Published in final edited form as:

Bone. 2013 March ; 53(1): 284–293. doi:10.1016/j.bone.2012.11.019.

Rapid Re-synostosis Following Suturectomy in Pediatric Mice is Age and Location Dependent

Christopher Hermann, M.S.^{1,2,3}, Kelsey Lawrence¹, Rene Olivares-Navarrete, D.D.S., Ph.D.^{1,2}, Joseph K. Williams, M.D.⁴, Robert E. Guldberg¹, Barbara D. Boyan, Ph.D.^{1,2,3}, and Zvi Schwartz, D.M.D., Ph.D.^{1,5}

¹Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA

²Wallace H. Coulter Department of Biomedical Engineering at Georgia Tech and Emory University, Georgia Institute of Technology, Atlanta, GA

³Emory University School of Medicine, Atlanta, GA

⁴Children's Healthcare of Atlanta, Atlanta, GA

⁵Department of Periodontics, University of Texas Health Science Center at San Antonio, San Antonio, TX

Abstract

Craniosynostosis is the premature fusion of the cranial sutures early in development. If left untreated, craniosynostosis can lead to complications resulting from cranial deformities or increased intracranial pressure. The standard treatment involves calvarial reconstruction, which in many cases undergoes rapid re-synostosis. This requires additional surgical intervention that is associated with a high incidence of life threatening complications. To better understand this rapid healing, a pediatric mouse model of re-synostosis was developed and characterized. Defects (1.5 mm by 2.5 mm) over the posterior frontal suture were created surgically in weanling (21 days post-natal) and adolescent (50 days post-natal) C57Bl/6J mice. In addition, defects were created in the frontal bone lateral to the posterior frontal suture. The regeneration of bone in the defect was assessed using advanced image processing algorithms on micro-computed tomography scans. The genes associated with defect healing were assessed by real-time PCR of mRNA isolated from the tissue present in the defect. The results showed that the weanling mouse healed in a biphasic process with bone bridging the defect by post-operative (post-op) day 3 followed by an increase in the bone volume on day 14. In adolescent mice, there was a delay in bone bridging across the defect, and no subsequent increase in bone volume. No bridging of the defect by 14 days post-op was seen in identically sized defects placed lateral to the suture in both a weanling and adolescent animals. This study demonstrates that bone regeneration in the cranium is both age and location dependent. Rapid and robust bone regeneration only occurred when the defect was created over the posterior frontal suture in immature weanling mice.

© 2012 Elsevier Inc. All rights reserved.

Corresponding Author Contact Information: Barbara D. Boyan, Ph.D. Wallace H. Coulter Department of Biomedical Engineering Georgia Institute of Technology 315 Ferst Drive NW Atlanta, GA 30332-0363 Phone: 404-385-4108 FAX: 404-894-2291 barbara.boyan@bme.gatech.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Craniosynostosis; Cranial defect; Micro-CT; Endochondral ossification; Re-synostosis

INTRODUCTION

Craniosynostosis is the pathologic premature fusion of the cranial sutures early in development, occurring in approximately one in 1500-2500 births [1-3]. There are over 100 syndromic mutations known to cause craniosynostosis known, but most commonly this premature fusion results from a non-syndromic cause [3]. If left untreated, craniosynostosis can result in complications resulting from cranial deformities and/or increased intracranial pressure [4]. These complications include blindness, deafness, developmental delays, seizures, and in extreme cases, death [5, 6].

Both non-syndromic and syndromic forms of craniosynostosis frequently require complex calvarial remodeling to prevent neurologic complications and restore normal cranial vault geometry [4, 7]. Patients undergoing this operation are usually under 12 months of age [7, 8]. The procedure often lasts between four and five hours, requires blood transfusions, and several days in the hospital, including time in the intensive care unit. Historically, surgical intervention for the treatment of craniosynostosis involved the removal of only the fused suture; however, this approach has been abandoned because the results were temporary. In many cases the bone reformed including synostosis of the pathologically fused suture.

This rapid re-growth is called re-synostosis and occurs in up to 40% of patients who undergo cranial vault reconstruction [9, 10]. Re-synostosis typically requires additional surgical intervention to reconstruct the skull in an attempt to restore a normal cranial vault and/or relieve the increase in intracranial pressure. While the complications from primary cranial vault surgery are relatively low, subsequent procedures to treat re-synostosis are associated with a high (~13%) incidence of life-threatening complications, which include encephalitis, meningitis, dural tears, intracranial hemorrhages, and cerebrospinal fluid leaks [5, 6, 11, 12]. Patients under six months of age and who have certain syndromic forms of craniosynostosis are at elevated risk for re-synostosis [5, 6].

In order to better understand the mechanisms responsible for this rapid re-synostosis, it is necessary to have clinically relevant animal models that exhibit this rapid bone growth. Rabbits displaying craniosynostosis have been used to study suture re-fusion because of their size advantage and because the craniosynostosis is naturally occurring, but unfortunately they are not commercially available [13-16]. There is a growing number of genetically engineered strains that display craniosynostosis [17-19], providing an opportunity to investigate mechanisms that contribute to rapid bone growth in the cranium following suturectomy. However, many instances of craniosynostosis are non-syndromic, indicating the need to develop an animal model that exhibits differences in bone regeneration in the cranium as a function of age and location of the defect in a manner comparable to children.

Mice are an attractive option because of the wealth of information related to cranial growth and development in this species. Previous studies used defects placed over the posterior frontal suture in adult mice as a model for re-synostosis; however, these defects did not completely heal [19]. One possibility for this is that age dependent differences in signaling via the dura alter both suture development and re-healing of calvarial defects [13, 20-25].

The overall goal of this study was to determine if healing of cranial defects is age and location dependent. To do this, we developed a pediatric murine model of rapid re-synostosis. Our hypothesis was that a defect over the posterior frontal suture in a weanling 21-day-old mouse will undergo rapid re-synostosis, whereas it will fail to do so in older mice. Moreover, defects not associated with the cranial suture will fail to heal with bone in weanling mice. Two time points were selected based on our previous work showing that at weaning (21 days post-natal), the posterior frontal suture in C57Bl/6J mice had fused but not mineralized whereas in adolescent mice (50 days post-natal) the posterior frontal suture has completed fusion and mineralization, but these mice have not reached sexual maturity [26]. Defect healing was assessed using image processing algorithms developed and validated previously [27]. Gene expression associated with defect healing was examined using real-time PCR.

METHODS

Surgical Model

Under the approval of the Georgia Tech IACUC, male C57Bl/6J mice were used for all surgeries (Jackson Labs, Bar Harbor, ME). All mice were housed in a light and temperature controlled environment with ad libitum access to food and water. At the time of surgery all mice were either 21 days old (weanling) or 50 days old (adolescent). Weanling mice were shipped with natural birth mothers and were weaned at the time of surgery. Mice were anesthetized to a surgical plane with isoflurane for all surgical procedures and preparation. Hair was removed from the top of the skull starting between the eyes and moving posterior 2 cm with depilatory cream (Nair, Churchill & Dwight, Princeton, NJ). The skin was washed with water taking care to ensure no cream or water came in contact with the animals' eyes. The skin was disinfected with three alternating washes of chlorhexidine and isopropanol.

A 1 cm midline incision was made beginning 5 mm posterior to the eyes. The skin and periosteum were elevated from the skull using blunt dissection. Under 28x magnification, the interfrontal ridge, the posterior frontal suture, and the bregma were identified (Figure 1A). Using these landmarks as a reference, a custom made template was placed above the posterior frontal suture and served as a size reference during the creation of the defect. The template contained a central 1.5 by 2.5 mm region, and centering notches that served as a reference for the appropriate defect size throughout surgery. All defects were created using a piezoelectric bone cutting instrument, ensuring that the underlying dura was not damaged (Piezotome, Saltec Acteon, Merignac, France). Piezoelectric instruments cut only through hard tissue by using very high frequency, low amplitude displacement vibrations [28]. This allows for a very controlled cut through bone, while minimizing the damage to the underlying dura. This is of particular concern as damaging the dura can impair the healing of the overlying bone.

For mice in the posterior frontal defect group, the suture was completely removed and the template was referenced to ensure the correct defect size during the procedure (Figures 1A-B). The defect was centered over the posterior frontal suture, posterior to the interfrontal ridge, and anterior to the bregma (Figure 1B). Care was taken to not damage the highly vascularized interfrontal ridge as damage to this structure leads to exsanguination. Intraoperative and μ CT images illustrate the ability to accurately create a defect with the appropriate dimensions and location of the defect, while maintaining the integrity of the underlying dura (Figures 1C-D). Any mice with a dural tear were withdrawn from the study and euthanized prior to recovering from anesthesia. The skin was closed with 5-0 silk sutures and the mice were allowed to recover from anesthesia. Post-operative (post-op) analgesia was provided via sub-cutaneous injection of buprenorphine (0.03-0.06 mg/kg) every 8-12 hours for the first 72 hours post-op. Mice were given access to ground,

moistened food for the first 24 hours after surgery to encourage food consumption and weight gain. Any mice displaying age adjusted weight loss greater than 10% or signs of distress were withdrawn from the study and euthanized.

Analysis of Bone Formation

Post-natal day 21 male mice were used to establish the time-course of posterior frontal defect healing in weanling animals. Following surgery, five mice per day were randomized for euthanasia on the following post-operative days: 1, 2, 3, 4, 5, 7, 14, and 21 days. Immediately following euthanasia the skull was dissected from soft tissues, imaged using μ CT, and snap frozen in liquid nitrogen for further analysis by real-time PCR or histology. The approximate region of the posterior frontal defect was identified by moving 4.5 mm posterior from the posterior most molars. The samples were imaged within a 21 mm sample holder using μ CT (isotropic voxels 21 μ m in size, an energy of 55 kVp [Viva CT 40, Scanco Medical, Bruttisellen, Switzerland]). The exact location of the defect was identified with the aid of 3D reconstructions created using a global threshold. These images were then converted to 2.5 mm by 3.5 mm DICOM images for analysis.

The extent of bone regeneration in the defect was quantified using our novel image processing algorithms previously validated with serial histology [27]. Briefly, the algorithm uses a combination of global threshold and the active contour snake algorithm to segment the heterogeneous bone from the μ CT images. Slight modifications to the algorithm allowed for segmentation of multiple bones in an image, as compared to the original algorithm that was limited to two bones. Segmentation boundaries identified by the algorithm were then used to quantify the average defect width, the average thickness of the bones of the defect, the volume of bone in the defect, and the defect mineral content. The defect mineral content was used as an indicator of bone mineralization and was calculated by converting pixel intensities to that of a known hydroxyapatite standard, giving a density value of each pixel relative to hydroxyapatite. The bone volume and mineral content contained within the defect were normalized to the amount of bone found in an intact suture at the time of surgery (21 days post-natal) and a fully mineralized suture (50 days post-natal). All evaluations were performed by a blinded reviewer.

Representative calvaria from the weanling posterior frontal suture defect group on post-op days 5 and 14 were fixed in 10% neutral buffered formalin and decalcified using 12% EDTA, changing the solution every 2-3 days. Complete decalcification was verified using plain x-ray (Faxitron X-ray, Lincolnshire, IL) before and after decalcification. The samples were trimmed, dehydrated using ethanol, and then embedded in paraffin. After establishing the location of the defect with histology, 7 μ m thick sections were made and stained with either haematoxylin and eosin (H&E) or Safranin-O with a fast green counter stain. The specimens were imaged at 10X and 60X magnification with a Leica DMLB microscope (Leica Microsystems, Bannockburn, IL).

To investigate whether rapid healing of the craniotomy defect was age and location specific, we created identically sized defects over the posterior frontal suture in weanling (21 days post-natal) mice, a posterior frontal suture defect in adolescent (50 days post-natal) mice, a defect lateral to the posterior frontal suture in weanling (21 days post-natal) mice, and a defect lateral to the posterior frontal suture in adolescent (50 days post-natal) mice. The lateral defects were identical in size to the posterior frontal defects and were placed 1 mm lateral to the posterior frontal suture. They were centered between the interfrontal ridge and the coronal sutures, taking care not to damage either structure. The side of the defect (left or right side) was randomized between mice. All mice were given the same post-operative care as described above and five mice in each group were randomized for euthanasia on post-operative days 2, 5, and 14. The skulls were immediately imaged with μ CT and snap frozen.

The extent of defect healing was quantified using the same image processing algorithms described above.

Analysis of Gene Expression

Real-time PCR was performed to assess expression of genes associated with rapid defect healing in the posterior frontal defect in weanling mice. At each time point, a 1.5 mm by 2.5 mm region of tissue at the defect site was removed under 4x magnification. The exact location of the defect was verified by comparing the appearance of the skull under magnification to 3D renderings of the μ CT scans. The dura was meticulously dissected from the defect bone and the tissues were ground using a mortar and pestle. RNA was extracted from the defect tissue, purified, and quantified from the defect tissue as described previously [29]. A cDNA library was made using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with 500 ng starting mRNA. The samples were diluted 1:20 in sterile nuclease free water. The starting quantity of the samples was calculated based on a 1:3 serially diluted standard pooled from samples of each time point. The starting quantity of all mRNAs was normalized by the housekeeping gene GAPDH.

Statistical Analysis

The Gaussian distributions of the real-time PCR and μ CT results were first verified using the Kolmogorov-Smirnov normality test. Statistical significance was determined using a 1 way ANOVA and the significance among groups was determined by a multiple comparison test with Bonferroni's modification where appropriate (GraphPad Prism, GraphPad Software, La Jolla, CA). Statistical significance for the normalized comparison to the intact suture was using the Mann Whitney U-test test. A $p < 0.05$ was considered to be significant. All results are represented as the mean $\pm$ the standard error of the mean. The sample size ($n=5$) for all experiments was determined by a prospective power analysis based on the variance of pilot surgeries.

RESULTS

Provided there were no intraoperative complications during the creation of the defect, all mice tolerated the surgical procedure well. A 5% intraoperative mortality rate occurred from complications with anesthesia or from aspiration of the irrigation solution. One mouse out of the over 100 included in this study was euthanized due to dural tear, and one was withdrawn because to failure to gain weight.

The analysis of the μ CT images using our image processing algorithms showed that the weanling posterior frontal defects healed rapidly in a biphasic manner. The width of the defect remained constant for the first two days post-op at approximately 1.2 mm and, then decreased to approximately 0.4 mm for the remainder of the time points (Figure 2A). The defect mineral content and bone volume remained constant through post-op day 7 and then increased to levels seen in an intact suture (Figures 2B-C). Both parameters more than doubled between days 7 to 14. Similarly, the average thickness of the defect remained constant at approximately 0.1 mm until post-op day 7, where it increased to over 0.3 mm on post-op days 14 and 21.

Normalizing the bone volume in the defect to the starting bone volume in an intact suture of a 21 day old mouse, showed that from days 1 to 5 post-op there was significantly less bone in the defect, no difference on post-op day 7, and significantly more bone in the defect on post-op days 14 and 21 (Figure 3A). Furthermore, the increase on days 14 and 21 was more than double the volume of bone in an intact suture at the time of surgery. Normalizing the mineral content in the defect to the intact post-natal day 21 suture showed identical trends

and significance (Figure 3B). Comparing the volume of bone in the defect to a fully developed suture (50 days post-natal) showed statistically less bone on days 1 through 7 post-op, but there was no difference on days 14 and 21 post-op (Figure 3C). Mineral content in the defect was normalized in the same manner to the day 50 intact suture and showed the same significance that was seen with bone volume (Figure 3D).

After establishing the timing of bone healing in the weanling posterior frontal suture defect, the effects of age and location on defect healing were assessed on days 2, 5, and 14 post-op. Analysis of the distance between the bones in the defect showed a decrease in the distances between the bones of the suture only for the defects created over the posterior frontal suture. In weanling mice, the defect width of the posterior frontal defect was 1.3 mm on day 2 post-op and then decreased sharply to approximately 0.4 mm on days 5 and 14 post-op (Figure 4A). Bone volume increased on post-op day 14 to over 0.6 mm³ (Figure 4B). In adolescent mice, the average defect width was approximately 1.3 mm on days 2 and 5 post-op, then decreased to less than 0.3 mm by 14 days post-op (Figure 4C). Despite the decrease in width, there were no changes in bone volume. Less than 0.1 mm³ of bone was present in the defect at all-time points examined (Figure 4D). The distance between bones in the lateral defects remained over 1.3 mm in the weanling mice through day 7. By day 14, this had decreased to 0.7 (Figure 4E). Bone increased on day 14 post-op (Figure 4F). In the adolescent lateral defects, there were no changes in the defect distance and all distances were approximately 1.3 mm (Figures 4G), and there were no changes in bone volume (Figure 4H).

Both the 2D and 3D images from the weanling posterior frontal defect show that by 14 days post-op the defect was nearly completely bridged with mineralized tissue (Figure 5A-B). The bone in the defect displayed a very thick trabecular pattern that was not seen with the normally intact suture (Figure 5A). No bone bridging was observed in the lateral defect, but the edges of the bone thickened substantially by 14 days post-op, as seen in the 2D μ CT image (Figures 5C-D). The adolescent posterior frontal defect showed a discontinuous thin shell of bone bridging the defect on post-op day 14 (Figure 5F). Examination of the 2D μ CT images revealed that this bridge of bone formed along the endocranial surface of the defect, but lacked the thick trabecular structure that was seen with the weanling defect (Figure 5E). The adolescent lateral defect did not show any bridging or an increase in bone volume (Figures 5G-H).

Analysis using real-time PCR showed distinct expression profiles for genes related to both osteoblastic and chondrogenic differentiation. Expression of transforming growth factor beta 2 (Tgfb2) was biphasic with peaks on days 2 and 14 post-op (Figure 6A). mRNAs for the extracellular matrix protein dentin matrix protein 1 (Dmp1) were increased on post-op days 3 and 4, corresponding to the time of the early bridging of the weanling defect (Figure 6B). There was a peak in expression for the early marker of chondrogenic expression, SRY-box containing gene 9 (Sox9) on post-op day 3 (Figure 6C). This was followed by an increase in expression of mRNAs for the chondrocyte extracellular matrix proteins: genes collagen 2 (Col2), collagen X (ColX), and cartilage oligomeric matrix protein (Comp). These mRNAs displayed peaks in expression during post-op days 4 and 5 (Figure 6D-F).

While mutations in genes related to bone morphogenic proteins (BMP) are not associated with any of the known mutations in craniosynostosis, mRNA for these proteins were elevated during with the rapid re-synostosis seen in the weanling posterior frontal defect. Bmp2 mRNAs remained constant until sharply increasing in expression on post-op day 7 and then gradually decreased for the remaining time points (Figure 6G). Bmp4 mRNAs were not expressed until day 3 and peaked on post-op days 5 and 7 (Figure 6H). Similar to what was seen with Tgfb2, Bmp6 expression was maximal on post-op day 2 and with a

second peak on day 14 (Figure 6I). Bmp7 expression was maximal on post-op day 2 with a second peak on day 4 (Figure 6J). mRNAs for the late marker of osteoblast differentiation, osteocalcin (Ocn), were present as early as day 2, but were at low levels until day 14 (Figure 6K). There was a 10 fold increase on post-day 14 that was maintained on day 21. Vitamin D receptor (Vdr) mRNAs were also present in the tissue at low levels through day 7 (Figure 6L). On day 14, expression increased 4 fold and on day 21, mRNAs doubled over the day 14 levels.

mRNAs for BMP inhibitors were differentially expressed. Sclerostin (Sost) mRNAs were present in the tissue at very levels through day 14, doubling on post-op day 21 (Figure 6M). Gremlin1 (Grem1) was expressed on days 4, 5, and 7 only, with peak levels on day 5 (Figure 6N). In contrast, mRNAs for Bmp3 were decreased on post-op days 5 and 7 (Figure 6O). Noggin (Nog) mRNAs were increased 4 fold on days 14 and 21 compared to day 7 (Figure 6P).

Cartilage was present in the healing defects at 5 days post-op. Staining with haematoxylin and eosin revealed a poorly organized but highly cellularized tissue within the defect at 5 days post-op (Figure 7A). By 14 days post-op, well-organized bone completely bridging the defect was observed (Figure 7B). The bone in and adjacent to the defect was thicker and more trabeculated than what is typically seen with an intact suture, as seen in the 2D μ CT images (Figure 5A). This thickening of the bone lateral to the defect was not seen on the 5 day post-op sections. Staining the 5 day post-op sections with Safranin-O showed the presence of small localized areas that stained red, indicating the presence of negatively charged glycosaminoglycans (Figure 7D-F). By 14 days post-op no red staining was observed, indicating fully mature bone throughout the section (Figure 7D).

DISCUSSION

This study demonstrates that rapid regeneration of bone in an immature cranium is both age and location specific. Complete healing was observed only in weanling mice and only when the defect was located over the posterior frontal suture. These defects healed via a biphasic process in which they were first bridged with a discontinuous shell of bone on the endocranial surface of the skull, followed by a robust mineralization of the defect by two weeks following surgery. This is similar to fusion of the normal posterior frontal suture, which also first closes along the endocranial surface of the suture and is followed later by a mineralization phase [26].

On post-op day 14 there was a complex, trabeculated network of mineralized tissue in the defect. This very complex trabecular network is not typically seen in the normal mineralization of the murine posterior frontal suture [30]. Normal sutures typically have fewer large bridges of bone spanning the endocranial to ectocranial surfaces of the suture which, increase in thickness as the animal ages.

Real-time PCR and histology also support the biphasic changes in defect healing, indicated by μ CT. Following the endocranial defect bridging on post-op day 3, there was an increase in the cartilage associated transcription factor, Sox9. This was then followed by an increase in mRNAs for proteins that are associated with cartilage extracellular matrix. The presence of this extracellular matrix was visible on sections stained with Safranin-O. The matrix was not as well organized as what is typically seen with cartilage in the growth plate or articular cartilage, but there were localized regions that stained positive for glycosaminoglycans. The presence of cartilage in healing of non-suture associated calvarial defects has been previously reported [31, 32].

The production of cartilage associated genes was followed by an increase in Bmp4 then Bmp2 expression, both of which are known to promote the transition from a chondrocyte to a mature osteoblast phenotype. The peak in expression of Bmps on post-op days 4 to 7 was followed by an increase in expression of the late marker of osteoblast differentiation, Ocn, on post-op days 14 and 21. This expression profile of Ocn was nearly identical to the changes in total defect mineral content and bone volume seen with μ CT. Both the Safranin-O and H&E sections of the 14 day post-op weanling defects showed organized bone by 14 days post-op, further supporting the changes in defect healing seen by μ CT and real-time PCR.

Comparing the healing of the weanling posterior frontal defect to the adolescent and lateral defects demonstrates that the rapid regeneration of bone within the defect is both age and location specific. The adolescent posterior frontal suture defect was the only other defect to bridge with a thin discontinuous shell of bone, as seen using 2D μ CT images at 14 post-op days. This bridged in an endocranial to exocranial manner, which is consistent with what is seen in normal suture development and fusion, suggesting a critical role of the dura in the regeneration of cranial defects. Unlike the posterior frontal weanling defect, the defects in the older adolescent mice displayed no significant increase in bone volume or defect mineralization by 14 days post-op. Furthermore, by 7 day post-op, the weanling posterior frontal defect contained as much bone in the defect as an intact suture at the time of surgery. By 14 days post-op, the amount of bone in the defect doubled compared to pre-operative levels. Additionally the defects contained as much bone as a fully developed 50 day old posterior frontal suture, even though the mice were two weeks younger. These data suggest that performing traditional craniotomies in mice actually accelerates the formation of bone in the region of the suture. The increase in bone volume seen was specific to both weanling groups and reflects what is seen clinically; children who are less than six months of age are at greater risk of post-operative re-synostosis [11, 12]. It is important to note that even though these mice were not genetically engineered to have craniosynostosis, they did still exhibit the robust post-operative bone growth. Creating the identical defect in a 21-day-old mouse model of Saethre-Chotzen syndrome showed impaired regeneration compared to their wild type littermates, most likely due to stress shielding of the PF suture region or from a more mature bone present in the PF suture at the time of surgery [33].

The defects lateral to the posterior frontal suture did not display any bone bridging by 14 days post-op. There were no changes in the percentage of the bone in the suture or defect distance until 14 days post-op in the weanling lateral defect. Even though there was a small decrease in these measurements, the 2D and 3D μ CT images clearly demonstrate that there was no bridging of the defect seen with the defects over the posterior frontal suture. Additionally, by 14 days post-op there was an increase in both bone volume and mineral content. Examination of the 2D μ CT images shows an increase in bone volume at the edges of the defect, but there was not complete bridging of the defect. In contrast, there were no changes in any of the algorithm measurements for the adolescent lateral defects. This indicates that this defect did not undergo any bridging or mineralization.

As with all animal models, the differences in size between an animal and human can pose significant challenges, especially in the case of weanling mice. At the time of surgery the mice were eating mostly solid food, but it is expected that there was some unavoidable stress that the weanling groups experienced as a result of being weaned and single-housed following surgery. However, it would be expected that the additional stress experienced by the weanling animals would result in a reduction in bone regeneration and not the very robust regeneration seen in the weanling mice [34]. Although our model accurately replicates what is seen clinically with re-synostosis, the size of the animals at the time of surgery and the location of the defect make this a technically challenging model. While our

surgical procedure can reliably and safely create the cranial defects, small variations in defect size necessitate using randomized groups and blinded reviewers. The discontinuity and heterogeneity seen with the both the weanling and adolescent defects further complicates analysis of the regenerating bone. While non-serial histology provides extremely detailed morphology of the bone, the discontinuity of the bone in the defect healing limits the validity of making generalizations from a limited number of sections. This same limitation has been reported in literature examining normal cranial suture fusion [35]. While it is possible to achieve complete analysis of the cranial defect using serial histology, this is prohibitively time and resource intensive for use in high throughput applications. The combination of μ CT and our imaging algorithms are well suited for examining the discontinuous bone regeneration as they allow for rapid nondestructive quantification of the bone regeneration and concomitant mineralization.

CONCLUSION

The results clearly demonstrate that rapid regeneration of bone occurs when a defect is placed over the posterior frontal suture of a weanling, 21 day old animal. To our knowledge this is the first animal model that replicates the rapid re-synostosis seen in very young children who undergo cranial vault reconstruction. Bridging of defects did not occur in non-suture associated regions, despite their being identical in size, and of the same embryonic origin. This difference in bone regeneration that was specific to the posterior frontal suture accurately models the rapid re-synostosis that is seen with cranial vault reconstruction to treat children with craniosynostosis. This model has tremendous potential to improve the understanding of the mechanisms responsible for re-synostosis in children.

REFERENCES

1. Aviv RI, Rodger E, Hall CM. Craniosynostosis. Clin Radiol. 2002; 57(2):93–102. [PubMed: 11977940]
2. Opperman LA. Cranial sutures as intramembranous bone growth sites. Dev Dyn. 2000; 219(4):472–85. [PubMed: 11084647]
3. Robin NH. Molecular genetic advances in understanding craniosynostosis. Plast Reconstr Surg. 1999; 103(3):1060–70. [PubMed: 10077104]
4. Panchal J, Uttchin V. Management of craniosynostosis. Plast Reconstr Surg. 2003; 111(6):2032–48. quiz 2049. [PubMed: 12711969]
5. Esparza J, Hinojosa J. Complications in the surgical treatment of craniosynostosis and craniofacial syndromes: apropos of 306 transcranial procedures. Childs Nerv Syst. 2008; 24(12):1421–30. [PubMed: 18769932]
6. Esparza J, et al. Surgical treatment of isolated and syndromic craniosynostosis. Results and complications in 283 consecutive cases. Neurocirugia (Astur). 2008; 19(6):509–29. [PubMed: 19112545]
7. Seruya M, et al. Long-term outcomes of primary craniofacial reconstruction for craniosynostosis: a 12-year experience. Plast Reconstr Surg. 2011; 127(6):2397–406. [PubMed: 21311385]
8. Fearon JA, Ruotolo RA, Kolar JC. Single sutural craniosynostoses: surgical outcomes and long-term growth. Plast Reconstr Surg. 2009; 123(2):635–42. [PubMed: 19182624]
9. Drake DB, et al. Calvarial deformity regeneration following subtotal craniectomy for craniosynostosis: a case report and theoretical implications. J Craniofac Surg. 1993; 4(2):85–9. discussion 90. [PubMed: 8324088]
10. Turner PT, Reynolds AF. Generous craniectomy for Kleeblattschadel anomaly. Neurosurgery. 1980; 6(5):555–8. [PubMed: 7413041]
11. Foster KA, Frim DM, McKinnon M. Recurrence of synostosis following surgical repair of craniosynostosis. Plast Reconstr Surg. 2008; 121(3):70e–76e.

12. Williams JK, et al. A longitudinal, statistical study of reoperation rates in craniosynostosis. *Plast Reconstr Surg.* 1997; 100(2):305–10. [PubMed: 9252595]
13. Mooney MP, et al. Correction of coronal suture synostosis using suture and dura mater allografts in rabbits with familial craniosynostosis. *Cleft Palate Craniofac J.* 2001; 38(3):206–25. [PubMed: 11386428]
14. Rice DP, et al. Gli3Xt-J/Xt-J mice exhibit lambdoid suture craniosynostosis which results from altered osteoprogenitor proliferation and differentiation. *Hum Mol Genet.* 2010; 19(17):3457–67. [PubMed: 20570969]
15. Wang Y, et al. Activation of p38 MAPK pathway in the skull abnormalities of Apert syndrome Fgfr2(+P253R) mice. *BMC Dev Biol.* 2010; 10:22. [PubMed: 20175913]
16. Perlyn CA, et al. The craniofacial phenotype of the Crouzon mouse: analysis of a model for syndromic craniosynostosis using three-dimensional MicroCT. *Cleft Palate Craniofac J.* 2006; 43(6):740–8. [PubMed: 17105336]
17. Rice DP, Rice R, Thesleff I. Molecular mechanisms in calvarial bone and suture development, and their relation to craniosynostosis. *Eur J Orthod.* 2003; 25(2):139–48. [PubMed: 12737212]
18. Carver EA, Oram KF, Gridley T. Craniosynostosis in Twist heterozygous mice: a model for Saethre-Chotzen syndrome. *Anat Rec.* 2002; 268(2):90–2. [PubMed: 12221714]
19. Cooper GM, et al. Ex vivo Noggin gene therapy inhibits bone formation in a mouse model of postoperative resynostosis. *Plast Reconstr Surg.* 2009; 123(2 Suppl):94S–103S. [PubMed: 19182668]
20. Bradley JP, et al. Studies in cranial suture biology: regional dura mater determines in vitro cranial suture fusion. *Plast Reconstr Surg.* 1997; 100(5):1091–9. discussion; 1100–2. [PubMed: 9326769]
21. Greenwald JA, et al. Immature versus mature dura mater: II. Differential expression of genes important to calvarial reossification. *Plast Reconstr Surg.* 2000; 106(3):630–8. discussion 639. [PubMed: 10987470]
22. Levine JP, et al. Studies in cranial suture biology: regional dura mater determines overlying suture biology. *Plast Reconstr Surg.* 1998; 101(6):1441–7. [PubMed: 9583471]
23. Opperman LA, et al. Cranial suture obliteration is induced by removal of transforming growth factor (TGF)-beta 3 activity and prevented by removal of TGF-beta 2 activity from fetal rat calvaria in vitro. *J Craniofac Genet Dev Biol.* 1999; 19(3):164–73. [PubMed: 10589398]
24. Slater BJ, et al. The role of regional posterior frontal dura mater in the overlying suture morphology. *Plast Reconstr Surg.* 2009; 123(2):463–9. [PubMed: 19182602]
25. Warren SM, et al. Regional dura mater differentially regulates osteoblast gene expression. *J Craniofac Surg.* 2003; 14(3):363–70. [PubMed: 12826808]
26. Hermann CD, et al. Biphase Fusion of the Murine Posterior Frontal Suture Part 1: Complete Time Course. *Plast Reconstr Surg.* 2012 Submitted.
27. Hermann CD, et al. Algorithm to Assess Cranial Suture Fusion with Varying and Discontinuous Mineral Density. *Annals of Biomedical Engineering.* 2012 In Press.
28. Gonzalez-Lagunas J, Mareque J. Calvarial bone harvesting with piezoelectric device. *J Craniofac Surg.* 2007; 18(6):1395–6. [PubMed: 17993887]
29. Hermann CD, et al. Biphase Fusion of the Murine Posterior Frontal Suture Part 2: Genes Associated with Early Cartilage Induction in Suture Fusion. *Plast Reconstr Surg.* 2012 Submitted.
30. Stadler JA 3rd, et al. A reinvestigation of murine cranial suture biology: microcomputed tomography versus histologic technique. *Plast Reconstr Surg.* 2006; 118(3):626–34. [PubMed: 16932170]
31. Schmitz JP, et al. Characterization of rat calvarial nonunion defects. *Acta Anat (Basel).* 1990; 138(3):185–92. [PubMed: 2389661]
32. Gurevitch O, et al. Reconstruction of cartilage, bone, and hematopoietic microenvironment with demineralized bone matrix and bone marrow cells. *Stem Cells.* 2003; 21(5):588–97. [PubMed: 12968113]
33. Hermann, Christopher D.; C.S.D.L.; Gadepalli, Siddharth; Lawrence, Kelsey A.; Richards, Megan A.; Olivares-Navarrete, Rene; Williams, Joseph K.; Schwartz, Zvi; Boyan, Barbara D. Interrelationship of Cranial Suture Fusion, Basicranial Development, and Re-synostosis following

Suturectomy in Twist1+/- Mice, a Murine Model of Saethre-Chotzen Syndrome. *Calcified Tissue International*. 2012 In Press.

34. Prado KF, et al. Prolonged immobilization-induced stress delays alveolar bone healing. A histometric study in rats. *Histol Histopathol*. 2001; 16(2):481–5. [PubMed: 11332704]
35. Sahar DE, Longaker MT, Quarto N. Sox9 neural crest determinant gene controls patterning and closure of the posterior frontal cranial suture. *Dev Biol*. 2005; 280(2):344–61. [PubMed: 15882577]

Highlights

- We developed a pediatric mouse model of re-synostosis
- Infant defect heals in rapid bi-phasic process
- Defect healing is age and location specific
- Healing occurs via endochondral ossification

Figure 1.

Images for the weanling posterior frontal defect. 3D μ CT rendering of mouse skull showing approximate locations of images and μ CT scans (red box) (A). Intraoperative images under 30X magnification prior to surgery showing the interfrontal ridge (white arrow), coronal suture (green arrow), and posterior frontal suture (yellow bracket). The template with centering notches was used as a reference throughout the procedure (B). The defect created after removing the posterior frontal suture, showing intact dura in the defect (C). 3D μ CT rendering of defect immediately after surgery (D).

Figure 2.

Quantification of weanling posterior frontal defect healing from μ CT images. Results from algorithm analysis of μ CT scans of defect. There was a decrease in the distance between the bones of the posterior frontal defect after post-op day 3 (A). The total mineral content and bone volume in the defect remained constant for post-op days 1-7 before increasing on days 14 and 21 (B, C). The average thickness of the bone in the defect showed no changes until an increase on post-op days 14 and 21 (D). $P < 0.01$ vs # = 1 day, % = 2 day, \$ = 14 day, & = 21 day.

Figure 3.

Normalized bone volume and mass of weanling posterior frontal defect to an intact posterior frontal suture. Results are from algorithm analysis of μ CT scans of defect and intact suture. The bone volume and mineral content of the defects was less than an intact 21 day old suture for post-op days 1-5 doubled by days 14 and 21 (A-B). Normalizing the bone volume and mass to the fully developed 50 day old posterior frontal suture showed no significant differences in either measure by days 14 or 21 post-op (C-D). * = $P < 0.05$ vs intact suture.

Figure 4.

The age and location dependence on defect healing. Results are from algorithm analysis of μ CT scans. The weanling posterior frontal defect was 1.3 mm wide on post-op day 2 then decreased on post-op days 5 and 14 (A). The increase in the volume of bone in the weanling posterior frontal defect occurred on 14 days post-op (B). The adolescent posterior frontal defect width showed no changes until decreasing on post-op day 14 (C). There was no change in volume of bone in the adolescent posterior frontal defect (D). There was a decrease in the width of the weanling lateral defect from post-op days 2 and 5 to 14 (E). There was a small increase in the volume of bone in the adolescent lateral defect on post-op day 14 (F). There were no changes in the bone volume of the adolescent lateral defect for any of the time points studied (G). There were no changes in the defect distance for the adolescent lateral defect (H). $P < 0.05$ vs * = day 2, \$ = day 5.

Figure 5.

Representative 2D and 3D μ CT images for the age and location defects on day 14 post-op. The weanling posterior frontal defect shows complete bridging and an increase in the thickness of the bones (A-B). The weanling lateral defect shows an increase in the thickness of the edges of the bones in the defect but no bridging (C-D). Adolescent posterior frontal defect shows thin discontinuous bridging along the endocranial surface of the defect, but no increase in the thickness of the bone in the defect (E-F). The adolescent lateral defect shows no increase in the thickness or bridging of bone in the defects (G-H).

Figure 6.

Real-time PCR analysis of genes associated with defect healing for Tgfb2 (A), Dmp1 (B), Sox9 (C), Col II (D), Co IX (E), Comp (F), Bmp2 (G), Bmp4 (H), Bmp6 (I), Bmp7 (J), Ocn (K), Vdr (L), Sost (M), Grem1 (N), Bmp3 (O), Nog (P). $p < 0.05$ vs # = day 2, \$ = day 3, % = day 4, ^ = day 5, & = day 7, * = day 14.

Figure 7.

Representative histology for weanling posterior frontal defect. H&E staining for post-op day 5 (A) and 14 (B) at 10X magnification. Safranin -O staining for post-op day 5 (C) and 14 (D) at 10X magnification. H&E (E) and Safranin -O (F) staining of 5 days post-op showing more organized tissue along the endocranial surface of the defects at 60X magnification.

Gene Name	Gene Abbreviation	Forward	Reverse
Bone morphogenic protein 2	Bmp2	TGGGTTTGTGGTGGAAGTG	TCGTTTGTGGAGCGGATG
Bone morphogenic protein 3	Bmp3	TATACGCCAACGATGCTG	CTTCTTCCTCCTCTCAACC
Bone morphogenic protein 4	Bmp4	TGGTTCTCCGTCCCTGATG	CGCTCCGAATGGCACTAC
Bone morphogenic protein 6	Bmp6	ACGAACAACAGCACAGCGAAG	AAGGCACCAGCGGGAGATG
Bone morphogenic protein 7	Bmp7	CCACAGCAAACGCCTAAG	CGATTACTCCTCAAAGACTCAG
Cartilage oligomeric matrix protein	Comp	GCGACGACGACATAGATG	GTCTTGGTCACTATCACAGG
Collagen X	ColX	TTCTGCTGCTAATGTTCTTGAC	CCTTTACTCTTTATGGCGTATGG
Gremlin1	Greml	AGAGAGCCACACCCAAAC	TGAAGCAGAGTAACAGGAAG
Noggin	Nog	GCCAGCACTATCTACACATCC	CAGCAGCGTCTCGTTCAG
Osteocalcin	Ocn	GTCTGTTCACCTTATTGC	TCTCTCTGCTCACTCTGA
Sclerostin	Sost	GTCGTCGTGCTGTCTCTG	ATTGTGGGTGGTGTCTGTGG
SRY-box containing gene 9	Sox9	CCACCAGTATCAGCGAGGAG	CCAAACAGGCAGGGAGATTC
Transforming growth factor beta 2	Tgfb2	GAGCGGAGCGACGAGGAG	TGTAGAAAGTGGCGGGATGG
Vitamin D receptor	Vdr	AATAAGCAGAGGAGGTGGTTC	GGGTGGGTGTGAGTAATGG
Dentin matrix protein 1	Dmpl	Pre Designed	
Collagen 2	Col2		