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OPG Deficiency Results in Disruption of Posterofrontal Suture Closure in Mice: Implications in Nonsyndromic Craniosynostosis

Maureen Beederman, MD^{a,b}, Stephanie H. Kim, PhD^c, M. Rose Rogers, MA^c, Sarah M. Lyon, BA^b, Tong-Chuan He, MD, PhD^c, and Russell R. Reid, MD, PhD^{a,b}

^aSection of Plastic and Reconstructive Surgery, Department of Surgery, University of Chicago Medical Center, Chicago, IL

^bThe Laboratory of Craniofacial Biology and Development, The University of Chicago Medical Center, Chicago, IL

^cMolecular Oncology Laboratory, Department of Orthopaedic Surgery, The University of Chicago Medical Center, Chicago, IL

Introduction

Craniosynostosis affects approximately 1 in 2,500 live births worldwide (1–3). This premature suture fusion restricts brain growth, which can cause increased intracranial pressure, craniofacial asymmetries, visual compromise, malocclusion, and developmental delay. The mainstay treatment available for these patients is cranial vault reconstruction, which is attendant with significant risks (4). Therefore, it is crucial to enhance our understanding of the molecular basis of craniosynostosis for the development of novel nonsurgical therapies.

Although linked with a variety of known genetic syndromes, the majority of craniosynostotic cases are termed nonsyndromic in nature and occur spontaneously. Our current understanding of the molecular and cellular aspects of cranial suture biology explains premature suture fusion from a dysregulated osteoblast perspective. However, given that proper bone formation and remodeling involves a complex interplay between both osteoblasts and osteoclasts, surprisingly little is known about the role of osteoclasts in cranial suture homeostasis.

Osteoclasts are predominantly regulated by tumor necrosis factor- α (TNF- α) superfamily members, receptor activator of NF- κ B (RANK) and its corresponding ligand (RANKL), both of which lead to osteoclast differentiation, activation, and maintenance.

Corresponding Author: Russell R. Reid, MD PhD, Associate Professor of Surgery, Section of Plastic and Reconstructive Surgery, 5841 South Maryland Avenue, MC 6035, Chicago, IL 60637, Ph: 773-702-6302, Fax: 773-702-1634, rreid@surgery.bsd.uchicago.edu.

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Osteoprotegerin (OPG) is a soluble inhibitor of RANK, and confers osteoclast apoptosis (5,6). Our laboratory has shown that suture fusion is correlated with a downregulation of RANK expression (7). This temporospatial relationship was shown in both murine and human suture samples. Of major interest, OPG protein expression was found to be significantly higher in fused human suture samples compared to nonfused internal controls from craniosynostotic patients (8). Furthermore, blockade of RANK signaling via siRNA technology in murine cranial suture explants produced osseous deposition within the suture fronts, further implicating this pathway in suture homeostasis (7).

Our past results suggest that the RANK-RANKL-OPG pathway and osteoclasts are important for proper suture fusion. We therefore hypothesized that perturbations in this axis affect normal cranial suture fusion. We aim to further define this pathway and its role in cranial suture biology through the use of an OPG knockout murine model.

Materials and Methods

Murine Colonies, Genotype Analysis and Suture Harvest

OPG heterozygous (+/-) mice (Jackson Laboratories, Bar Harbor, ME) were obtained and maintained, anesthetized, and euthanized in accordance with the University of Chicago Institutional Animal Care and Use Committee guidelines. Food and water were provided ad libitum. OPG +/- mice were bred to obtain litters that contained OPG wildtype (WT), OPG +/- and OPG -/- (KO) mice. These mice were genotyped using a common reverse primer for OPG, as well as WT and OPG KO forward primers (5'-GGT CCT CCT TGA TTT TTC TAT GCC-3' (common); 5'-TGC CCT GAC CAC TCT TAT ACG GAC-3' (WT); 5'-TGA CCG CTT CCT CGT GCT TTA C- 3' (KO)). Hey1 primers (5'-ACC TTT TCA GAG GAG CTG TGA G; TCC CGT CTA TGA AGC AAA ACT T-3') were used for a positive control to ensure adequate DNA extraction. Mice over 16 weeks of age were euthanized accordingly. Their calvaria were dissected and dura was stripped off the bone. The posterofrontal (PF) suture of each mouse was isolated and fixed in formalin.

Micro-Computerized Tomography (microCT) Analysis

OPG+/+ (WT), OPG+/- and OPG-/- mice were imaged by serial micro-CT scans at postnatal weeks 3, 5, 7, 9, and 16. CT imaging was performed at the Integrated Small Animal Imaging Research Resource, micro-PET/SPECT/CT subcore on a Trifoil Triumph trimodality. Craniometric analysis and PF suture density measurements were performed in triplicate on three-dimensional reconstructions of the murine skulls using Amgen image analysis software (Amira 5.2.1; Visage Imaging Inc, Andover, MA). Three-dimensional volumetric renderings of the sutures were created, and relative bone density was measured in Hounsfield Units (HU), at a threshold of 850 HU. For each suture, a consistent three-dimensional volume was sectioned into at least 50 segmental slices. Samples were standardized to both air and water standards, which were included in the raw microCT image. Craniometric analysis included anterior-posterior (AP) skull length, interorbital distance, and skull width measurements as previously described (9-11). Specifically, AP distance was measured at the midline from nasale to the intersection of the interparietal and occipital bones, interorbital distance was measured between the intersections of the frontal

processes of the maxilla with the frontal and lacrimal bones, and skull width was measured between the joining of the squamosal body to the zygomatic process of the squamosal bilaterally. Data was analyzed using t-test and ANOVA functions in Microsoft Excel.

Gross Light Microscopic Analysis

Formalin-fixed murine sutures were decalcified in DeCalX (Fisher Scientific, Pittsburgh, PA) overnight at room temperature and subsequently washed for several hours with water. The samples were paraffin embedded, and 5- μ m sections were mounted onto slides. Slides were warmed, deparaffinized, and rehydrated with sequential xylene, ethanol, and water washes. Slides were stained using Mayer hematoxylin (Sigma Aldrich, St. Louis, MO) and eosin washes (H&E). Slides were mounted with Permount (Fisher Scientific). The slides were analyzed using a Leica LM DB microscope (Leica Microsystems, Germany).

Results

Genotype and Weight Analysis of OPG KO Mice

C57BL/6J OPG +/- mice were bred to create OPG KO mice. Three separate litters were generated, containing a total of six WT mice, six OPG het mice, and five KO mice. All litters maintained viability and harbored no gross defects. Genotype PCR analysis on a small sample of DNA obtained from tail specimens confirmed homozygous deficiency in the OPG gene (OPG-/-). PCR products for WT mice showed a single band at 249 bp. Heterozygous mice demonstrated two bands at 249 and 600 bp, while OPG KO mice had one band at 600 bp. These findings were consistent with previous studies (12) (Figure 1). There was no statistically significant difference in the weights of WT, OPG het, and OPG KO mice at Weeks 3–9, during normal PF suture fusion.

MicroCT Imaging Demonstrates Persistent Posterofrontal Suture Patency in OPG KO Mice

Under normal physiologic conditions, the murine posterofrontal suture begins to fuse at Day 25 and is fully closed by day 45. Micro-CT images were taken at 3 weeks (prior to suture closure), 5 weeks (during suture closure), and 7, 9, and 16 weeks (after suture closure). While there were no gross differences in the overall shape of the skulls of WT, OPG +/-, and OPG KO mice, radiographic examination of the PF suture demonstrated an attenuation in suture fusion in the OPG KO sutures compared to WT and heterozygous littermates (Figure 2). This difference was seen when examining the skulls from both an ectocranial and endocranial perspective (Figure 2B). This reduction of fusion persisted through Week 16, long after the physiologic closure of the PF suture at Day 45.

OPG KO Mice Display Reduced Cranial Suture Density

Given these qualitative gross differences that were observed in WT and OPG KO mice with respect to posterofrontal suture fusion, we then quantified these differences by measuring the bone density of the posterofrontal suture over time. Intracutaneous bone density was used as an index of suture fusion, with the expectation of bone density to increase as the suture fused over time. The bone density measurements at Week 3 prior to suture closure were not statistically different between the three groups ($p>0.05$) (Figure 3A); the relative bone densities for WT, OPG het, and OPG KO mice were similar (857.50 ± 53.83 , 967.03 ± 62.42 ,

and 807.90 ± 56.26 , respectively, in $HU \pm SEM$). However, after this time point, a statistically significant difference was seen between both the WT and OPG KO and between OPG het and OPG KO ($p < 0.05$) (Figure 3A). At Week 5, bone densities for WT, OPG het, and OPG KO were $1272.16 (\pm 37.25)$, $1176.47 (\pm 53.28)$, and $979.16 (\pm 36.55)$ HU, respectively. Average bone density values for OPG KO mice were markedly reduced compared to the other groups through Week 16, long after the expected closure of the PF suture (WT, 1545.60 ± 64.10 , OPG het, 1590.34 ± 63.56 , and OPG KO, 1203.58 ± 33.07 HU). No statistically significant difference existed between the WT and OPG +/- groups at any time point.

No Difference in Bone Density Exists in Non-Suture Containing Bone

To correct for global effects of OPG deficiency on overall bone mineralization, we looked at overall bone density in non-suture containing calvarial bone between the three groups. The same area of bone was measured, from at least 50 consecutive slices. The extrasutural differences in bone density between the three groups were negligible at all time points and no statistically significant difference was found between the three groups (Figure 3B).

Craniometric Analysis Shows Differences in Anterior Posterior Skull Length

To assess if alterations in cranial suture bone density affected cranial growth and morphology, craniometric analysis on these mice was performed, using the three-dimensional reconstructions obtained from micro-CT imaging. Specifically, anterior-posterior skull distance, interorbital distance, and skull width were measured to assess craniometric differences. Interestingly, there was a statistically significant reduction in AP skull length in OPG KO mice when compared to WT and OPG +/- littermates (Figure 4A) at all timepoints. Moreover, mature OPG KO mice had an interorbital distance of 5.77 ± 0.047 at Week 16, which was statistically higher when compared to the interorbital distance measured for WT controls, 5.45 ± 0.010 (millimeters $\pm SEM$). This difference may point to the role of murine posterofrontal suture closure in interorbital spacing (Figure 4B). There were no significant differences between the three groups for skull width measurements.

Gross Light Microscopic Results

We performed H&E staining on the posterofrontal suture of adult mice past the week 16 timepoint to assess whether our microCT results were consistent with the histological appearance of the suture. Our results show a difference in the amount of bone at the posterofrontal suture (Figure 5) between the WT and OPG KO mice. While WT mice have an abundance of bone at the posterofrontal suture junction, the KO mice displayed an absence of bone at this site. The fusion of the posterofrontal suture is reduced in the OPG KO mouse when compared with WT controls.

Discussion

To date, we still do not have a complete understanding of the pathogenesis of craniosynostosis and the molecular mechanisms that govern premature suture fusion in nonsyndromic patients. Many efforts have focused on the role of osteoblasts in early suture fusion. Several transcription factors and signaling pathways have been shown to contribute

to early cranial suture fusion. Fibroblast growth factor receptor (FGFR) and transforming growth factor beta (TGF- β) mutations have been implicated in cases of both syndromic and nonsyndromic craniosynostosis. Both mutations lead to increased osteoblast activity and subsequent bone formation, and have been implicated in early closure of cranial sutures in several animal models (13–15).

Osteoclasts are predominantly regulated by the RANK-RANKL-OPG axis. Although they are important for normal maintenance of bone, the function of osteoclasts in cranial suture biology has not been thoroughly investigated. Lee and Spiguel et al. found that if RANK expression is knocked down using si-RNA, bone density is increased in a murine, explanted cranial suture model (7). Thus, perturbation in the RANK-RANKL-OPG axis affects murine cranial suture fusion, implicating a role for osteoclasts in cranial suture biology.

Past research on RANK and OPG knockout mice has predominantly investigated effects on axial skeletal bone formation and mineralization. A study on RANK KO mice showed osteopetrosis in the long bones and vertebrae of these animals, as well as an increase in cranial density observed on imaging (16). Another study by Dougall et al. noted that RANK KO mice exhibit small body size, shortened limbs, and “doming” of the skull, in addition to osteopetrosis (17). However, the authors did not further investigate the skull morphology in these mice. In terms of OPG, several other studies have characterized OPG KO mice. Bucay et al. used a combination of radiographic imaging and histological analysis to show that adolescent and adult mice deficient in OPG display decreased total bone density, increased bone porosity, early-onset osteoporosis, and a higher incidence of fractures (18). The observed bone thinning was most prominent in the long bones of the skeleton, as well as the lumbar vertebrae. They also reported thinning of the parietal bones in the skulls of these mice. Interestingly, this same group also studied the effect of OPG overexpression in a mouse model, and did not detect any changes in the skull bones of these animals (19). Despite the mention of the thinned parietal bones of the skull, there were no other cranial findings reported in these studies. Thus, while mice deficient in OPG have been studied before, this current report to our knowledge is the first that investigates specifically cranial suture biology in OPG KO mice.

Revisiting such animals with a new focus, we looked specifically at posterofrontal suture fusion in OPG KO mice. Murine suture biology mimics human suture biology and is a useful model for studying cranial suture development. The sutures of the mouse skull remain patent throughout its lifetime, with the exception of the posterofrontal suture, which is analogous to the human metopic suture (20). The results reported herein support our hypothesis that changes in osteoclast function have an effect on cranial suture biology. While no difference was observed in the bone density measurements of the PF suture at Week 3, prior to suture closure, differences in bone density were seen at all subsequent time points. Our radiographic findings, providing longitudinal morphologic analyses, were corroborated by H&E staining of adult posterofrontal suture microsections after the 16-week time point, which clearly revealed impaired PF suture bridging and reduced bone density in OPG-deficient mice. Furthermore, loss of OPG had a suture-specific effect on bone density, as non-suture containing calvarial bone did not exhibit a similar trend from unchecked osteoclast activation. It follows that such reduced closure would secondarily alter

interorbital distance, demonstrated by craniometric analysis (Figure 4). Conversely to a child with premature metopic suture fusion (hypotelorism), our findings demonstrate that delayed or failed closure of the posterofrontal suture yields increased dacryon-dacryon distance (hypertelorism) in the adult mice. The increased interorbital distance in the knockout cohort did not appear to negatively impact the visual function of this group compared to its WT or heterozygous counterparts, and seemed restricted to sheer morphologic differences. In contrast, we do not have a reasonable explanation for the relative brachycephaly in the genetically altered animals; however, this may point to a secondary effect of OPG deficiency on as to yet uninvestigated suture systems (e.g., cranial base) or on overall bony deposition of the craniofacial skeleton. In regards to this latter point, however, we did not find a difference in cranial bone mineralization among the three groups tested.

Our current study examined the effect of OPG deficiency on murine posterofrontal suture fusion. However, it remains to be seen whether OPG deficiency also has an effect on fusion of other calvarial sutures. To this end, treatment of murine cranial suture culture explants with adenovirus-encoding OPG (adOPG) led to increased intrasutural bone density (data not shown), similar to our findings with siRANK (7). This may have direct clinical implications as some current anti-osteoporotic therapies are based on targeted RANK blockade (e.g., denosumab, Priolia®). Future investigations should examine differences between the PF suture and other, typically patent, sutures of the murine skull, which will ultimately provide us with a better understanding of the role of this soluble RANK inhibitor in suture fusion.

Given that craniofacial development and suture fusion are dynamic processes that occur over an extended period of time, it was especially useful to use micro-CT imaging to assess the same animals at different time points. However, this longitudinal approach precluded molecular analysis of the dynamics of suture closure, which may give us more insight into the molecular switches critical in the suture fusion process. Further examination of the crosstalk between osteoblasts and osteoclasts, specifically the role of FGF-2 in osteoclastogenesis, as described by Chikazu et al. (21), would be especially interesting. Additionally, while our results show that perturbations in OPG affect normal cranial suture biology of the murine skull, it remains to be seen whether these findings translate into humans.

One limitation of this study was its small sample size, with only five to six mice per group. Given this small sample size, there was also variation in the number of male and female mice per group. Despite this, there was no statistically significant difference in weight between the three groups during physiologic suture fusion from Weeks 3–9, and previous studies have failed to note any differences in cranial suture fusion related to gender (22). There were several other limitations to our study. Our mice were all followed for a period of 16 weeks, at which point no differences were observed between WT mice and their OPG +/- counterparts. As previously reported studies (18) observed differences between OPG +/- and wildtype mice as late as six months of age, it is unclear whether additional differences between mice would have been observed past week 16 of life. Our craniometric data, which showed a significantly increased interorbital distance in the OPG KO mouse compared to WT at week 16, suggests that we might have discovered additional trends and differences if we had followed our litters for a longer period of time.

Finally, although the OPG KO mice had lower cranial suture bone densities when compared to WT and OPG het littermates, there was still some evidence of partial suture fusion, both from our microCT measurements as well as light microscopic analysis. These results suggest that the RANK-RANKL-OPG axis is one of many systems involved in cranial suture homeostasis, and ongoing work needs to be done in order to fully elucidate all the specific factors at work.

Conclusions

To our knowledge, this is the first study to demonstrate that OPG deficiency results in reduced murine posterofrontal suture closure. Such perturbations led to targeted defects in suture mineral deposition, increased interorbital distance and brachycephaly in deficient animals compared to WT and heterozygotic counterparts, with the absence of global cranial mineralization defects. Further studies are required to elucidate the molecular switches responsible for suture homeostasis from this RANK/osteoclast perspective. Such analyses may be foundational for therapies targeting craniosynostosis.

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References

1. Robin NH. Molecular genetic advances in understanding craniosynostosis. *Plast Reconstr Surg.* 1999; 103:1060. [PubMed: 10077104]
2. French LR, Jackson IT, Melton LJ 3rd. A population-based study of craniosynostosis. *J Clin Epidemiol.* 1990; 43:69–73. [PubMed: 2319283]
3. Singer S, Bower C, Southall P, et al. Craniosynostosis in Western Australia, 1980–1994: a population-based study. *Am J Med Genet.* 1999; 83:382–387. [PubMed: 10232748]
4. Czerwinski M, Hopper RA, Gruss J, et al. Major morbidity and mortality rates in craniofacial surgery: an analysis of 8101 major procedures. *Plast Reconstr Surg.* 2010; 126:181–186. [PubMed: 20220557]
5. Leibbrandt A, Penninger JM. Novel functions of RANK(L) signaling in the immune system. *Adv Exp Med Biol.* 2010; 658:77–94. [PubMed: 19950018]
6. Leibbrandt A, Penninger JM. RANK/RANKL: regulators of immune responses and bone physiology. *Ann N Y Acad Sci.* 2008; 1143:123–150. [PubMed: 19076348]
7. Lee JC, Spiguel L, Shenaq DS, Zhong M, Wietholt C, He TC, Reid RR. Role of RANK-RANKL-OPG axis in cranial suture homeostasis. *J Craniofac Surg.* 2011 Mar; 22(2):699–705. [PubMed: 21415639]
8. Lee JC, Spiguel L, Shenaq D, Zhong M, Wietholt C, He TC, Reid RR. Abstract 66: Rank Signaling in Cranial Suture Biology. *Plastic & Reconstructive Surgery.* 2012 Jul. 130(1S):52.
9. Perlyn CA, DeLeon VB, Babbs C, Govier D, Burell L, Daryann T, Kreiborg S, Morriss-Kay G. The craniofacial phenotype of the Crouzon mouse: analysis of a model for syndromic craniosynostosis using three-dimensional MicroCT. *Cleft Palate Craniofac J.* 2006 Nov; 43(6):740–8. [PubMed: 17105336]

10. Kawakami M, Yamamura K. Cranial bone morphometric study among mouse strains. *BMC Evol Biol.* 2008 Feb 29;8:73. [PubMed: 18307817]
11. Richtsmeier JT, Baxter LL, Reeves RH. Parallels of craniofacial maldevelopment in Down syndrome and Ts65Dn mice. *Dev Dyn.* 2000 Feb; 217(2):137–45. [PubMed: 10706138]
12. Yun TJ, Tallquist MD, Aicher A, Rafferty KL, Marshall AJ, Moon JJ, Ewings ME, Mohaupt M, Herring SW, Clark EA. Osteoprotegerin, a crucial regulator of bone metabolism, also regulates B cell development and function. *J Immunol.* 2001 Feb 1; 166(3):1482–91. [PubMed: 11160187]
13. Most D, Levine JP, Chang J, Sung J, McCarthy JG, Schendel SA, Longaker MT. Studies in cranial suture biology: up-regulation of transforming growth factor-beta1 and basic fibroblast growth factor mRNA correlates with posterior frontal cranial suture fusion in the rat. *Plast Reconstr Surg.* 1998 May; 101(6):1431–40. [PubMed: 9583470]
14. Mathy JA, Lenton K, Nacamuli RP, Fong KD, Song HM, Fang TD, Yang GP, Longaker MT. FGF-2 stimulation affects calvarial osteoblast biology: quantitative analysis of nine genes important for cranial suture biology by real-time reverse transcription polymerase chain reaction. *Plast Reconstr Surg.* 2003 Aug; 112(2):528–39. [PubMed: 12900611]
15. Warren SM, Brunet LJ, Harland RM, Economides AN, Longaker MT. The BMP antagonist noggin regulates cranial suture fusion. *Nature.* 2003 Apr 10; 422(6932):625–9. [PubMed: 12687003]
16. Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, McCabe S, Elliott R, Scully S, Van G, Kaufman S, Juan SC, Sun Y, Tarpley J, Martin L, Christensen K, McCabe J, Kostenuik P, Hsu H, Fletcher F, Dunstan CR, Lacey DL, Boyle WJ. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc Natl Acad Sci U S A.* 2000 Feb 15; 97(4):1566–71. [PubMed: 10677500]
17. Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, Daro E, Smith J, Tometsko ME, Maliszewski CR, Armstrong A, Shen V, Bain S, Cosman D, Anderson D, Morrissey PJ, Peschon JJ, Schuh J. RANK is essential for osteoclast and lymph node development. *Genes Dev.* 1999 Sep 15; 13(18):2412–24. [PubMed: 10500098]
18. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ, Simonet WS. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev.* 1998 May 1; 12(9):1260–8. [PubMed: 9573043]
19. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Lüthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell.* 1997 Apr 18; 89(2):309–19. [PubMed: 9108485]
20. Bradley JP, Levine JP, Roth DA, McCarthy JG, Longaker MT. Studies in cranial suture biology: IV. Temporal sequence of posterior frontal cranial suture fusion in the mouse. *Plast Reconstr Surg.* 1996 Nov; 98(6):1039–4. [PubMed: 8911474]
21. Chikazu D, Katagiri M, Ogasawara T, Ogata N, Simoaka T, Takato T, Nakamura K, Kawaguchi H. Regulation of osteoclast differentiation by fibroblast growth factor 2: stimulation of receptor activator of nuclear factor κ B ligand/osteoclast differentiation factor expression in osteoblasts and inhibition of macrophage colony-stimulating factor function in osteoclast precursors. *J Bone Miner Res.* 2001 Nov; 16(11):2074–81. [PubMed: 11697804]
22. Cray J Jr, Cooper GM, Mooney MP, Siegel MI. Ectocranial suture fusion in primates: patterns and phylogeny. *J Morphol.* 2014 Mar; 275(3):342–7. [PubMed: 24142900]

Figure 1. PCR Genotype Analysis, Gross Anatomical Comparison, and Weight Comparison of Wildtype C57BL6J mice (OPG +/+), OPG heterozygous mice (OPG +/-), and OPG knockout mice (OPG -/-)

A) Representative PCR genotyping results showing WT, OPG het, and OPG KO mice.

These results were consistent for all 3 litters that were analyzed in this study.

B) OPG knockout mice were viable and reached adulthood, similar to both wildtype and OPG heterozygote animals. These mice did not show any gross anatomical abnormalities upon visual inspection. The difference in size between the OPG +/+ mouse and the OPG +/- and OPG -/- mice is likely due to the WT mouse being male, while the OPG het and OPG KO mice were both female.

C) Analysis of murine weights between the WT, OPG het, and OPG KO mice. No statistically significant difference in weight was detected at Weeks 3–9, during the time of PF suture fusion. Although there was a trend towards reduced weight in OPG KO mice, this is most likely due to the higher number of female mice in this group. An analysis of murine weights between WT, OPG het, and OPG KO mice by gender showed increased similarity in weights between these three groups. No statistically significant difference was detected in the female group. Statistical analysis could not be performed on the male group, given $n=1$ for OPG KO male mice.

Figure 2. OPG KO Mice Show Reduced Posterofrontal Suture Fusion at Weeks 5, 7, 9, and 16

The posterofrontal suture of the mouse normally begins to fuse around Day 25 and is completely fused by Day 45. MicroCT imaging of the skulls of OPG KO mice shows a reduction in suture fusion. This reduced suture fusion can be seen both from ectocranial and endocranial views of the suture. Red box denotes the location of the posterofrontal suture. OPG heterozygous mice (data not shown) displayed results that were similar to those of WT mice regarding skull shape and suture fusion.

Figure 3. OPG KO Mice Display Lower Posterofrontal Suture Density

A) Bone density analysis of the posterofrontal suture was performed in order to quantify the degree of posterofrontal suture fusion. The relative bone density (measured in Hounsfield Units) is not statistically different at week 3 prior to suture closure. However, a significant ($p < 0.05$) difference in bone density does exist between WT (blue) and KO (red) and between OPG het (yellow) and KO mice at all subsequent timepoints. This difference persists through week 16.

B) To correct for global effects of OPG deficiency on overall bone mineralization, bone density was measured in non-suture containing calvarial bone. The differences in bone density were not statistically significant between the three groups.

Figure 4. OPG KO Mice Have Significant Craniometric Differences When Compared to Wildtype and OPG Heterozygous Littermates

To determine whether alterations in suture fusion affected cranial morphology, craniometric analysis was performed. Specifically, anterior-posterior distance (A), interorbital distance (B), and skull width (C), were assessed. Results show a statistically significant reduction in anterior-posterior distance in OPG KO mice compared to WT and OPG heterozygous littermates (A). Interorbital distance was statistically higher for mature OPG KO mice compared to WT adult mice (B). No differences were observed in skull width (C).

**Wildtype
>16 weeks**

**OPG KO
>16 weeks**

Figure 5. OPG KO Mice Show Reduced Posterofrontal Suture Closure On Histological Analysis
H&E staining was performed on mice after the 16-week time point to assess whether microCT results were consistent with the histological appearance of the suture. Representative image demonstrates reduced ossification at the PF suture in OPG KO mice compared to WT mice. Images shown were acquired at 10x magnification. Arrow denotes the location of the posterofrontal suture.