

Introduction

Biglycan (Bgn) and decorin (Dcn) 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 Dcn 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 Dcn are unknown, however both can bind and modulate members of the TGF-beta superfamily (11).

Bgn deficient mice have impaired postnatal 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). *Dcn* deficient mice do not have obvious skeletal defects, but instead, have skin fragility which is hypothesized to be due to the ability of Dcn 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 Dcn 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 craniofacial 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 lifespan of the animal, except for the posterior frontal suture (PFS), which fuses in a predictable fashion between 21-45 days postnatal (25). In humans the equivalent metopic suture fuses within the first three years postnatal (30, 34). Therefore, the mouse PFS serves as a model of post-natal human sutural growth (33). *Bmp-2/ 4* signaling has been linked to murine PFS fusion by Warren *et al.*, who showed that inhibition 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 S 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 decreased expression of *Dlx-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 (#NIDCR-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*^{-/-} / *Dcn* ^{+/+}) with an *Dcn* heterozygous deficient male (*Bgn*^{+/0} / *Dcn*^{+/-}); *Bgn*^{-/-} deficient males are designed as *Bgn* ^{-/0} 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* ^{0/-} / *Dcn*^{+/-} males were bred to *Bgn* ^{+/-} / *Dcn*^{+/-} 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 21-45 days postnatal) 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 (5). PCR products were resolved by electrophoresis through 1.8% agarose gels, yielding bands of 212 bp for the Wt *Bgn* allele, 310 bp for the disrupted *Bgn* allele, 161 bp for the Wt *Dcn* allele, and 238 bp for the disrupted *Dcn* allele.

Alizarin Red and Alcian Blue Staining of Mice Littermates

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* +/- / *Dcn*+/+) mice as our controls. 18.5 d.p.c. littermates obtained from 5 litters were euthanized, and then their skin, muscle, and fat were removed. The animals were fixed in 100 % ethanol for 4 days and then placed in acetone for 3 days. Mice were then stained with alizarin red (0.09%) and alcian 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, Wt, *Bgn* deficient, *Dcn* deficient and *Bgn*/*Dcn* double deficient, with an n=3 for each group. The mice were sacrificed and the soft tissues on their heads were removed. The calvaria were radiographed using a Faxitron MX-20 Specimen Radiography System (Faxitron X-ray Corp., Wheeling, IL, USA) at energy of 90 kV for 20 s. The images were captured with Eastman Kodak Co.

X-OMAT TL (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 Wt, *Bgn* deficient, *Dcn* deficient and *Bgn/ Dcn* double deficient mice was first measured. The mean length \pm the standard error of the PFS was 11.0 \pm .6 mm for Wt, 10.3 \pm 0.3 mm from *Bgn* deficient, 10.7 \pm 1.2 mm from *Dcn* deficient and 11.7 \pm 0.3 mm for *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 quantitated by using Image J NIH IMAGE.

Histology and Immunohistochemistry

Wt, *Dcn* deficient, *Bgn* deficient, and *Bgn/ Dcn* double deficient mice were sacrificed by CO₂ 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 minutes, 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) 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 minutes to block endogenous peroxidase activity. In order to expose the antigen, sections were predigested in chondroitinase ABC (cat# KE01502, Seikagaku Corp, Tokyo, Japan) at a concentration of 0.015 units/ml for 1 hour at 37 C. Incubating the sections in 10% goat serum for 30 minutes reduced non-specific binding. Sections were then incubated overnight at 4° C with primary antibody at a 1: 200 dilution. Dcn and Bgn antibodies (respectively LF 113 and LF 106) were kind gifts from Larry Fisher (NIDCR, NIH). Biotinylated rabbit anti-goat secondary antibody 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 IgE.

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 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-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>). The primers used for RT-PCR were for *Gapdh* 5'gagaggccctatcccaact3' (forward) 5'gtgggtgcagcgaactttat3'(reverse), for *Bmp-4* 5'ggtgggtttgcttcactta3' (forward) and 5'atgggagcgaagttgtgtacc3' (reverse), for *Dlx-5* 5'ccgggacgctttattagatg3' (forward) and 5'tggacactatcaatggtgcc3' (reverse), for biglycan 5'acctgtcccccttcactct3' (forward) and 5'ccgtgtgtgtgtgtgtgt3' (reverse), for decorin 5'ccaacataactcgatccct3'(forward) and 5'tgtccaagtgagttccctc3'(reverse) and for 18s 5'catgtggtgtgaggaaa3'(forward) and 5'gccagagactcatttct3'(reverse). 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 50 cycles (Dlx-5 and Bmp-4) of 1 min at 94°C, 20 seconds at 57°C and 30 seconds at 72°C followed by a 7 minute extension at 70°C. The reaction was chilled to 4°C afterwards 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 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 *Dlx-5*, *Bgn*, *Dcn* and *Bmp-4* were 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-Alcian Blue Staining of E 18.5 Littermates

Alizarin red-alcian 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 (Figure 1 A-D). Alizarin red and alcian 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 (Figure 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 (Figure 2 B-E). In the PFS from the Wt and *Bgn* deficient mice, Dcn was found in the underlying dura (Figure 2 C, E). In the Wt mice, Dcn appeared to have a more restricted 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 *Dcn* 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, Dcn deficient, Bgn/ Dcn Double Deficient and Wt Mice

In order to determine what role Bgn and Dcn deficiency had on later developmental stages, 60 day-old Wt and *Bgn/ Dcn* double deficient mice were examined. Faxitron analysis showed that the *Bgn/ Dcn* 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 *Dcn* deficient mice, all had PFS that were 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/ Dcn* double deficient mice compared to the other groups ($p < 0.001$) (Figure 3 a). Besides a decrease in mineralization of the PFS, the decrease in radio-opacity in the PFS from the *Bgn/Dcn* 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 (Figure 3C), *Dcn* deficient (Figure 3D), *Bgn/ Dcn* double deficient (Figure 3E) and Wt mice (Figure 3B) were examined. This analysis revealed PFS patency only in the *Bgn/ Dcn* double deficient samples compared to the other groups.

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 semiquantitative 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 (Figure 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 sutural 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 Bmp-4 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 up regulated 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 the ability of Bmp-2 to induce mineralization, as measured by alizarin red accumulation, was decreased 59% 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 marked reduction of *Dlx-5* expression 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 BMP2/ 4 regulation of the osteoblast differentiation markers, *Runx-2*, *Osterix*, 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).

In conclusion, we show that the alteration in the extracellular matrix of the posterior frontal suture causes impaired fusion. These new data provide the foundation for the identification of novel candidate genes for patients who have sutural growth disturbances.

Acknowledgments:

This research was supported by the National Institute of Dental and Craniofacial Research in the Intramural Program of the NIH.

References

1. Ameye, L., and Young, M. F. Mice deficient in small leucine-rich proteoglycans: novel in vivo models for osteoporosis, osteoarthritis, Ehlers-Danlos syndrome, muscular dystrophy, and corneal diseases. *Glycobiology* 12:107R-16R; 2002.
2. Bi, Y., Stuelten, C. H., Kilts, T., Wadhwa, S., Iozzo, R. V., Robey, P. G., Chen, X. D., and Young, M. F. Extracellular matrix proteoglycans control the fate of bone marrow stromal cells. *J Biol Chem*; 2005.
3. Chen, L., Li, D., Li, C., Engel, A., and Deng, C. X. A Ser250Trp substitution in mouse fibroblast growth factor receptor 2 (Fgfr2) results in craniosynostosis. *Bone* 33:169-78; 2003.
4. Chen, X. D., Fisher, L. W., Robey, P. G., and Young, M. F. The small leucine-rich proteoglycan biglycan modulates BMP-4-induced osteoblast differentiation. *Faseb J* 18:948-58; 2004.
5. Corsi, A., Xu, T., Chen, X. D., Boyde, A., Liang, J., Mankani, M., Sommer, B., Iozzo, R. V., Eichstetter, I., Robey, P. G., Bianco, P., and Young, M. F. Phenotypic effects of biglycan deficiency are linked to collagen fibril abnormalities, are synergized by decorin deficiency, and mimic Ehlers-Danlos-like changes in bone and other connective tissues. *J Bone Miner Res* 17:1180-9; 2002.
6. Dabovic, B., Chen, Y., Colarossi, C., Zambuto, L., Obata, H., and Rifkin, D. B. Bone defects in latent TGF-beta binding protein (Ltbp)-3 null mice; a role for Ltbp in TGF-beta presentation. *J Endocrinol* 175:129-41; 2002.
7. Danielson, K. G., Baribault, H., Holmes, D. F., Graham, H., Kadler, K. E., and Iozzo, R. V. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J Cell Biol* 136:729-43; 1997.
8. Funderburgh, J. L., Hevelone, N. D., Roth, M. R., Funderburgh, M. L., Rodrigues, M. R., Nirankari, V. S., and Conrad, G. W. Decorin and biglycan of normal and pathologic human corneas. *Invest Ophthalmol Vis Sci* 39:1957-64; 1998.
9. Gutierrez, J., Osses, N., and Brandan, E. Changes in secreted and cell associated proteoglycan synthesis during conversion of myoblasts to osteoblasts in response to bone morphogenetic protein-2: Role of decorin in cell response to BMP-2. *J Cell Physiol*; 2005.
10. Harris, S. E., Guo, D., Harris, M. A., Krishnaswamy, A., and Lichtler, A. Transcriptional regulation of BMP-2 activated genes in osteoblasts using gene expression microarray analysis: role of Dlx2 and Dlx5 transcription factors. *Front Biosci* 8:s1249-65; 2003.
11. Hildebrand, A., Romaris, M., Rasmussen, L. M., Heinegard, D., Twardzik, D. R., Border, W. A., and Ruoslahti, E. Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. *Biochem J* 302 (Pt 2):527-34; 1994.
12. Hocking, A. M., Shinomura, T., and McQuillan, D. J. Leucine-rich repeat glycoproteins of the extracellular matrix. *Matrix Biol* 17:1-19; 1998.
13. Holleville, N., Quilhac, A., Bontoux, M., and Monsoro-Burq, A. H. BMP signals regulate Dlx5 during early avian skull development. *Dev Biol* 257:177-89; 2003.

14. Iozzo, R. V. The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins. *J Biol Chem* 274:18843-6; 1999.
15. Iozzo, R. V. The family of the small leucine-rich proteoglycans: key regulators of matrix assembly and cellular growth. *Crit Rev Biochem Mol Biol* 32:141-74; 1997.
16. Kamiya, N., Shigemasa, K., and Takagi, M. Gene expression and immunohistochemical localization of decorin and biglycan in association with early bone formation in the developing mandible. *J Oral Sci* 43:179-88; 2001.
17. Kim, Y. J., Lee, M. H., Wozney, J. M., Cho, J. Y., and Ryoo, H. M. Bone morphogenetic protein-2-induced alkaline phosphatase expression is stimulated by *Dlx5* and repressed by *Msx2*. *J Biol Chem* 279:50773-80; 2004.
18. Kuwabara, M., Takuma, T., Scott, P. G., Dodd, C. M., and Mizoguchi, I. Biochemical and immunohistochemical studies of the protein expression and localization of decorin and biglycan in the temporomandibular joint disc of growing rats. *Arch Oral Biol* 47:473-80; 2002.
19. Lee, M. H., Kim, Y. J., Kim, H. J., Park, H. D., Kang, A. R., Kyung, H. M., Sung, J. H., Wozney, J. M., Kim, H. J., and Ryoo, H. M. BMP-2-induced *Runx2* expression is mediated by *Dlx5*, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of *Dlx5* expression. *J Biol Chem* 278:34387-94; 2003.
20. Lee, M. H., Kwon, T. G., Park, H. S., Wozney, J. M., and Ryoo, H. M. BMP-2-induced *Osterix* expression is mediated by *Dlx5* but is independent of *Runx2*. *Biochem Biophys Res Commun* 309:689-94; 2003.
21. Matheson, S., Larjava, H., and Hakkinen, L. Distinctive localization and function for lumican, fibromodulin and decorin to regulate collagen fibril organization in periodontal tissues. *J Periodontal Res* 40:312-24; 2005.
22. Matsuura, T., Duarte, W. R., Cheng, H., Uzawa, K., and Yamauchi, M. Differential expression of decorin and biglycan genes during mouse tooth development. *Matrix Biol* 20:367-73; 2001.
23. Miura, M., Chen, X. D., Allen, M. R., Bi, Y., Gronthos, S., Seo, B. M., Lakhani, S., Flavell, R. A., Feng, X. H., Robey, P. G., Young, M., and Shi, S. A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells. *J Clin Invest* 114:1704-13; 2004.
24. Nacamuli, R. P., Song, H. M., Fang, T. D., Fong, K. D., Mathy, J. A., Shi, Y. Y., Salim, A., and Longaker, M. T. Quantitative transcriptional analysis of fusing and nonfusing cranial suture complexes in mice. *Plast Reconstr Surg* 114:1818-25; 2004.
25. Opperman, L. A. Cranial sutures as intramembranous bone growth sites. *Dev Dyn* 219:472-85; 2000.
26. Opperman, L. A., and Rawlins, J. T. The extracellular matrix environment in suture morphogenesis and growth. *Cells Tissues Organs* 181:127-35; 2005.
27. Parisuthiman, D., Mochida, Y., Duarte, W. R., and Yamauchi, M. Biglycan modulates osteoblast differentiation and matrix mineralization. *J Bone Miner Res* 20:1878-86; 2005.
28. Tenorio, D. M., Santos, M. F., and Zorn, T. M. Distribution of biglycan and decorin in rat dental tissue. *Braz J Med Biol Res* 36:1061-5; 2003.

29. Theocharis, A. D., Karamanos, N. K., Papageorgakopoulou, N., Tsiganos, C. P., and Theocharis, D. A. Isolation and characterization of matrix proteoglycans from human nasal cartilage. Compositional and structural comparison between normal and scoliotic tissues. *Biochim Biophys Acta* 1569:117-26; 2002.
30. Vu, H. L., Panchal, J., Parker, E. E., Levine, N. S., and Francel, P. The timing of physiologic closure of the metopic suture: a review of 159 patients using reconstructed 3D CT scans of the craniofacial region. *J Craniofac Surg* 12:527-32; 2001.
31. Wadhwa, S., Embree, M. C., Bi, Y., and Young, M. F. Regulation, regulatory activities, and function of biglycan. *Crit Rev Eukaryot Gene Expr* 14:301-15; 2004.
32. Warren, S. M., Brunet, L. J., Harland, R. M., Economides, A. N., and Longaker, M. T. The BMP antagonist noggin regulates cranial suture fusion. *Nature* 422:625-9; 2003.
33. Warren, S. M., Greenwald, J. A., Spector, J. A., Bouletreau, P., Mehrara, B. J., and Longaker, M. T. New developments in cranial suture research. *Plast Reconstr Surg* 107:523-40; 2001.
34. Weinzwieg, J., Kirschner, R. E., Farley, A., Reiss, P., Hunter, J., Whitaker, L. A., and Bartlett, S. P. Metopic synostosis: Defining the temporal sequence of normal suture fusion and differentiating it from synostosis on the basis of computed tomography images. *Plast Reconstr Surg* 112:1211-8; 2003.
35. Wilda, M., Bachner, D., Just, W., Geerkens, C., Kraus, P., Vogel, W., and Hameister, H. A comparison of the expression pattern of five genes of the family of small leucine-rich proteoglycans during mouse development. *J Bone Miner Res* 15:2187-96; 2000.
36. Xu, T., Bianco, P., Fisher, L. W., Longenecker, G., Smith, E., Goldstein, S., Bonadio, J., Boskey, A., Heegaard, A. M., Sommer, B., Satomura, K., Dominguez, P., Zhao, C., Kulkarni, A. B., Robey, P. G., and Young, M. F. Targeted disruption of the biglycan gene leads to an osteoporosis-like phenotype in mice. *Nat Genet* 20:78-82; 1998.

Figure Legends

Figure 1. Alizarin red/alcian blue staining of E18.5 littermates. a, c. Control *Bgn* +/- *Dcn* +/- mice. b, d. *Bgn*/*Dcn* double deficient mice.

Figure 2. Expression of *Bgn* and *Dcn* in cranial sutures. A. Semiquantitative RT-PCR analysis performed for *Dcn* and *Bgn* on RNA extracted from the sagittal (S) suture and the posterior frontal suture (PFS) from 21, 25 and 35 day-old wildtype (Wt) mice. b-e . Immunohistochemistry (brown) for biglycan (B, D) 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).

Figure 3. Analysis of radio-opacity and histology of the PFS from Wt, *Bgn* deficient, *Dcn* deficient, and *Bgn*/*Dcn* 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, *Dcn* deficient, and *Bgn*/*Dcn* 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*/*Dcn* 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), *Dcn* deficient (d), and *Bgn*/*Dcn* double deficient (e) mice.

Figure 4. Semi-quantitative RT-PCR performed for *Bmp-4* and *Dlx-5* on RNA extracted from the sagittal (S) suture and the posterior frontal suture (PFS) from 35 day-old

wildtype (Wt) and Bgn/ *Dcn* double deficient mice. RT-PCR of Gapdh served as a normalizing control.

Summary of Craniofacial Anomalies in Bgn, Dcn and Bgn/Dcn Deficient Mice Compared to Wt

Genotype	E 18.5 Calvaria	Post-natal PF fusion	Suture Gene/protein expression
Wt	normal	normal	Dlx-5 and Bmp-4 mRNA higher in PF vs. S
Bgn Deficient	ND	normal	Dcn protein higher in Bgn Deficient
Dcn Deficient	ND	normal	Bgn protein unaffected
Bgn/Dcn Deficient	Hypomineralized	patent suture	Dlx-5 mRNA absent in PF and S sutures

Table 1. Normal Wt mice and mice deficient in Bgn, Dcn or both (Genotype, column 1) were analyzed embryonic day 18.5 (E 18.5) by alizarin red/ alcian blue staining (column 2) or post-natally (60 days after birth) by Faxitron 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.

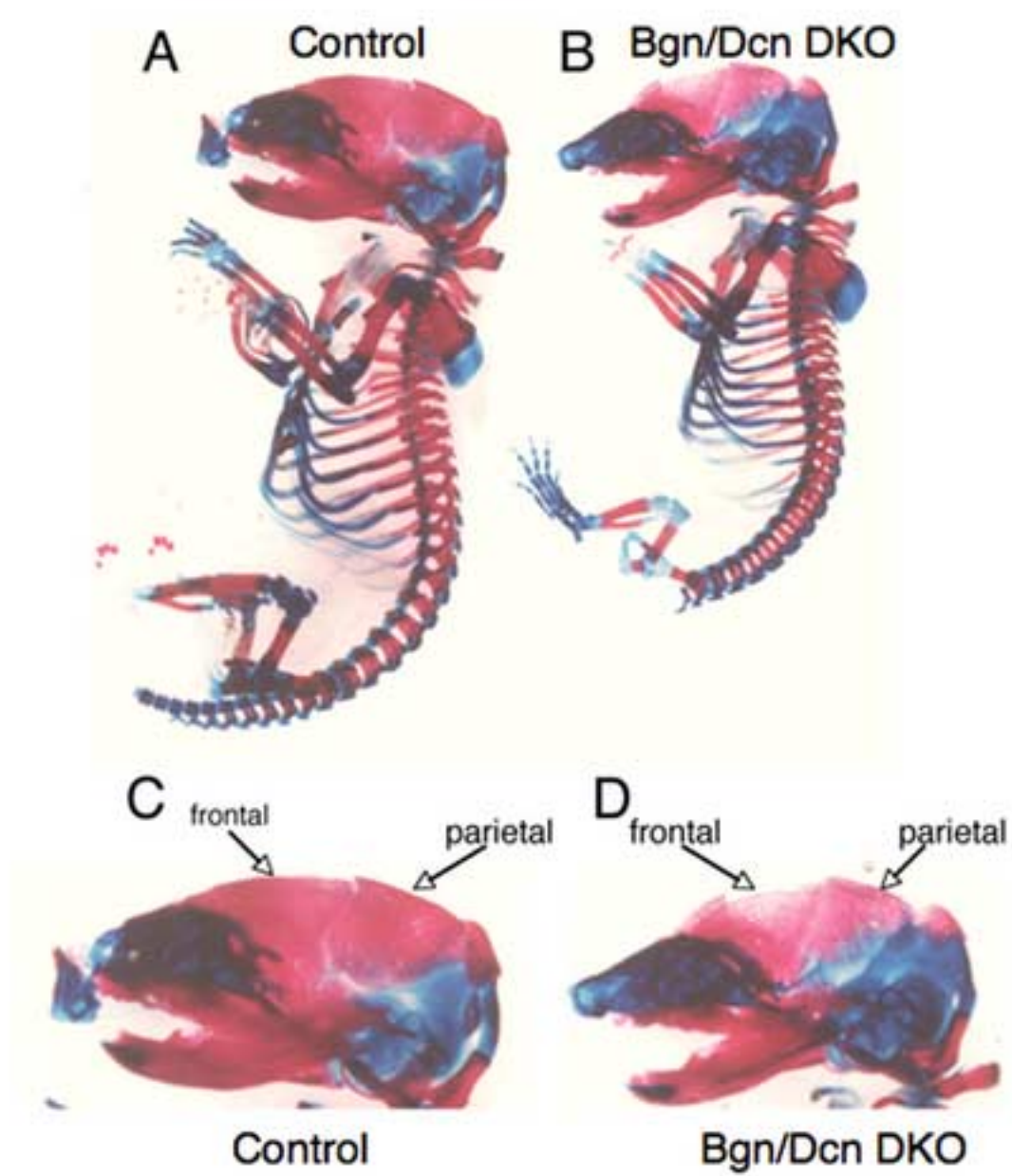


Figure 1

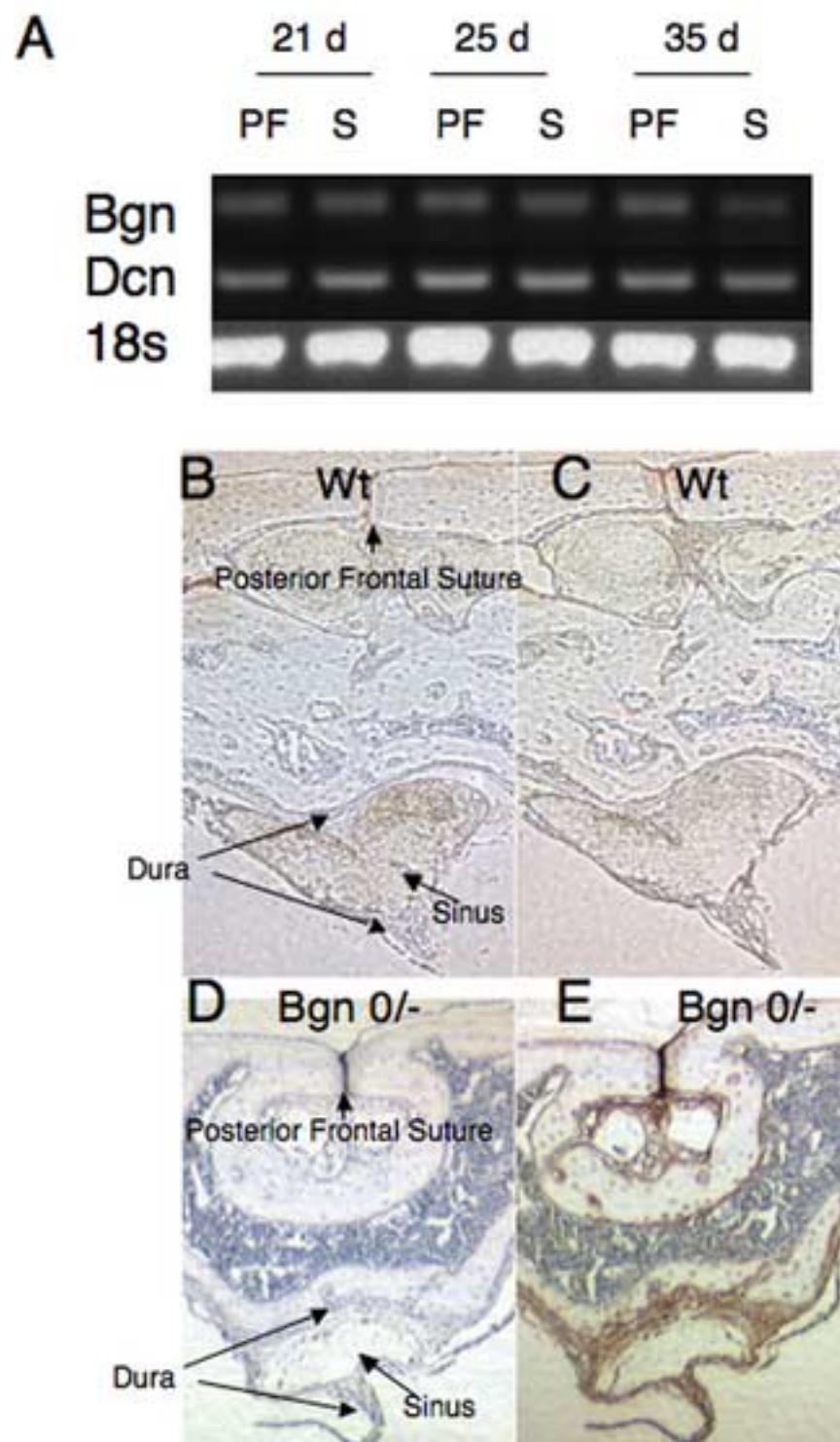


Figure 2

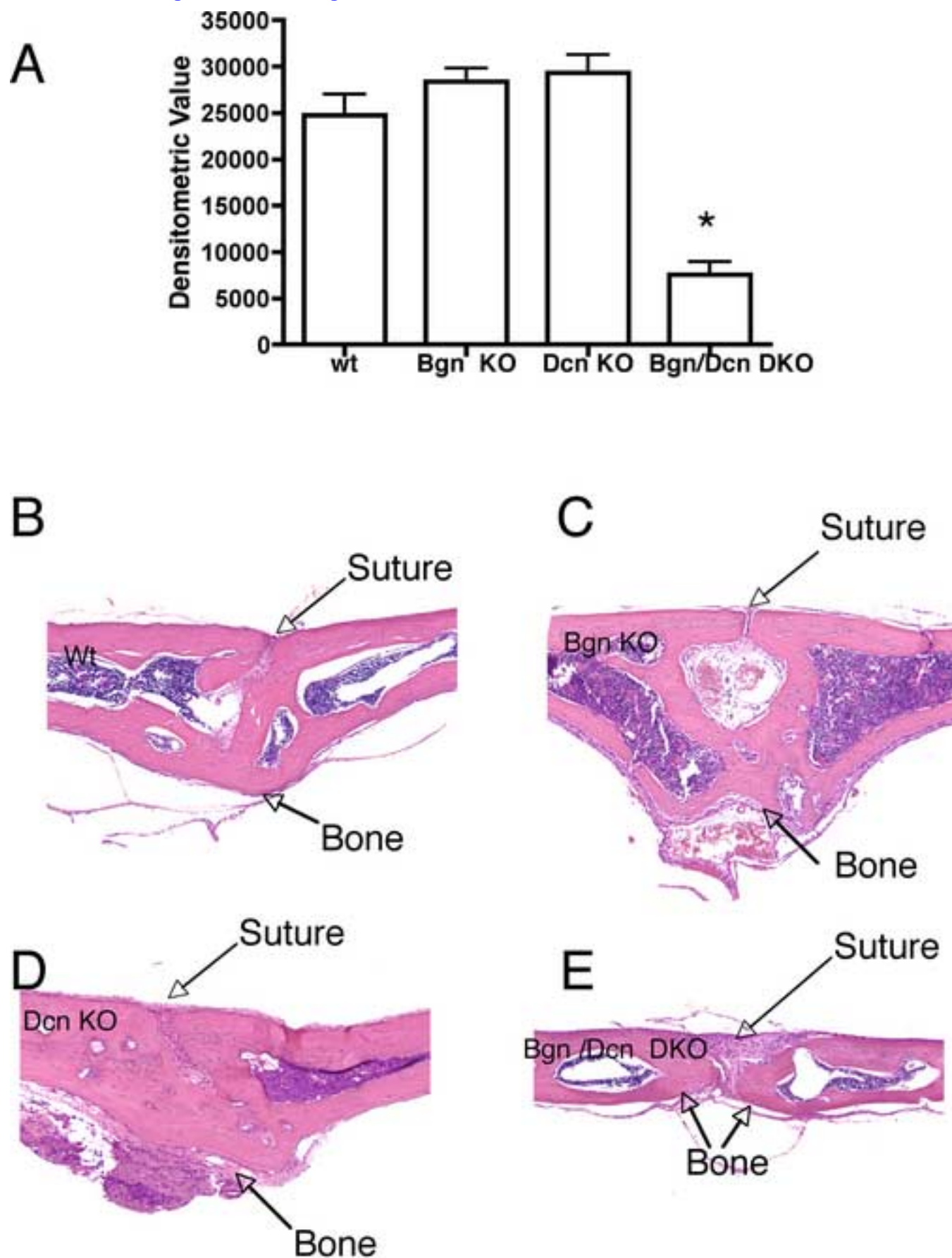


Figure 3

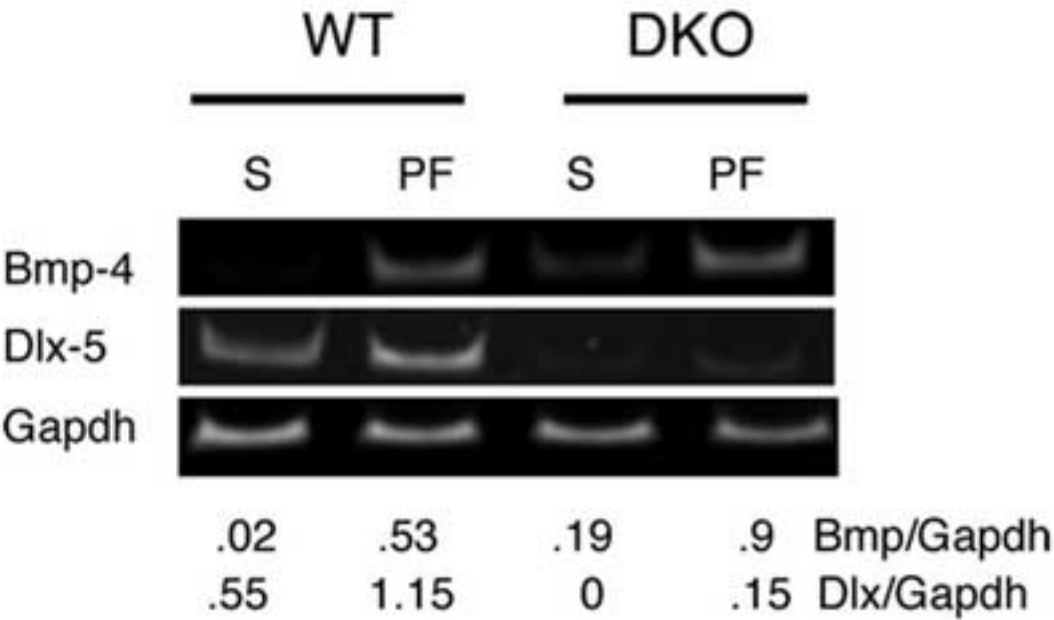


Figure 4