

# The Role of RANK-RANKL-OPG Axis in Cranial Suture Homeostasis

Justine C. Lee, MD, PhD,\* Lisa Spiguel, MD,\* Deana S. Shenaq, BA,\*† Ming Zhong, BS,\*†  
Christian Wietholt, PhD,‡ Tong-Chuan He, MD, PhD,† and Russell R. Reid, MD, PhD\*

**Abstract:** Craniosynostosis is a significant disorder affecting 1 in 2500 live births worldwide. Although a large body of work has focused on dural regulation and the contributions of molecular mediators such as fibroblast growth factor, bone morphogenetic protein, and transforming growth factor  $\beta$ , minimal attention has been directed toward osteoclast function in cranial suture biology. Receptor activator of nuclear factor  $\kappa$ B (RANK) is an essential mediator of osteoclastogenesis and osteoclast activation. In this study, physiologic fusion of posterior frontal sutures in murine development correlated with decreasing protein expression of RANK in comparison to age-matched coronal and sagittal sutures via immunohistochemical survey. However, RANK mRNA did not exhibit a similar pattern suggesting that RANK is regulated at the protein level. Fused cranial sutures in nonsyndromic craniosynostotic children also showed decreased levels of RANK staining in immunohistochemistry in comparison to patent sutures from the same patients. Immunohistochemistry with a RANK ligand antibody did not show differences in fused or patent sutures. Moreover, RANK knockdown in calvarial strip suture cultures displayed increased bone density specifically in the suture line after infection with small interfering RANK viruses. Cranial suture biology, similar to bone biology in general, likely depends on a complex interplay between osteoblasts and osteoclasts. We now report a temporospatial correlation between RANK expression and suture morphology that suggests that osteoclast activity is important in maintenance of cranial suture patency in normal physiology and disease. Furthermore, RANK down-regulation promoted suture fusion establishing a causal relationship between the presence of RANK and patency.

**Key Words:** RANK, craniosynostosis, cranial suture fusion

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Craniosynostosis, the premature ossification of cranial sutures, is a heterogeneous condition affecting approximately 1 in 2500 live births worldwide.<sup>1–3</sup> Consequences of cranial suture fusion are directly related to the inability of the cranial vault to accommodate physiologic brain growth, resulting in potential elevation of intracranial pressure and the possibility of permanent neurologic dysfunction. Currently, the only therapeutic measure for craniosynostosis is surgical correction. Despite advances in technique and safety since its initial description by Lannelongue in 1890, cranial vault remodeling remains a major surgical procedure with complications including low rates of mortality,<sup>4–6</sup> warranting the need for alternative or adjunctive therapies.

Syndromic and nonsyndromic craniosynostoses differ in epidemiology and etiology. Population-based studies document that nonsyndromic isolated fusion comprises approximately 84% of patients diagnosed with craniosynostosis, with sagittal sutures most commonly affected.<sup>3,7</sup> A number of syndromic craniosynostoses have autosomal dominant inheritance patterns with defined mutations in several fibroblast growth factor receptor (FGFR) genes, Twist1 transcription factor, EFNB1, MSX2, and RAB23.<sup>8–10</sup> In contrast, nonsyndromic craniosynostoses have both genetic and environmental associations. Familial nonsyndromic craniosynostoses have been reported for multiple sutures including lambdoid,<sup>11</sup> coronal,<sup>12–14</sup> and sagittal.<sup>14</sup> Despite the known heritability, genetic associations for nonsyndromic fusion are controversial. Whereas some investigators have correlated FGFR3 P250R and Twist box heterozygous mutations to nonsyndromic coronal and sagittal synostoses,<sup>13–15</sup> others have found no somatic FGFR or Twist mutations.<sup>16</sup> Environmental contributions to nonsyndromic fusion are supported by twin studies that show less than 100% concordance rate in monozygotic twins.<sup>17,18</sup> Suggested risk factors include maternal and paternal age,<sup>3,12</sup> ethanol use, certain medications such as valproic acid,<sup>19</sup> and parental education.<sup>20</sup> In addition, mechanical forces in murine models have been able to reproduce suture fusion both in utero and in postnatal development.<sup>21–23</sup> Furthermore, the male predominance of nonsyndromic craniosynostosis may have an association with the proproliferative effects of dihydrotestosterone on osteoblasts.<sup>24</sup>

Molecular mechanisms involved in cranial suture biology are complex and have been described largely from a dysregulated osteoblast perspective.<sup>25</sup> Significant contributions by Longaker and colleagues have elucidated that crosstalk between regional dura mater, the osteogenic fronts, and the suture mesenchyme is important in the decision to progress to ossification and fusion.<sup>26</sup> Extracellular profusion signals are centered on the presence of specific growth factors, including the differential temporal expression of basic fibroblast growth factor<sup>27</sup> and spatial expression of transforming growth factor  $\beta$ 1.<sup>28</sup> In contrast, bone morphogenetic protein 3 (BMP-3) and noggin are expressed as propatency signals.<sup>29</sup> Intracellularly, syndromes have contributed insights into involved transcription factors such as Twist1, which regulates FGFR2 expression,<sup>30,31</sup> as well as MSX2, which promotes sagittal fusion in overexpression.<sup>32,33</sup> Despite such advances, bone biology is based on a balance between osteoblasts

**AQ1** From the \*Laboratory of Craniofacial Biology and Development, †Molecular Oncology Laboratory, Department of Surgery, and ‡Preclinical SPECT/PET/CT Laboratory, Department of Radiology, University of Chicago Medical Center, Chicago, Illinois.

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Address correspondence and reprint requests to Dr. Russell R. Reid, MD, PhD, 5841 S Maryland Ave, MC 6035, Chicago, IL 60637;  
E-mail: rreid@surgery.bsdc.uchicago.edu

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J.C.L. and L.S. contributed equally to work.

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and osteoclasts. The latter cell type has received little attention in the craniosynostosis arena and may be equally important in maintaining patency.

Osteoclast regulation intimately involves the receptor activator of nuclear factor  $\kappa$ B (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) axis.<sup>34,35</sup> Receptor activator of NF- $\kappa$ B, a tumor necrosis factor superfamily receptor originally identified in T lymphocytes and osteoblasts, is essential to osteoclast differentiation and activation upon binding to its cognate ligand.<sup>36–39</sup> Knockout mice generated in these studies show that both RANK and RANKL deficiencies resulted in osteopetrosis due to a complete absence of osteoclasts.<sup>40,41</sup> Osteoprotegerin, the soluble decoy receptor for RANKL, is a potent inhibitor of osteoclast activation. In contrast to the RANK and RANKL-deficient mice, OPG knockouts exhibit profound osteoporosis.<sup>41,42</sup> We postulate that nonsyndromic craniosynostosis, on a cellular level, is a perturbation of the delicate balance between osteoblast and osteoclast function. The results herein demonstrate for the first time that the RANK-RANKL-OPG axis is also a regulator of cranial suture biology in both physiologic and pathologic development.

## MATERIALS AND METHODS

### Murine Calvaria and Suture Harvest

CD1 mice (Charles River Laboratories, Wilmington, MA) were obtained and maintained in accordance with the University of Chicago Institutional Animal Care and Use Committee. Food and water were provided ad libitum. Mice at 2, 5, 7, and greater than 12 weeks of age were anesthetized using CO<sub>2</sub> asphyxiation and cervical dislocation followed by calvaria dissection and dural stripping. Posterior frontal, coronal, and sagittal sutures were isolated and snap frozen for RNA and protein extraction or fixed in formalin for immunohistochemistry.

### Patient Samples

Nonsyndromic craniosynostosis patients and their families were enrolled after informed consent was obtained in the preoperative setting in accordance with the University of Chicago institutional review board guidelines (institutional review board no. 16045B). Suture samples obtained intraoperatively during cranial vault reconstruction were immediately fixed in formalin at room temperature overnight.

### Immunohistochemistry

Formalin-fixed murine and human sutures were decalcified in DeCaX (Fisher Scientific, Pittsburgh, PA) overnight at room temperature and paraffin embedded, and 5- $\mu$ m sections were mounted onto slides. Slides were warmed, deparaffinized, and rehydrated with sequential xylene, ethanol, and water washes. Slides were boiled for 10 minutes in 10 mM sodium citrate with 0.05% Tween-20, pH 6.0, for antigen retrieval, and endogenous peroxidase was quenched using the Dako EnVision System solution (Dako, Carpinteria, CA) under manufacturer's directions. Samples were blocked in 3% bovine serum albumin for 20 minutes at room temperature. Primary antibodies specific for RANK (1:25; Santa Cruz Biotechnology, Santa Cruz, CA) and RANKL (1:25; Santa Cruz Biotechnology) were incubated on the samples overnight at 4°C. Slides were washed, incubated with anti-rabbit horseradish peroxidase (Pierce Biotechnology, Rockford, IL) at 1  $\mu$ g/mL, and developed using the Dako EnVision System substrate and chromogen (Dako). Samples were counterstained with Mayer hematoxylin (Sigma Aldrich, St Louis, MO) and dehydrated with sequential ethanol to xylene washes. Slide covers were mounted with Permount (Fisher Scientific). Slides were visualized and photographed with the Olympus DP25 microscope camera (Olympus Imaging America, Center Valley, PA).

### RNA Extraction and Real-Time Reverse Transcriptase Polymerase Chain Reaction

Pooled coronal, sagittal, and posterior frontal sutures from 2-, 5-, 7-, and greater than 12-week-old mice were decalcified in DeCaX (Fisher Scientific) overnight at room temperature and morselized with an electronic tissue mincer with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) on ice. Total RNA was isolated according to manufacturer's instructions. cDNA was generated with the Superscript II (Invitrogen) or M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA) first-strand synthesis protocol with 10  $\mu$ g of total RNA using manufacturer's guidelines. Real-time polymerase chain reaction (PCR) reactions were performed with DyNAmo SYBR Green (Finnzymes, Espoo, Finland) and 2.5 ng/ $\mu$ L of sense and antisense PCR primers. Reactions were cycled and analyzed using the DNA Engine Opticon 2 Real-Time Cycler (GMI Inc, Ramsey, MN). The relative concentration of RANK RNA was corrected with GAPDH RNA run in parallel reactions. The following primers were used: RANK sense (5'-CCAGCAGGGAAGC AAATCTA-3'), RANK antisense (5'-CAGTGAAGTCACAGCCCT CA-3'), GAPDH sense (5'-GGCTGCCAGAACATCAT-3'), and GAPDH antisense (5'-CGGACACATTGGGGGTAG-3').

### Adenoviral Infection and Micro-Computed Tomography Analysis

Recombinant adenoviruses were generated using AdEasy technology as previously described.<sup>43–46</sup> Adenovirus expressing small interfering RNAs that target mouse RANK (siRANK) coding sequence was constructed using our recently developed pSOS system.<sup>47</sup> Four pairs of siRNA targeting sites (CTA CAG GAA GGG AGG GAA A and TTT CCC TCC CTT CCT GTA G; CCA AGG AGG CCC AGG CTT A and TAA GCC TGG GCC TCC TTG G; CGG ACA ATG TGC AGA ATG A and TCA TTC TGC ACA TTG TCC G; and CAA AAG AAA TAG AAG GTG A and TCA CCT TCT ATT TCT TTT G) were designed using *siDESIGN* (Dharmacon, Lafayette, CO), cloned into an adenoviral shuttle vector, and subsequently used to generate recombinant adenoviruses that coexpress RFP in HEK293 cells. Analogous adenovirus expressing only monomeric RFP (AdRFP) was used as a control. Viruses were serially titrated to determine dosage with optimal infection and cell viability.

Three-week-old CD1 mice were killed as described above. Calvarial strips containing posterior frontal, sagittal, or coronal sutures were dissected free of dura mater. The suture pieces were cultured in High Glucose Gibco DMEM (Invitrogen) containing 0.5% bovine serum albumin with 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin at 37°C in 95% humidified air and 5% CO<sub>2</sub>. After 24 hours of culture, sutures were infected with AdRFP or siRANK with 6  $\mu$ L/mL polybrene (Sigma Aldrich). The infection efficiency was confirmed and monitored by red fluorescence.

Serial micro-computed tomography (CT) imaging of calvarial explants was performed at 0-, 1-, and 2-week time points after infection. Three-dimensional volumetric renderings of the sutures were created at a threshold of 400 Hounsfield units, which signifies the density of immature bone (Amira 5.2.1; Visage Imaging Inc, Andover, MA). Suture density of the treatment groups was then assessed and compared with RFP controls.

## RESULTS

### Physiologic Fusion of Calvarial Sutures Demonstrates Differential Expression of RANK

The CD1 murine model for cranial suture biology has been well characterized<sup>48–50</sup> and shown to be analogous to human sutures. Similar to the human metopic suture, CD1 posterior frontal sutures close in vivo around 5 weeks of age, whereas coronal and sagittal

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sutures remain patent. Using this model, coronal, sagittal, and posterior frontal sutures at 2, 5, 7, and greater than 12 weeks of age were harvested, and immunohistochemical analysis was performed with anti-RANK (Fig. 1). Whereas coronal and sagittal sutures remained patent and demonstrated strong RANK staining at the suture junction at all ages, posterior frontal sutures fused and decreased in RANK staining at 5, 7, and greater than 12 weeks of age. Interestingly, sagittal sutures appeared to undergo narrowing in older mice, suggestive of early fusion, and display lower intensity of RANK staining. Thus, the sagittal suture may have an intermediate level of RANK correlating to an intermediate phenotype.

**Differential RANK Expression Occurs at the Protein Level in Physiologic Suture Fusion**

Given the differences in RANK protein found in immunohistochemistry, we then quantitated RANK mRNA and protein levels in murine sutures. Mouse calvaria containing coronal, sagittal, or posterior frontal sutures were harvested at 2, 5, 7, and greater than 12 weeks of age, and mRNA was isolated from each of the sutures (Fig. 2). In addition, calvarial bone without suture was used as a control. Real-time reverse transcriptase PCR showed that relative RANK mRNA levels, corrected against GAPDH mRNA, uniformly decreased in a temporal manner that is independent of suture identity. Unlike suture-containing calvarium, sutureless bone contained low to moderate amounts of RANK mRNA at 2, 5, and 7 weeks of age. Sutureless bone also demonstrated low levels of RANK mRNA at greater than 12 weeks of age. These data suggest that differential RANK expression is not regulated on the transcriptional level.

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**Pathologic Fusion of Human Calvarial Sutures Demonstrates Differential Expression of RANK**

Four nonsyndromic craniosynostotic patients requiring cranial vault reconstruction were enrolled between June 2008 and July 2009 (Table 1). The patients ranged between 5 and 8 months of age (mean, 6.25 months). Patients were diagnosed based on the presence of scaphocephaly in 3 patients and cloverleaf deformity in the multisutural synostotic patient. None of the patients exhibited any preoperative symptoms concerning for elevated intracranial pressure

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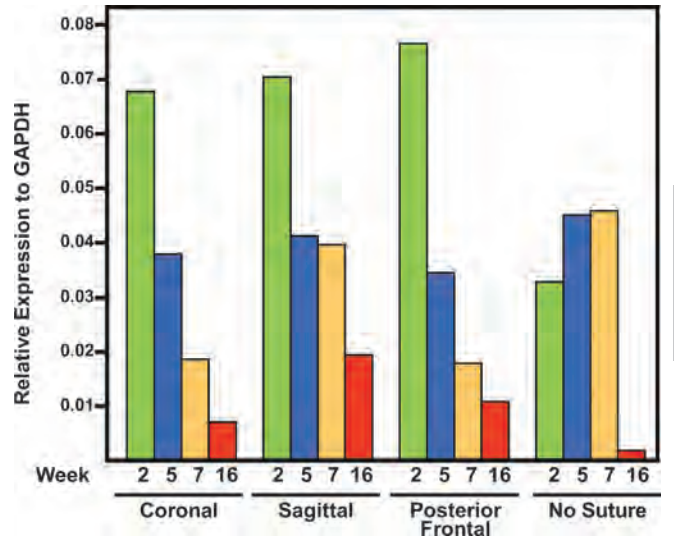


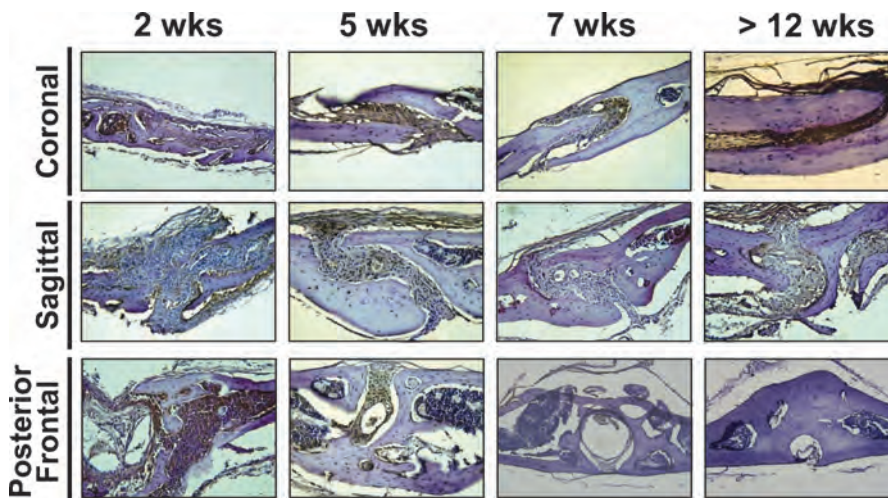
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**FIGURE 2.** RANK mRNA decreases in maturing coronal, sagittal, and posterior frontal sutures. RANK real-time PCR of pooled coronal, sagittal, and posterior frontal murine sutures and sutureless calvaria at 2, 5, 7, and greater than 12 weeks of age.

including headaches, nausea, vomiting, visual changes, or papilledema. The birth histories for 2 patients were significant in that 1 patient was delivered at 40 weeks and another patient had a cephalohematoma and was born by cesarean delivery. All patients met developmental milestones, and none of the patients had any history suggestive of familial inheritance. Formal testing of FGFR and Twist mutations performed in the multisuture synostotic patient was negative. In 1 patient, CT findings did indicate evidence of thumbprinting concerning for elevated intracranial pressure despite the lack of clinical symptoms.

Samples of fused sutures and, if available, patent sutures were obtained during cranial vault reconstruction and immediately fixed in formalin. Sutures were subsequently prepared for

Fig 1 4/C



**FIGURE 1.** RANK staining decreases in fusing posterior frontal sutures. CD1 murine coronal, sagittal, and posterior frontal sutures at 2, 5, 7, and greater than 12 weeks of age were harvested and stained with anti-RANK. Coronal and sagittal sutures remained patent and demonstrated strong staining at all ages. Posterior frontal sutures fused and decreased in RANK staining at 5, 7, and greater than 12 weeks of age.

TABLE 1. Patient Data

Age, mo	Sex	Fused Suture	Birth History	Family History	Preoperative Symptoms	Concerning CT Findings
5	Male	Sagittal	40 wk, NSVD	None	None	None
6	Male	Sagittal	Full term, cesarean delivery, maternal hypertension, cephalohematoma	None	None	None
6	Female	Sagittal, Right Coronal	Full term, NSVD	None	None	Thumbprinting of parietal bones
8	Male	Sagittal	Full term, NSVD	None	None	None

AQ2

immunohistochemistry and stained with anti-RANK (Fig. 3). Like patent murine sutures, patent human coronal sutures displayed high levels of RANK staining at the suture junction (Fig. 3, top panels).

Similar to physiologic suture fusion in mice, pathologically fused human sutures uniformly exhibited lower levels of RANK protein (Fig. 3, bottom panels). This expression pattern does not appear to

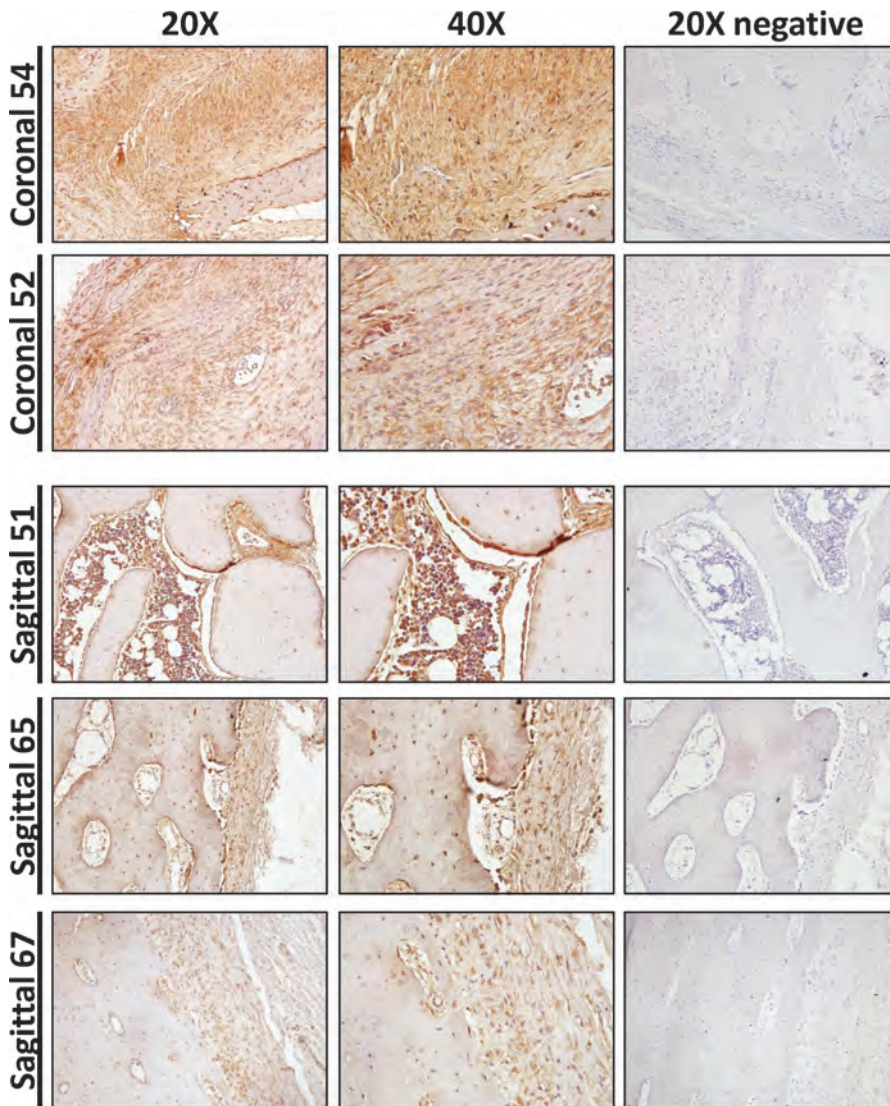


Fig 3 4/C

FIGURE 3. RANK staining decreases in stenotic sutures. Sutures obtained from nonsyndromic craniosynostotic patients during cranial vault reconstruction were stained with anti-RANK. Patent coronal sutures were also obtained from these patients when available. Stenotic sutures uniformly exhibited less RANK staining in comparison to open sutures.

**F4** be paralleled in RANKL (Fig. 4). Both stenotic and patent sutures appear to express RANKL with minimal differences.

### RANK Down-Regulation Increases Bone Density in Murine Calvarial Sutures

Calvarial strips containing coronal, sagittal, or posterior frontal sutures were infected with adenovirus expressing RFP (control) or a siRANK RNA sequence. Infection and cell viability were confirmed by following red fluorescence (Fig. 5).

**F5** Micro-CT scanning was performed 24 hours ( $t = 0$ ), 1, and 2 weeks after successful infection. For each suture sample, a defined three-dimensional volume corresponding to approximately  $5.3 \times 4.3$  mm for at least 40 segmented slices was designed and used to analyze the images at each time point. Without changing the defined volume, the postinfection density of the calvaria was averaged in Hounsfield units, and the  $t = 0$  density averages were subtracted (Fig. 6). For both the posterior frontal and sagittal suture strip cultures, the density of the sutures increased significantly more than the

control samples at both 1 and 2 weeks after infection. The coronal suture, however, demonstrated a modest increase in bone density. Sutures in complete calvaria demonstrated similar increases in sagittal and posterior frontal bone densities after infection (data not shown), albeit the differences were smaller possibly because of the lower efficiency of infecting the entire calvarium versus small strips of calvarium. Using the paired Student's  $t$ -test, the increase in densities between siRANK-infected and control infected sutures yielded  $P$  values of 0.04, 0.03, and 0.09 for coronal, sagittal, and posterior frontal sutures, respectively.

### DISCUSSION

Cranial vault remodeling has received multiple technical and technological advancements in its history, transforming a once dangerous procedure into one with relatively low mortality rates. However, complications such as restenosis and need for reoperation remain and are reported at 5.9% for nonsyndromic and 27.3% for

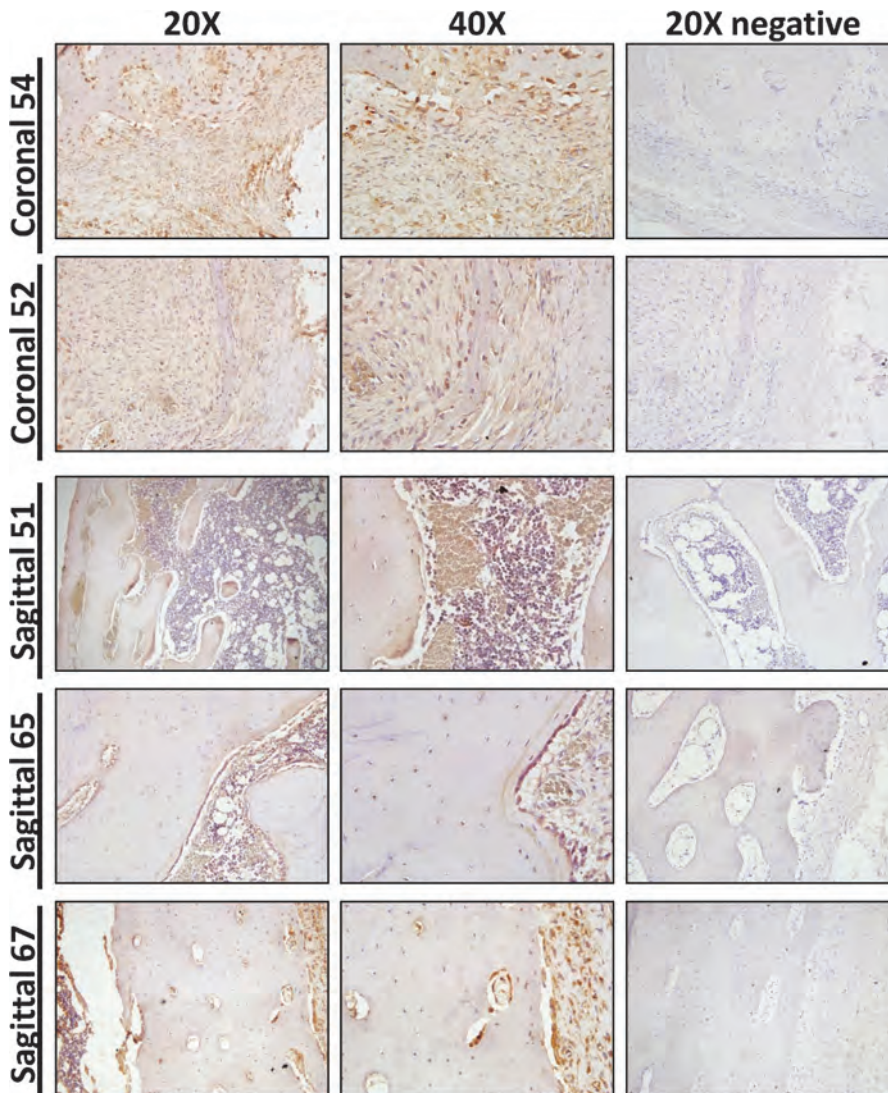


Fig 4 4/C

**FIGURE 4.** RANKL is unaffected in stenotic sutures. Sutures obtained from nonsyndromic craniosynostotic patients during cranial vault reconstruction were stained with anti-RANKL. Patent coronal sutures were also obtained from these patients when available. Both stenotic and open cranial sutures stain positively for RANKL with minimal differences.

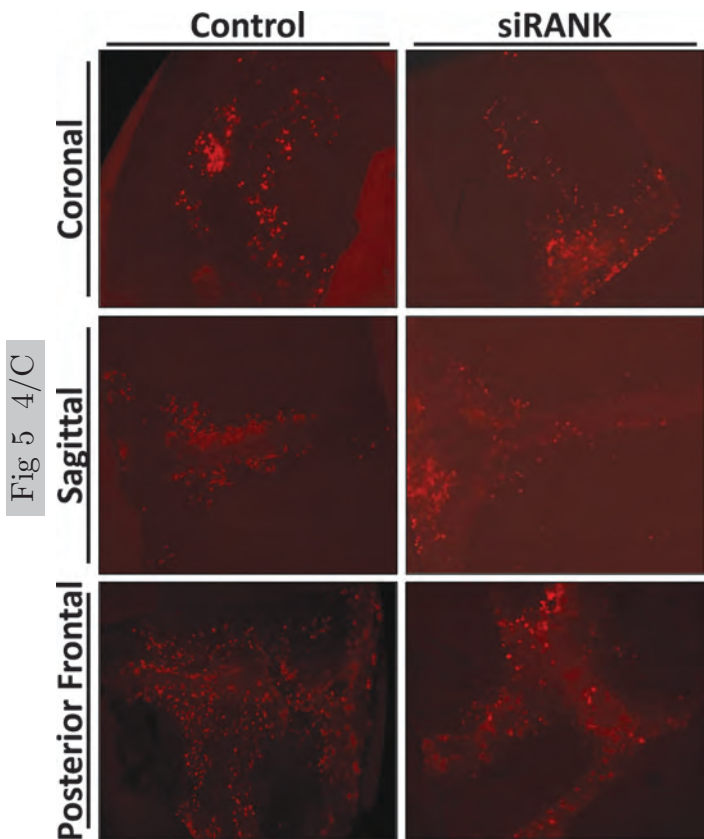


Fig 5 4/C

**AQ3** FIGURE 5. AdRFP and AdsiRANK infect calvarial suture strips with similar efficiencies. Suture-containing calvarial strips from 3-week-old mice were infected with AdRFP (control, left) or AdsiRANK (right). Red fluorescence was used to confirm infection efficiency and cell viability.

syndromic.<sup>51,52</sup> A number of investigators also describe long-term consequences such as cognitive and speech delays.<sup>53</sup> The limitations and resulting sequelae of surgery provide an impetus to identify methods to prevent craniosynostosis.

The past decade elucidated many important mechanisms in cranial suture biology including osteoblast dysfunction and the participation of the regional dura mater in regulating suture fusion.<sup>54,55</sup> These studies have been the basis for progress toward developing molecular therapeutics. Recently, Noggin, a BMP antagonist, applied postoperatively in a rabbit model appeared to decrease restenosis.<sup>56</sup> However, bone biology depends on the interaction of both osteoblasts and osteoclasts. It is therefore likely that bone pathology also involves both cell types.

The RANK-RANKL-OPG axis is a major pathway in osteoclast survival, development, and activation (reviewed in Takayanagi<sup>57</sup>). In this study, we identified a lower level of RANK protein expression in cranial suture fusion in both physiological and pathologic situations, suggesting a mechanism for osteoclast activation in the maintenance of patency. Unlike previous work on molecules expressed on osteoblasts and in the dura mater, RANK levels appeared to be regulated at a posttranslational rather than transcriptional level, which is in accordance to the reported genomic microarray studies that did not detect differences in RANK.<sup>26</sup> Furthermore, RANK down-regulation is necessary and sufficient to induce ex vivo increases in bone density consistent with fusion. Given the known effect of RANK on osteoclast activation and maturation, we propose a model for RANK function

in cranial suture biology (Fig. 7). Receptor activator of NF-κB receptors expressed on osteoclasts trimerize to interact with RANKL, thereby delivering survival and activating signals to osteoclasts. In addition, either the receptor-ligand complex or posttranslational modifications of RANK prevent degradation of the receptor. Conversely, we propose that a reduction of RANK signaling in cranial suture fusion results in a lack of survival or activating signals inducing apoptosis.

Regulation of RANK signaling in cranial suture biology remains to be elucidated. Our study did not demonstrate differences in the expression of RANKL. Whereas RANK is a known osteoclast receptor, RANKL may be expressed by several cell types such as osteoblasts and T cells. Given that osteoblasts are members of the cranial suture microenvironment, osteoblasts represent an obvious candidate. Immunohistochemical analysis of CD3<sup>+</sup> T cells did not yield an appreciable influx of lymphocytes in patent or fused sutures in the patients reported in this study (data not shown). However, RANKL is also a secreted protein and may act in a paracrine manner after secretion by other cell types such as the regional dura mater. Inhibition of RANK signaling is known to involve the OPG decoy receptor, which sequesters RANKL. Again, our immunohistochemical analyses showed no indication of differences in OPG expression in either fused or patent sutures of craniosynostosis patients (data not shown). Because of their secreted nature, OPG and RANKL may be molecules that require other means for detection such as in vivo fluorescence labeling. Finally, strip suture cultures infected with siRANK displayed elevations in bone density. Whereas both posterior frontal and sagittal cultures exhibited sizable increases in density, coronal cultures showed a much smaller difference from control infections, suggesting that mechanisms controlling fusion are suture dependent. These regional differences in osteoclast regulation and downstream molecules from RANK are intriguing and deserve further attention.

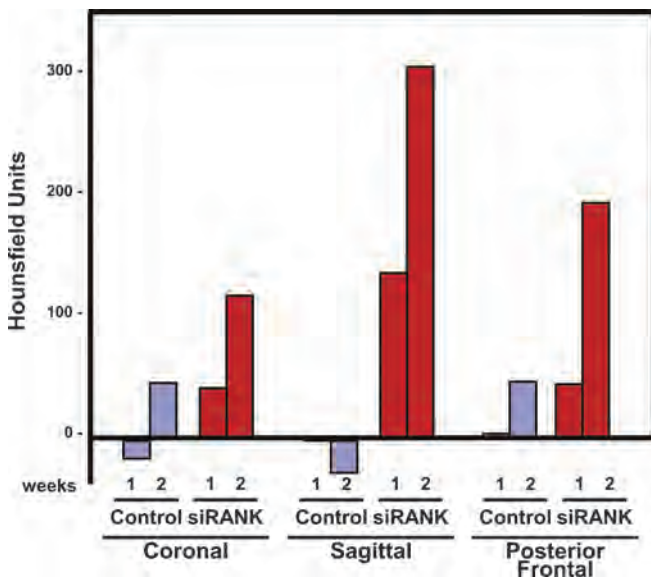
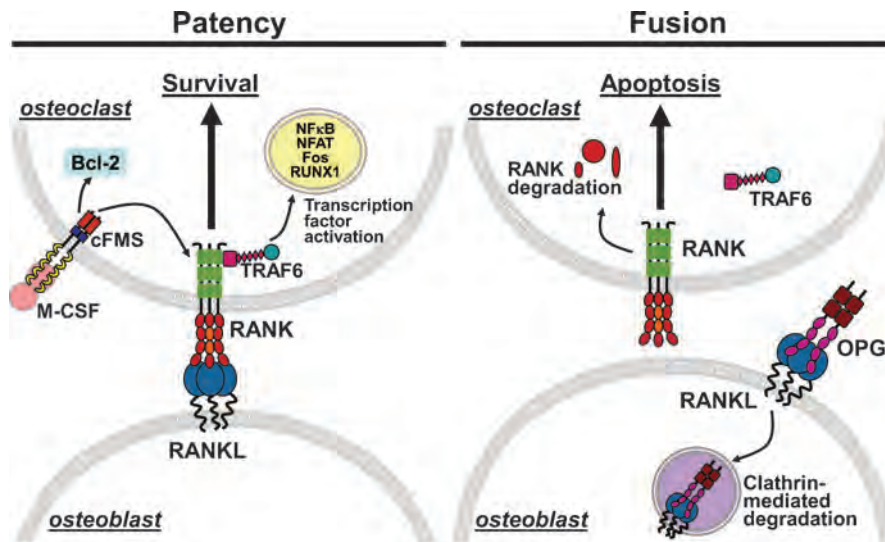


Fig 6 4/C

**FIGURE 6.** siRANK expressing calvarial sutures exhibit increases in bone density consistent with fusion. Micro-CT scans were performed on control and siRANK-infected suture-containing calvarial strips 24 hours after successful infection ( $t = 0$ ), 1, and 2 weeks after infection. Identical three-dimensionally rendered images corresponding to an approximately  $5.3 \times 4.3$ -mm strip of suture were duplicated on each sample. The average Hounsfield units in these strips were calculated and  $t = 0$  densities subtracted.



**FIGURE 7.** Model for RANK function in cranial suture biology. RANK receptors expressed on osteoclasts trimerize to interact with RANKL on osteoblasts or other involved cell types delivering survival signals to osteoclasts. In addition, the receptor-ligand complex or posttranslational modifications of RANK prevent degradation of the receptor. A secondary role of macrophage colony-stimulating factor potentiates RANK signaling by increasing the expression of RANK and Bcl-2. Decreased activity downstream of RANK from protein degradation induces apoptosis. One possible mechanism involves the presence of the decoy receptor OPG.

Murine models deficient in RANK and RANKL display osteopetrotic phenotypes, whereas OPG-deficient mice are osteoporotic.<sup>37–39,41,42</sup> Further *in vivo* studies in cranial suture development in these models to delineate any phenotypic differences in comparison to wild-type mice and other murine craniosynostosis models are needed. Mice with FGFR pathway disruptions found in syndromic craniosynostosis recapitulate the human disease phenotype through increasing osteoblast proliferation and activity.<sup>58,59</sup> Our study now complements these results by suggesting that disruptions in osteoclast proliferation and activity also promote craniosynostosis. Future studies will be directed toward unraveling the mechanisms regulating the RANK signaling pathway, as well as the molecular switches responsible for osteoclast function within the suture front.

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