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The Effect of NELL1 and Bone Morphogenetic Protein-2 on Calvarial Bone Regeneration

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Abstract

Purpose—Most craniofacial birth defects contain skeletal components that require bone grafting. Although many growth factors have shown potential for use in bone regeneration, bone morphogenetic proteins (BMPs) are the most osteoinductive. However, supraphysiologic doses, high cost, and potential adverse effects stimulate clinicians and researchers to identify complementary molecules that allow a reduction in dose of BMP-2. Because NELL1 plays a key role as a regulator of craniofacial skeletal morphogenesis, especially in committed chondrogenic and osteogenic differentiation, and a previous synergistic mechanism has been identified, NELL1 is an ideal molecule for combination with BMP-2 in calvarial defect regeneration. We investigated the effect of NELL1 and BMP-2 on bone regeneration in vivo.

Materials and Methods—BMP-2 doses of 589 and 1,178 ng were grafted into 5-mm critical-sized rat calvarial defects, as compared with 589 ng of NELL1 plus 589 ng of BMP-2 and 1,178 ng of NELL1 plus 1,178 ng of BMP-2, and bone regeneration was analyzed.

Results—Live micro-computed tomography data showed increased bone formation throughout 4 to 8 weeks in all groups but a significant improvement when the lower doses of each molecule were combined. High-resolution micro-computed tomography and histology showed more mature and complete defect healing when the combination of NELL1 plus BMP-2 was compared with BMP-2 alone at lower doses.

Conclusion—The observed potential synergy has significant value in the future treatment of patients with craniofacial defects requiring extensive bone grafting that would normally entail extraoral autogenous bone grafts or doses of BMP-2 in milligrams.

Congenital and acquired craniofacial defects are not uncommon. Congenital defects such as craniosynostosis affect 1 in 3,000 live births, and cleft lip and palate comprise the second most common congenital birth defect after cardiac anomalies.¹⁻³ In addition, acquired defects from simple tooth extractions to radical tumor resections appear daily in our practices and present difficult reconstructive treatment plans. Most craniofacial defects have skeletal components and require extensive surgeries and bone grafting. Although these patients undergo multiple invasive surgeries, treatment outcomes often yield suboptimal results.

In addition to long-term treatment outcomes, surgical morbidity is a concern. Bone grafting procedures are often performed with autogenous bone, which is associated with limited volume and significant donor-site morbidity.⁴⁻⁷ Although alloplasts or allografts may be used, these materials have other risks and have limited use based on defect volume, location, or host factors.⁸⁻¹⁰ With the availability of growth factors, including potent osteoinductive molecules such as bone morphogenetic protein (BMP) 2, a new era in reconstructive surgery is apparent. The identification of BMPs has significantly advanced the study of growth factors in bone healing.¹¹ BMP-2 released from various carriers has been shown to completely regenerate calvarial defects in the rodent model.¹²⁻¹⁵ In humans BMP-2 recombinant protein can regenerate mandibular continuity defects and cleft palate defects with results comparable to autogenous particulate bone and marrow.¹⁶⁻¹⁸ However, supraphysiologic doses required for osteoinductive effects in humans create concern regarding adverse effects and high cost.¹⁹⁻²⁴

NELL1 is a novel gene that is upregulated in human craniosynostosis. One of the putative mechanisms of the effect of NELL1 in craniosynostosis is through its ability to increase osteoblast differentiation and mineralization, thus contributing to osteoblastic function.²⁵ Upon investigation of NELL1 signaling, we showed that NELL1 transcription was increased on transforming growth factor β_1 and fibroblast growth factor 2 stimulation but not BMP-2. In an evaluation of downstream regulation of NELL1, it was found to decrease expression of early markers of osteoblastic differentiation and increase expression of intermediate and late markers, which is markedly different from the effects of BMP-2 on osteoblastic differentiation.¹² Moreover, we showed a significant increase in bone regeneration in vivo after rat calvarial defects were implanted with a scaffold containing either NELL1 or BMP-2. In fact, there was no apparent difference between the 2 molecules in bone regeneration on micro-computed tomography (microCT) or histologic evaluation.¹² These in vivo data combined with investigation into the signaling cascades regulating and regulated by NELL1 and BMP-2 suggest that these molecules may be inducing osteoblastic differentiation through different signaling pathways.^{12,26} Indeed, synergistic induction of osteoblastic differentiation and bone formation has been shown when BMP-2 and NELL1 were combined in a rodent intramuscular model of bone formation.²⁶ On the basis of its importance in craniofacial development and its effect on osteoblast differentiation in vitro and bone formation in vivo, NELL1 may be an ideal molecule to use in combination with BMP-2 for craniofacial bone regeneration to reduce the required dose of BMP-2.

This study investigated the effect of NELL1 plus BMP-2 on bone formation in vivo, as compared with BMP-2 alone. We hypothesized that NELL1, when administered together with BMP-2, will enhance regenerative activity compared with administration of BMP-2 alone and will require lower doses than BMP-2 alone. In this study suboptimal doses of BMP-2 showed enhanced bone formation with the addition of NELL1 in 5-mm critical-sized

rat calvarial defects as analyzed through live microCT, high-resolution cadaveric microCT, and histologic methods. The data showed potential synergy in vivo with the combination of BMP-2 and NELL1, which may decrease potential adverse effects of a higher dose of either molecule alone.

Materials and Methods

FABRICATION OF MULTILAYER CARRIER SCAFFOLDS

Five hundred-micron-thick 85:15 poly(lactic-co-glycolic acid) (PLGA) scaffolds were fabricated by solvent casting and a particulate leaching process (inherent viscosity, 0.62 dL/g) (Absorbable Polymers, Pelham, AL) as previously described.²⁷ The porogen (sugar, 200-300 μ m in diameter) and PLGA/chloroform solution were packed into a Teflon mold to achieve 92% porosity (volume fraction). After porogen leaching and ethanol sterilization, scaffolds were verified by use of a scanning electron microscope (FEI/Philips XL-30; FEI/Philips, Hillsboro, OR). For growth factor coating, recombinant human BMP-2 (Sigma-Aldrich, St Louis, MO) was diluted in 0.025% type I collagen solution (Cohesion, Toronto, Ontario, Canada) and then incorporated onto the PLGA scaffold. The groups were as follows, with 5 scaffolds in each group: control (0 ng), 30 ng/mm³ (589 ng) of BMP-2, 60 ng/mm³ (1,178 ng) of BMP-2, 30 ng/mm³ (589 ng) of BMP-2 plus 30 ng/mm³ (589 ng) of NELL1, and 60 ng/mm³ (1,178 ng) of BMP-2 plus 60 ng/mm³ (1,178 ng) of NELL1 (Sigma-Aldrich²⁸). Scaffolds were then dried and stored at -20°C before implantation into animals.

RAT CALVARIAL DEFECT SURGERIES

All animals and surgical procedures were handled in accordance with the guidelines of the Chancellor's Animal Research Committee of the Office for Protection of Research Subjects at the University of California, Los Angeles. Sprague-Dawley rats, purchased from Charles River Laboratories (Wilmington, MA), were housed in light- and temperature-controlled facilities and given food and water ad libitum. Five-millimeter-diameter trephine defects were created unilaterally in the calvaria of male 3-month-old Sprague-Dawley rats under constant irrigation and with care to avoid injury to the underlying dura. Five-millimeter defects have been deemed critical sized because they heal with fibrous tissue.²⁹ Each defect was flushed with saline solution to remove bone debris; implanted with scaffolds containing saline solution (control), 589 ng of BMP-2, 1,178 ng of BMP-2, 589 ng of BMP-2 plus 589 ng of NELL1, or 1,178 ng of BMP-2 plus 1,178 ng of NELL1; and sutured closed.

LIVE MICROCT IMAGING

Live microCT (Imtek, Knoxville, TN) was used to examine bone formation over time in individual rats.³⁰ Rats were anesthetized with 2% isoflurane while heated with a heat-recirculating water pad to prevent hypothermia. Live microCT imaging was performed at weeks 4, 6, and 8 for each animal, as previously described.¹⁴ No x-ray contrast medium was used in this study. Image reconstruction and density measurements were performed with the visualization software AVS/Express (version 5.1; Advanced Visual Systems, Waltham, MA). AVS software allowed for density measurements at each 50- μ m distance throughout the thickness of the calvaria. Data were plotted by use of Microsoft Excel (Microsoft, Redmond, WA).

HIGH-RESOLUTION MICROCT IMAGING

Animals were sacrificed after 12 weeks, and calvaria were harvested for analysis. Calvaria were fixed in 10% formalin for 48 hours and rinsed in phosphate-buffered saline solution before imaged with high-resolution microCT (μ CT 40; Scanco USA, Wayne, PA) as

previously described.²⁵ A microCT threshold of 275 was used for analysis of mineralization and was consistent with previous publications.¹² Visualization, reconstruction, and volume analysis of the data were performed with the MetaMorph Imaging System (Universal Imaging, Downingtown, PA). In addition, computed tomography-based morphometric analyses of defects were used to determine area and volume within the defect covered by new bone formation by use of Image-Pro Plus, version 5.0 (Media Cybernetics, Carlsbad, CA).³¹

HISTOLOGIC ANALYSIS

Decalcified (Fisher Scientific, Houston, TX) samples were embedded in paraffin, and 10- μ m-thick sections were stained with hematoxylin-eosin (H&E)²⁷ and Masson-Goldner trichrome stain according to standard protocols. Photomicrographs were taken with a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) and analyzed by use of BioQuant software (R&M Biometrics, Nashville, TN).

Results

INCREASED BONE REGENERATION WITH NELL1 AND BMP-2 IN RAT CALVARIAL DEFECTS

After inhalational general anesthesia, the critical-sized rat calvarial defect model was used. 29 Defects were created unilaterally to eliminate any effect of scaffold or growth factor from the contralateral side (Fig 1A). Either saline solution (control) or growth factors were adsorbed onto PLGA scaffolds (Fig 1B) and then grafted into the defect (Fig 1C).

The ability of both NELL1 and BMP-2 to regenerate bone in rat calvarial defects has been previously shown.^{12,14} However, in vivo bone regeneration with BMP-2 often requires supraphysiologic doses, with risks of potential adverse effects.¹⁹⁻²⁴ Therefore we used the combination of NELL1 and BMP-2 recombinant proteins adsorbed onto PLGA scaffolds to decrease the required dose of BMP-2 in regenerating critical-sized calvarial defects. Live microCT analysis, which can evaluate the same animal over time, showed increasing bone regeneration after for both high-dose and low-dose BMP-2 treatments compared with minimal healing over the 8-week time period after implantation of PLGA scaffolds alone. The addition of 589 ng of NELL1 to 589 ng of BMP-2 significantly increased bone regeneration over either BMP-2 dose at 4, 6, and 8 weeks (Fig 2). By 4 weeks, approximately 90% of the defect was regenerated, which continued to mature, reaching almost 100% by 8 weeks (Fig 2B). Although both the low dose and high dose of BMP-2 showed a decrease in the defect size, morphology of new bone formed after regeneration with NELL1 and BMP-2 is significantly different, with the BMP-2-treated sites showing islands of mature lamellar bone surrounding large amounts of fatty marrow tissue.³² Therefore high-resolution microCT and histology are required to determine both the quality and quantity of newly formed bone.

HIGH-RESOLUTION MICROCT IMAGING AND BONE QUANTIFICATION

To quantitate the extent of bone regenerated with BMP-2 and with NELL1 plus BMP-2, new bone volume was investigated. We have previously shown that advances in microCT imaging are used to acquire 3-dimensional data nondestructively, where these data may accurately substitute for conventional histology.^{14,33-35} In this study we used high-resolution microCT to evaluate new bone regeneration qualitatively and quantitatively after 12 weeks. Both low and high doses of BMP-2 showed significant bone regeneration compared with control defects. When NELL1 was added, again the dose of both factors was greatly reduced to show an equivalent or improved effect than with either factor alone (Fig 3). Interestingly, when the volume of new bone was quantified, only up to 70% of bone was

regenerated as compared with the uninjured calvaria (Fig 3B). High-resolution microCT evaluation after sacrifice is more accurate at 20 μ m of resolution, as compared with live microCT at 100 μ m of resolution, which accounts for significant differences in bone regeneration seen with these techniques.

HISTOLOGIC ANALYSIS OF BONE REGENERATION

Conventional H&E histology showed an increase in mature lamellar bone regeneration after 12 weeks in all treated defects, as compared with minimal bone healing from the margins of defects in controls. After treatment with both high and low BMP-2 doses, bone regeneration occurred from the margins and at the center of the defect, as previously reported.^{12,14} Defects treated with the combination of NELL1 and BMP-2 showed greater bridging of the defect, with near-complete healing with 589 ng of NELL1 plus BMP-2 and 1,178 ng of NELL1 plus BMP-2. In fact, bone regeneration with 589 ng of NELL1 plus BMP-2 was equivalent to the higher doses of either molecule alone or the higher doses of the combination (Fig 4). This suggests that NELL1 and BMP-2 may be working through distinct mechanisms for their osteoinduction. Although almost complete regeneration was seen on histology, volume regeneration on microCT reached only 70% (Fig 4). The visualization of osseous tissue throughout the defect does not necessarily correlate with the degree of mineralization, especially because the samples are demineralized before embedding for the histologic evaluation.¹⁴ These data suggest that histology for evaluation of bone regeneration should be combined with high-resolution microCT to acquire accurate 3-dimensional information, both qualitatively and quantitatively.

Importantly, mature lamellar bone found histologically correlated with the increased bone density found via microCT. Trichrome staining showed a combination of osteoid and mineralized bone within all regenerated bone (Fig 5). The 589-ng dose of BMP-2 resulted in both osteoid and mature bone throughout the thickness of the defect, with fibrous tissue between the regenerated bone, whereas the 1,178-ng BMP-2 dose resulted in a larger fraction of mature and highly mineralized bone in the center of the defect. When NELL1 and BMP-2 are used in combination, both low and high doses show both osteoid and mature lamellar bone throughout the defects. High- and low-dose combinations show bridging bone, beginning with less mineralized bone in the center of the defect, with mineralized mature bone on the inner and outer surfaces of the calvaria. In addition, combined NELL1 and BMP-2 show an increased thickness in the regenerated bone when the 2 factors are used in combination. Although conventional H&E histology shows osseous tissue throughout the thickness of the calvaria, it does not give information on the maturity of the regenerated bone. Thus histologic evaluations, both H&E and trichrome staining, and microCT density measurements are a useful combination.

Discussion

The use of growth factors for hard tissue reconstruction is gaining increased popularity and acceptance as clinicians, scientists, and patients search for less invasive therapies for craniofacial and orthopedic indications.^{17,18,36-40} Currently, most bone regenerative strategies using growth factors have focused on BMP-2, a potent osteoinductive molecule.^{41,42} However, concerns about the supraphysiologic doses required for effective bone regeneration, as well as adverse effects such as heterotopic bone formation and high cost, are raised when such large doses are used.^{21,23,43,44} Therefore a significant challenge to identify molecules effective in inducing bone healing remains.

NELL1 (NEL-like molecule 1) (NEL is a protein strongly expressed in neural tissue encoding epidermal growth factor–like domain) is a novel secretory protein originally identified in active bone-forming sites in craniosynostosis patients.⁴⁵ One of the putative

mechanisms of the effect of NELL1 in craniosynostosis is through its ability to increase osteoblast differentiation and mineralization, thus contributing to osteoblastic function.^{25,45,46} Upon investigation of NELL1 signaling, we previously showed that NELL1 transcription is increased on transforming growth factor β_1 and fibroblast growth factor 2 stimulation but not after BMP-2 stimulation, suggesting that BMP-2 is not directly upstream from NELL1. NELL1 also decreases the expression of early markers of osteoblastic differentiation such as alkaline phosphatase and increases the expression of intermediate and late markers, a markedly different pattern from the effects of BMP-2 on osteoblastic differentiation.¹² Because BMP-2 induces osteogenic differentiation through both *core binding factor- $\alpha 1$* -dependent and -independent pathways⁴⁷⁻⁴⁹ and NELL1 signals downstream of *core binding factor- $\alpha 1$* ,^{50,51} a potential interaction is not surprising.

In vivo we previously found that NELL1 significantly increases bone regeneration in rat calvarial defects. In fact, neither microCT nor histologic evaluation of bone regeneration showed apparent differences between NELL1 and BMP-2.¹² These in vivo data combined with investigation into the signaling cascades regulating and regulated by NELL1 and BMP-2 suggest that these molecules may be inducing osteoblastic differentiation through different signaling pathways and that a potential synergistic effect on osteoblast function exists.^{12,26} Moreover, unlike BMP-2, the effects of NELL1 are primarily osteochondral, targeting cells already committed to the osteogenic or chondrogenic lineage.^{25,26,45,46,50,52} Because differences in morphology of bone regenerated with NELL1 and BMP-2 exist, complementarity of the 2 factors is not surprising.^{26,32} This investigation evaluated the combined effect of NELL1 and BMP-2 recombinant protein technology on bone regeneration in the well-established rat calvarial defect model. This mode of delivery, adsorbed onto a biodegradable PLGA scaffold, was chosen because of its clinical relevance, where currently, BMP-2 is approved for use on an absorbable collagen sponge carrier. Live microCT, which is used to maximize results from a single animal to obtain sequential data of the bone regeneration process, showed an increase in healing when 589 ng of BMP-2 and NELL1 were combined, as compared with 589 ng and 1,178 ng of BMP-2 alone.

Although live microCT showed almost complete healing with combined NELL1 and BMP-2 at 589 ng, high-resolution microCT indicated approximately 60% volume regeneration with 589 ng or 1,178 ng of BMP-2 and up to 70% with 589 ng and 1,178 ng of NELL1 plus BMP-2. In fact, no apparent differences were seen between the low- and high-dose combination, suggesting that the synergistic action of the 2 molecules was effective at a low dose, not requiring an increase in either factor. The lack of complete volume regeneration to that of uninjured calvaria may represent a suboptimal carrier. In this study we used PLGA scaffolds because PLGA is a common synthetic polymer with a proven safety record in humans. In addition, PLGA is neither osteoinductive nor osteoconductive, which allows the evaluation of bone healing directly correlated to the molecules used and not the scaffold.^{14,53} However, both in vitro and in vivo release profiles of both molecules must be evaluated to determine the ideal scaffold to produce the desired regenerative effect. Studies evaluating sustained release of BMP-2 to increase the bioactivity led to improved osteoblastic differentiation in vitro and bone formation in vivo.^{13,54-56} Upon histologic evaluation, marked bone formation was seen after treatment with both low- and high-dose BMP-2. The addition of NELL1 allowed a lower dose of BMP-2 to be used and resulted in increased bone regeneration as compared with BMP-2 alone at both doses. The combination not only improved bone architecture and overall formation but also enabled the use of decreased doses of BMP-2. Because concerns about supraphysiologic doses and adverse effects of a single factor are clinical realities, decreasing the dose of individual factors to obtain equivalent or even superior effects is ideal.

In this new era of reconstructive biotechnology, developing osteoinductive molecules with divergent but complementary pathways of bone formation will maximize biological efficiency, which will, in turn, improve clinical efficacy, lower dose requirements and costs, and minimize potential adverse effects of current osteogenic therapeutics. Although, currently, BMP-2 and BMP-7 are the only molecules approved for use in orthopedic indications and only BMP-2 is approved for oral and maxillofacial indications, the potential for alternative molecules in combination with BMPs is apparent. In vitro, molecules including osteogenic oxysterols,⁵⁷ NELL1,²⁶ C-type natriuretic peptide,⁵⁸ interleukin 6,⁵⁹ cartilage-derived morphogenetic proteins,⁶⁰ statins,⁶¹⁻⁶³ vascular endothelial growth factor,⁶⁴ and parathyroid hormone-related peptide⁶⁵ have been shown to be either synergistic with or supportive of BMPs' induction of osteogenic differentiation. In vivo studies have also shown synergistic improvement in bone formation when BMPs are combined with vascular endothelial growth factor⁶⁶ and NELL1.²⁶ It has been shown that morphology of regenerated bone differs between BMP-2 and other osteoinductive materials including NELL1. BMP-2-regenerated bone showed extensive amounts of fatty tissue and less densely packed woven and lamellar bone.³² In contrast, NELL1 formed smaller but more solid bone masses.³² These variations in morphology will reflect choices in bone grafting for patients requiring augmentation. Future studies to evaluate the combination of molecules that have shown favorable results with BMP-2 in vitro that complement each other to achieve the most effective and precise bone regeneration in vivo must now be undertaken. The data presented offer a new strategy to advance regenerative therapeutics for patients with craniofacial disorders using NELL1 and BMP-2 while minimizing potential adverse effects of high doses of each individual factor alone.

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FIGURE 1.

A, Unilateral critical-sized rat calvarial defect. B, Saline solution or growth factor adsorbed onto PLGA scaffold. C, PLGA scaffold implanted into calvarial defect.

FIGURE 2.

Live microCT analysis of bone formation over time. *A*, Qualitative live microCT analysis in rat calvarial defect 4, 6, and 8 weeks after implantation of PLGA scaffolds coated with control (saline solution), 589 ng of BMP-2, 1,178 ng of BMP-2, or 589 ng of NELL1 plus BMP-2. *B*, Quantitative measurements of percent bone regeneration after implantation of scaffolds alone or with 589 ng of BMP-2, 1,178 ng of BMP-2, or 589 ng of NELL1 plus BMP-2 (N+B). Asterisk, Significant increase compared with control ($P < .05$); pound sign, significant increase compared with both doses of BMP-2 alone ($P < .05$).

FIGURE 3.

High-resolution microCT and quantification of regenerated bone volume. *A*, Three-dimensionally reconstructed high-resolution microCT images of defects implanted with control, 589 ng of BMP-2, 1,178 ng of BMP-2, 589 ng of NELL1 plus BMP-2, and 1,178 ng of NELL1 plus BMP-2 after 12 weeks of healing. *B*, Quantification of percent volume bone regeneration in calvarial defects after 12 weeks of healing compared with uninjured calvaria. All groups produced significantly more regenerated bone than control samples ($P < .05$).

FIGURE 4.

Histologic analysis of bone regeneration in calvarial defects after 12 weeks of healing. H&E staining of calvarial defects (*arrows* represent defect margins) shows fibrous tissue in control samples, as compared with more woven bone formation in groups with BMP-2 alone and more lamellar bone formation in groups with NELL1 plus BMP-2.

FIGURE 5.

Masson trichrome staining of bone healing in calvarial defects. After 12 weeks of healing, the maturity of the regenerated bone was evaluated. Combined NELL1 and BMP-2 show areas of osteoid (*blue*) and mature lamellar (*red*) bone formation, with an increased thickness in the regenerated bone when the 2 factors are used in combination.