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Growth Factors, Cytokines, Cell Cycle Molecules

Nell-1-Induced Bone Regeneration in Calvarial Defects

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Many craniofacial birth defects contain skeletal components requiring bone grafting. We previously identified the novel secreted osteogenic molecule NELL-1, first noted to be overexpressed during premature bone formation in calvarial sutures of craniosynostosis patients. *Nell-1* overexpression significantly increases differentiation and mineralization selectively in osteoblasts, while newborn *Nell-1* transgenic mice significantly increase premature bone formation in calvarial sutures. In the current study, cultured calvarial explants isolated from *Nell-1* transgenic newborn mice (with mild sagittal synostosis) demonstrated continuous bone growth and overlapping sagittal sutures. Further investigation into gene expression cascades revealed that fibroblast growth factor-2 and transforming growth factor-β1 stimulated *Nell-1* expression, whereas bone morphogenetic protein (BMP)-2 had no direct effect. Additionally, Nell-1-induced osteogenesis in MC3T3-E1 osteoblasts through reduction in the expression of early up-regulated osteogenic regulators (*OSX* and *ALP*) but induction of later markers (*OPN* and *OCN*). Grafting Nell-1 protein-coated PLGA scaffolds into rat calvarial defects revealed the osteogenic potential of Nell-1 to induce bone regeneration equivalent to BMP-2, whereas immunohistochemistry indicated that Nell-1 reduced osterix-producing cells and increased bone sialoprotein, osteocalcin, and BMP-7 expression. Insights into Nell-1-regulated osteogenesis coupled with its ability to stimulate bone regeneration revealed a potential therapeutic role and an alternative to the currently accepted techniques for bone regeneration. ***(Am J Pathol 2006, 169:903–915; DOI: 10.2353/ajpath.2006.051210)***

Clinical problems requiring bone regeneration are diverse and especially challenging within the craniofacial complex. The majority of craniofacial defects have skeletal components and require extensive surgery and bone grafting procedures.[1] Although autografts are the gold standard, they are clinically limited by availability and donor site morbidity.[2] Therefore, clinicians and researchers are continuously looking for ways to treat bony defects without autogenous grafting. Growth factors such as bone morphogenetic proteins (BMPs) have demonstrated great *in vivo* osteogenic potential[3] and are already approved for spinal fusion and long bone fractures in humans. Unfortunately, these applications currently require superphysiological doses[4] and have been reported to induce undesirable heterotopic bone formation away from sites of administration as well as pleiotropic non-bone-specific effects.[5] These concerns have limited the surgical techniques, site selection, and reinforce the need for osteoblast-specific stimulants. Core-binding factor α 1/runt-related transcription factor 2 (Cbfa1/Runx2), an essential transcription factor in osteoblast differentiation and bone formation,[6] has been actively investigated for potential translational applications because it signals downstream of BMPs and is osteoblast-specific. However, because Cbfa1/Runx2 is a transcription factor, its use is restricted to gene therapy approaches as opposed to recombinant protein delivery.

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Thus, osteogenic proteins that are downstream of Cbfa1/Runx2 and more specific to bone formation may be viable alternatives to BMPs.

NELL-1 [NEL-like molecule-1; NEL (a protein strongly expressed in neural tissue encoding *e*pidermal growth factor-like domain)], isolated and characterized in craniosynostosis patients as specifically up-regulated within prematurely fusing sutures,[7,8] may represent such an exciting alternative. NELL-1 is highly conserved across species, with human and rat Nell-1 being 90% homologous at the genetic level and 93% homologous at the protein level.[9,10] The phenotype of the *Nell-1* transgenic overexpression mouse revealed cranial suture overgrowth similar to human craniosynostosis,[7] suggesting a distinct role for Nell-1 in bone formation. Conversely, a mouse model with mutated *N*-ethyl-*N*-nitrosourea-induced alleles, including Nell-1, resulted in cranial and other skeletal defects.[11] Furthermore, *Nell-1* is preferentially expressed in neural crest-derived tissues, suggesting its specificity for the craniofacial region. In osteoblasts, Nell-1 up-regulation accelerates differentiation and bone formation, whereas Nell-1 down-regulation inhibits osteoblast differentiation.[7,8,10,12,13] Interestingly, we have recently shown that human *NELL-1* is directly regulated by Cbfa1/Runx2,[14] confirming its osteochondral specificity. Finally, because Nell-1 is a secreted protein, controlled delivery of Nell-1 may be a possible modality to regenerate craniofacial bony defects.[15]

In the current research, wild-type and transgenic *Nell-1* newborn (with mild sagittal synostosis) mouse calvarial explants demonstrated patent and dramatic bone overlap within sagittal sutures, respectively. Parietal bone overgrowth and overlap within the sagittal suture has been described by other transgenic mouse models as murine craniosynostosis and has been compared to the human disease of craniosynostosis.[16,17] Investigations into Nell-1 signaling pathways revealed a transcriptional regulation by transforming growth factor (TGF)-β1 and fibroblast growth factor (FGF)-2, but not bone morphogenetic protein (BMP)-2. Although all three growth factors are known to regulate bone formation[18–21] and the protein kinase C (PKC) activity[22] that is indicated for Nell-1 activation,[12] only increased FGF-2 signaling, which has multiple defined pathways leading to PKC activation, has been clearly associated with craniosynostosis.[23] Furthermore, Nell-1 reduced the transcription of early regulators and induced that of intermediate and late markers of osteogenic differentiation. Finally, bioactive recombinant trimeric Nell-1 protein, with a modified signaling peptide, was generated using the baculoviral expression system. Grafting Nell-1-coated PLGA scaffolds into 3-mm calvarial defects revealed the osteogenic potential of Nell-1 to induce *in vivo* bone regeneration equivalent to BMP-2. Insights into Nell-1 regulated osteogenesis coupled with its ability to induce specific localized bone regeneration *in vivo*, indicates a potential alternative therapeutic role to the currently accepted techniques for bone regeneration.

Materials and Methods

Organ Culture

B6C3 F1 wild-type mice were purchased from Charles River (Wilmington, MA), and *Nell-1* transgenic mice were generated as previously described.[7] Animals were housed and experiments were performed in accordance with guidelines of the Chancellor's Animal Research Committee of the Office for Protection of Research Subjects at the University of California, Los Angeles. For the calvarial explant stimulation, calvaria of wild-type and *Nell-1* transgenic newborn littermates (p1) were harvested and cultured in osteogenic differentiation media containing 50 μg/ml of ascorbic acid and 10 mmol/L of β-glycerol phosphate in BGJb media without serum.[13] The calvarial explants from newborn *Nell-1* transgenic mice ($n = 3$) and wild-type littermates ($n = 4$) were cultured for 9 days and harvested for histological analysis. Calvaria cultured for more than 9 days became nonviable. Calvaria were fixed and stained with Alizarin Red and Alcian Blue. Five-μm-thick sections of the sagittal sutures were analyzed histologically using a fluorescent microscope.

Cell Culture

Fetal rat calvarial cells were harvested as previously described.[7] Subconfluent fetal rat calvarial cells were stimulated with rhBMP-2 (100 ng/ml), FGF-2 (10 ng/ml), or TGF-β1 (5 ng/ml) (all from Sigma-Aldrich, St. Louis, MO) for 24 and 48 hours. Total RNA was purified in Trizol reagent (Invitrogen, Carlsbad, CA). MC3T3-E1 cells were purchased from the American Type Culture Collection (Rockville, MD) for plasmid transfection and adenoviral transduction. Cells were transfected with pcDNA-Cbfa1 or pcDNA3.1 (Invitrogen) for 3 hours with lipofectamine reagent and cultured in standard media for 24 hours before total RNA was harvested in Trizol reagent. Additional subconfluent MC3T3-E1 cells were infected with an adenovirus overexpressing rat *Nell-1* (*AdNell-1*) driven by a CMV promoter[7] at 20 PFU/cell. Cells were then maintained for 48 hours before switched to osteogenic differentiation medium.[13] *AdLacZ* was used as a control. RNA was harvested on days 0, 3, and 6.

RNA Extraction and Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted by Trizol reagent, and DNase-treated RNA was tested for its integrity by agarose gel electrophoresis. One μg of DNase I-treated total RNA was used for reverse transcription as previously described.[7] The product of reverse transcription was used for both conventional PCR and real-time PCR. PCR products were separated on an agarose gel. The primers for mouse genes are as follows: Cbfa1/Runx2 (forward: 5′-CCGCACGACAACCGCACCAT-3′, reverse: 5′-CGCTCCGGCCCACAAATCTC-3′), Nell-1 (forward: 5′-TGCCACTGTGAGAAGACCTG-3′, reverse: 5′-TGCACAGGA-

AGTGAGTCTGG-3′), osteocalcin (OCN) (forward: 5′-CAAGTCCCACACAGCAGCTT-3′, reverse: 5′-AAAGCCGAGCTGCCAGAGTT-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5′-CCATCCACAGTCTTCTGA-3′, reverse: 5′-CACCACCATGGAGAAGGC-3′). For protein-stimulated fetal rat calvarial cells, PCR products were then blotted to nitrocellulose membrane for hybridization with product-specific oligonucleotide probe labeled with ^{32}P. The specific primer and probe sequences for *Nell-1* were designed according to accession number NM031096 (forward: 5′-CTGTGTGGCTCCTAACAAGTGTG-3′, reverse: 5′-GGATTCTGGCAATCACAAGCTGCT-3′, probe: 5′-CCTACTCACTGTCCGGGGAGTCCTGC-3′).

Real-time PCR analysis of bone marker genes, including alkaline phosphatase (ALP), osteopontin (OPN), OCN, Nell-1, osterix (OSX), and GAPDH, performed for MC3T3-E1 transduction with *AdNell-1* and *AdLacZ* with ABI Prism 7300 real-time PCR system, and the primers and probes were purchased as TaqMan primer-probe sets (Applied Biosystems, Foster City, CA). Analysis was based on calculating the relative expression level of the gene of interest compared to GAPDH,[24] then normalized to the expression induced by *AdLacZ* control at corresponding time points ($n = 4$). Student's *t*-test was used to assess significant differences of gene expression on days 3 or 6 as compared to day 0 with $P \leq 0.05$ considered significant.

Scaffold Fabrication and Implantation

Five hundred-μm-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.[25] The porogen (NaCl, 200 to 300 μm) and PLGA/chloroform solution were packed into a 3-mm diameter Teflon mold to achieve 92% porosity (volume fraction). After porogen leaching and ethanol sterilization, scaffolds were verified by scanning electron microscope (FEI/Phillips XL-30; New York, NY). For growth factor coating, 200 ng/scaffold of Nell-1[10] or BMP-2 (Sigma-Aldrich) was diluted in 0.025% type I collagen solution (Cohesion, Palo Alto, CA) and adsorbed onto each scaffold. For production of the C-terminally FLAG-tagged Nell-1 protein, a pIZT-FLC-Nell-1 plasmid was constructed by inserting the rat Nell-1 cDNA linked to a FLAG epitope sequence derived from the pTB701-FLC-Nell-1 plasmid into baculoviral vector pIZT/V5-His (Invitrogen).[12] The recombinant rat Nell-1 protein was purified from the culture medium of zeocin-resistant high five cells by anion-exchange chromatography using a UNO Q-6 column (Bio-Rad, Hercules, CA).

Sprague-Dawley rats were purchased from Charles River Laboratories. Calvaria of male 3-month-old Sprague-Dawley rats were exposed to a trephine drill, under constant irrigation, to create 3-mm full-thickness craniotomy defects in each parietal bone with care to avoid injury to the underlying dura mater. Each defect was flushed with saline to remove bone debris and then implanted with scaffolds.

Live and High Resolution Microcomputed Tomography (microCT) Imaging

A small animal microCT imaging system was used to examine bone formation throughout time in individual rats, as previously described.[26] Rats containing control ($n = 9$), Nell-1 ($n = 3$)-, or BMP-2 ($n = 2$)-coated scaffolds were anesthetized and placed in a microCT scanner (Imtek Inc., Knoxville, TN) to acquire three-dimensional morphometric data. Images were acquired with the X-ray source biased at 35 kVp and 400 μA. Data sets for rats were acquired and reconstructed with resolutions of 100 μm. Visual analyses of the CT data were performed using AVS/Express (version 5.1; Advanced Visual Systems Inc., Waltham, MA) Density measurements were taken at each 50 μm distance throughout the thickness of the calvaria. Analyses performed included mineralization density and calvarial thickness.

A high-resolution microCT (μCT40; Scanco USA, Inc., Southeastern, PA) was used as previously published.[7] MicroCT data were collected at 50 kVp and 160 μA. Visualization, reconstruction, and volume analysis of the data were performed using the MetaMorph Imaging System (Universal Imaging Corp., Downingtown, PA) of defects containing control ($n = 10$ per time point), Nell-1 ($n = 5$ per time point)-, or BMP-2 ($n = 5$ per time point)-coated scaffolds. Bone-specific analyses included: new bone area using Image Pro Plus version 5.0 (Media Cybernetics, Carlsbad, CA) and new bone volume/tissue volume, number of bone voxels in the volume of interest divided by the total number of tissue voxels in the volume of interest.[27]

Histological and Histochemical Analysis

Ten-μm-thick paraffin sections (microtome; McBain Instruments, Chatsworth, CA) of decalcified samples were stained with hematoxylin and eosin[25] and Masson-Goldner trichrome stain according to standard protocols. Additional sections were incubated with anti-bone sialoprotein (BSP), anti-OSX, anti-BMP-7, anti-OCN, and anti-calcitonin receptor (anti-CTR) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and biotinylated anti-rabbit or anti-goat IgG secondary antibody (Vector Laboratories, Burlingame, CA). Positive immunoreactivity was detected using Vectastain ABC and AEC kits (Vector Laboratories). Controls for each antibody consisted of incubation with secondary antibody in the absence of primary antibody. In addition, tartrate-resistant acid phosphatase staining was used to examine osteoclast activity within frozen sections using an acid phosphatase, leukocyte kit (Sigma Diagnostics, St. Louis, MO) according to the manufacturer's protocol. Photomicrographs were taken with a Leica DMLB microscope (McBain Instruments) and using BioQuant software (R&M Biometrics, Nashville, TN).

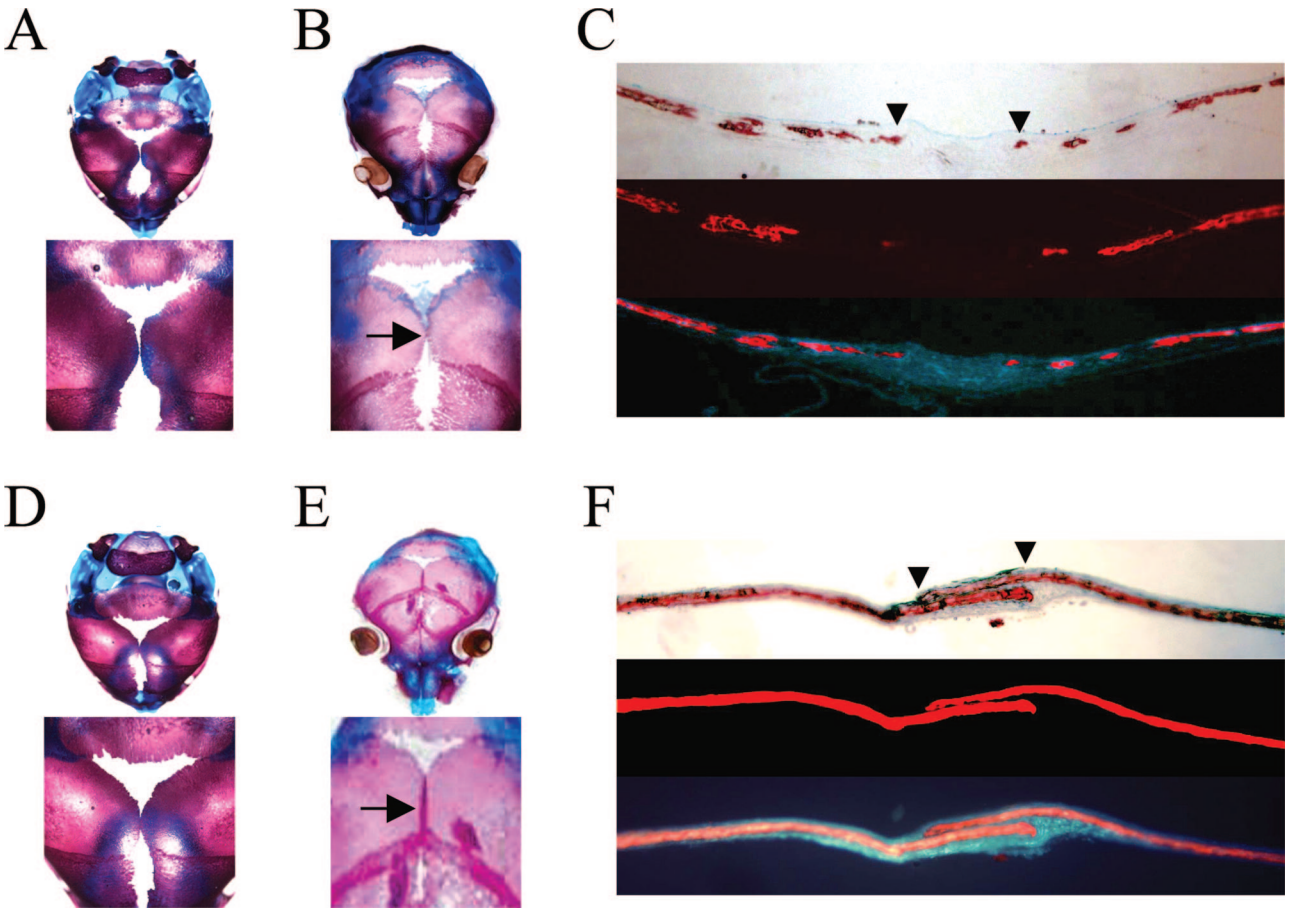


Figure 1. Sagittal suture overgrowth in Nell-1 transgenic calvarial explants. **A:** Newborn wild-type mouse calvarial explant demonstrating patent sagittal suture in low magnification (top) and high magnification (bottom). **B:** Wild-type mouse calvarial explant after 9 days in culture showing patent sagittal suture in low magnification (top) and high magnification (bottom). **Arrow** marks area of histological sections. **C:** Coronal sections of the wild-type sagittal suture after 9 days in culture under white light (top), Texas red filter (middle), or DAPI filter (bottom). **Arrowheads** indicate the edge of the calvaria within the suture. **D:** Newborn Nell-1 transgenic mouse calvarial explant demonstrating mild synostosis of the sagittal suture (top) and high magnification (bottom). **E:** Nell-1 transgenic mouse calvarial explant after 9 days in culture showing an overlapping sagittal suture in low magnification (top) and high magnification (bottom). **Arrow** marks area of histological sections. **F:** Coronal sections of the Nell-1 transgenic mouse sagittal suture after 9 days in culture under white light (top), Texas red filter (middle), or DAPI filter (bottom). **Arrowheads** indicate the osteogenic fronts of parietal bones. Note the overgrowth of the sagittal suture in Nell-1 transgenic calvarial explants. All specimens are stained with Alizarin Red and Alcian Blue. Original magnifications, ×100 (**C**, **F**).

Results

Nell-1 Stimulates Sagittal Suture Overgrowth ex Vivo

To investigate Nell-1-induced sagittal suture overgrowth, wild-type and *Nell-1* transgenic newborn mouse calvaria were harvested and cultured *ex vivo* for 9 days with the dura mater detached. Whole mount staining revealed that newborn wild-type mice had patent sagittal and posterior frontal sutures (Figure 1A), which remained patent after 9 days in culture (Figure 1B). *Nell-1* transgenic mice displayed mild synostosis of the sagittal suture at the time of harvest (Figure 1D) with further sagittal suture overgrowth after 9 days of culture (Figure 1E). Compared to wild-type controls, the sagittal suture of Nell-1 transgenic mice demonstrated overlapping calvaria osteogenic fronts that extended into the suture mesenchyme, and increased mineralization after 9 days in culture (Figure 1, C and F). These observations demonstrate the osteogenic potential of Nell-1 in calvarial tissues.

Nell-1 Stimulates Osteogenic Differentiation in Vitro

Because the Nell-1 osteoinductive pathway is primarily unknown, our first goal was to determine whether Nell-1 was regulated by common osteoinductive growth factors, such as BMPs and other members of the TGF-β and FGF superfamilies, which reportedly play major roles in the regulation of osteogenesis.[18–21,28,29] Because BMP-2, FGF-2, and TGF-β1 are known to directly regulate the expression of human Cbfa1/Runx2,[6,30] which has been demonstrated to directly regulate the expression of human Nell-1,[14] we hypothesized that BMP-2, FGF-2, and TGF-β1 may regulate the expression of downstream genes including Nell-1. Figure 2A demonstrated that whereas BMP-2 did not regulate *Nell-1* expression at 24 and 48 hours, both FGF-2 and TGF-β1 increased the expression of *Nell-1* at these time points. This difference is interesting because both BMP-2 and TGF-β1 belong to the TGF-β superfamily[31] yet have different effects on

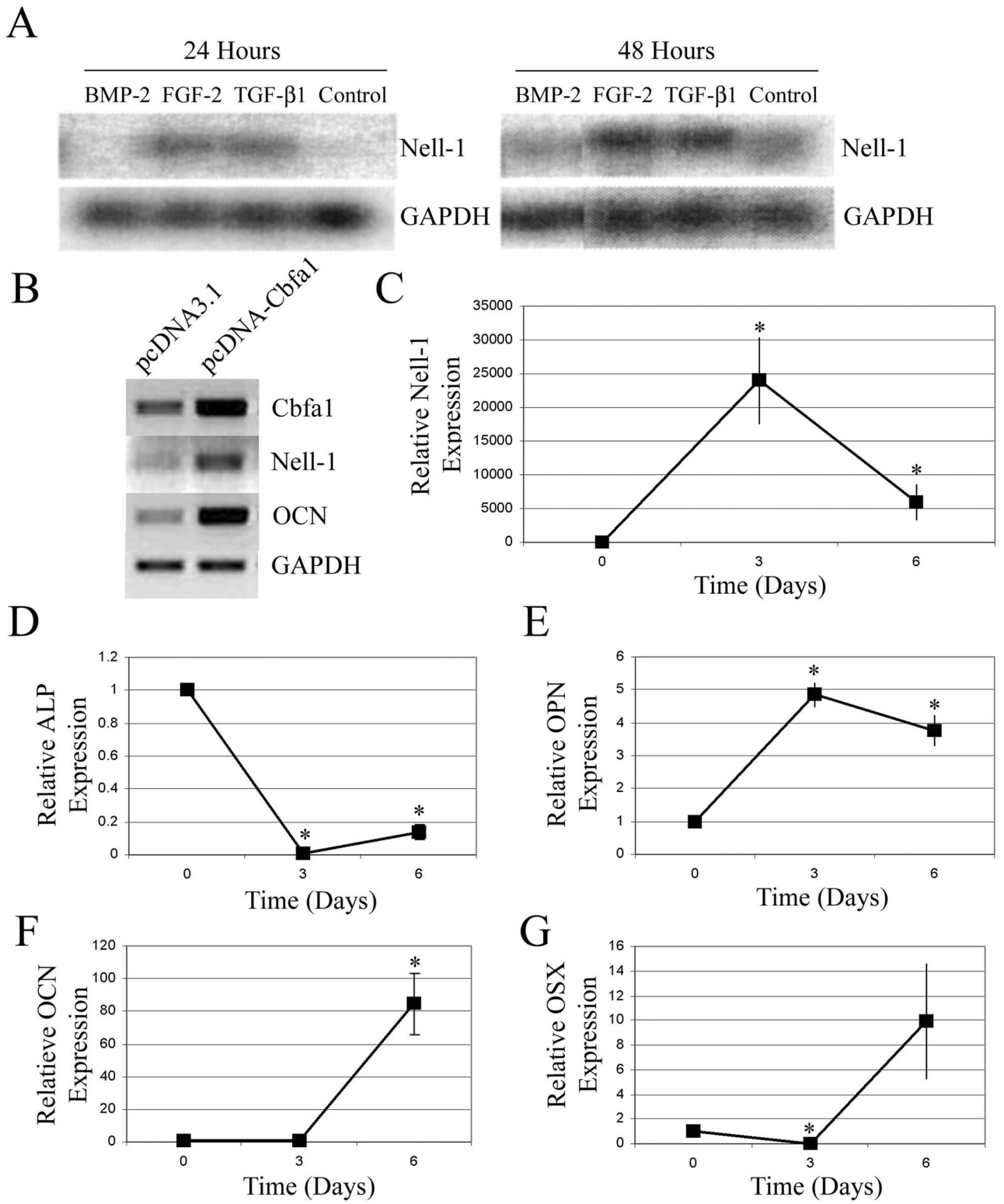


Figure 2. *In vitro* analysis of Nell-1 signaling. **A:** Radioisotope hybridization analysis of *Nell-1* expression in fetal rat calvarial cells stimulated with rhBMP-2, rhFGF-2, or rhTGF-β1 *in vitro* after 24 and 48 hours. **B:** Ethidium bromide staining of an agarose gel of PCR products for Cbfa1, Nell-1, OCN, and GAPDH on transfection in MC3T3-E1 cells with pcDNA3.1 or pcDNA-Cbfa1 plasmids after 24 hours. Real-time PCR analysis of *Nell-1* (**C**), *ALP* (**D**), *OPN* (**E**), *OCN* (**F**), and *OSX* (**G**) transcription in MC3T3-E1 cells on infection with *AdNell-1* for 0, 3, and 6 days. *Significant differences from control samples; $P \leq 0.05$.

Nell-1 expression. Furthermore, Cbfa1/Runx2 may be an intermediate regulator of FGF-2- or TGF-β1-induced *Nell-1* expression. To confirm Cbfa1/Runx2 regulation of murine *Nell-1*, mouse MC3T3-E1 osteoblasts were transfected with a plasmid to overexpress *Cbfa1/Runx2*. The data demonstrated that *Cbfa1/Runx2* overexpression in-

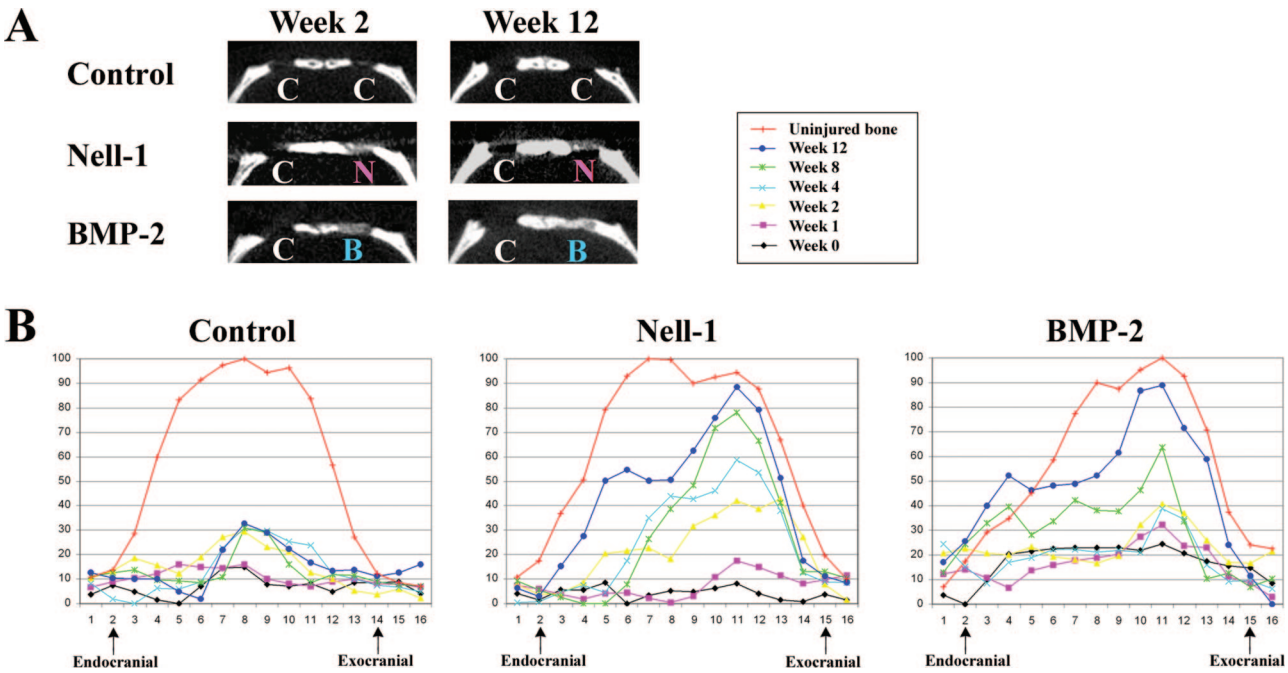


Figure 3. Live microCT analysis of bone formation and density throughout time. **A:** Live microCT analysis of control (C), Nell-1-implanted (N), or BMP-2-implanted (B) defects at weeks 0, 1, 2, 4, 8, and 12. Note the increasing bone formation throughout time in defects implanted with either Nell-1 or BMP-2. **B:** Density measurements through the center of calvarial defects from the endocranial to exocranial sides. Sixteen points were measured including the thickness of the calvaria and background on either side in 50-μm increments. Background density measured less than 20% of the maximal density of uninjured bone. Note the increasing bone density throughout time in defects implanted with either Nell-1 or BMP-2.

creased the expression of *Nell-1* and *OCN* (Figure 2B). This suggests that, in addition to the Cbfa1/Runx2 pathway, other growth factor-induced pathways may be involved in the regulation of *Nell-1* expression.

To investigate the downstream targets of Nell-1, osteogenic markers were analyzed in MC3T3-E1 osteoblasts infected with *AdNell-1*. *AdNell-1* infection resulted in a high level of *Nell-1* expression that peaked on day 3 but exhibited a fourfold reduction on day 6 (Figure 2C). *AdNell-1* infection resulted in a significant decrease in the expression level of *ALP*, a marker that is expressed early in osteoblast differentiation but continues to be up-regulated at later stage,[32–34] on day 3 (100-fold) and day 6 (7.4-fold) (Figure 2D). The expression of *OPN*, an intermediate marker of osteoblast differentiation, was significantly increased by *AdNell-1* infection on day 3 (4.8-fold) and day 6 (3.8-fold) (Figure 2E). Finally, the expression of *OCN*, a late marker of osteogenic differentiation, was significantly increased in *AdNell-1*-infected cells on day 6 (85-fold) (Figure 2F). As previously published, *OCN* was expressed at a later time point than *OPN*.[35] Because OSX is a critical regulator of osteogenesis,[36] we examined the effects of Nell-1 on *OSX* expression. On *AdNell-1* infection, *OSX* expression was significantly repressed 200-fold on day 3, a time when *Nell-1* expression peaked, then *OSX* expression increased to 10-fold on day 6, corresponding to a time when *Nell-1* expression was reduced (Figure 2G). Although differences exist in the timeline of OCN up-regulation after Cbfa1/Runx2 transfection versus *AdNell-1* infection of MC3T3-E1 osteoblasts, altered patterns of gene expression may result from transfection efficiency, culture conditions, Nell-1 secretion profile, and/or Nell-1-independent pathways. Thus, the data suggest that Nell-1 stimulation may decrease critical regulators of osteogenesis (OSX) and early markers of osteogenic differentiation (ALP), while increasing the expression of later markers (OPN and OCN).

Increasing Bone Formation in Nell-1 Stimulated Calvarial Defects

To analyze the ability of Nell-1 to induce bone regeneration *in vivo*, rat calvarial defects were implanted with control, Nell-1, or BMP-2 protein-coated PLGA scaffolds and analyzed via live imaging at weeks 0, 1, 2, 4, 8, and 12. PLGA is a common synthetic polymer with an established safety record in humans and not considered osteoinductive.[37] These characteristics allow the results to be correlated to the effects of the growth factor. PLGA membranes incorporated with continuously released growth factors have shown to be effective in healing bone defects in animal models.[38–40] The current experiments used a dose of 200 ng of Nell-1 or BMP-2 per defect; previous publications had successfully used this minimal concentration of BMP-2 to induce bone regeneration.[41]

MicroCT analysis demonstrated increasing bone formation throughout a 12-week time period for both Nell-1- and BMP-2-treated defects, whereas control scaffolds induced minimal bone formation (Figure 3A). Additionally, the density of newly formed bone was quantitated at each time point, through the defect from the endocranial side to the exocranial side in 50-μm increments (Figure

3B). Control defects slightly increased mineralization (maximum 30% the density of uninjured bone) within some areas of the defect after 12 weeks of healing. Defects implanted with either Nell-1 or BMP-2 protein greatly increased bone density within defects, as early as 2 weeks after implantation. Mineralization continued to increase through 12 weeks at which time they reached almost 90% of the density of uninjured bone. Interestingly, samples implanted with Nell-1, as compared to BMP-2, consistently demonstrated greater mineralization within defects at weeks 2, 4, and 8, although they were equivalent at week 12.

Nell-1 Stimulated Calvarial Defect Healing

To quantitate the extent of Nell-1-induced bone regeneration, new bone area and volume were investigated. Analysis of regenerated bone demonstrated significantly increased bone formation within defects implanted with Nell-1 or BMP-2 protein, as compared to control, at 2- and 4-week time points, at which time complete bridging was achieved (Figure 4A). Closer investigation into the percent volume healed revealed that, although the area was covered, new bone had only regenerated half the volume of the calvaria on the exocranial side. These data indicate a protective strategy of covering the defect before filling in the nonweight-bearing defect. Corresponding microCT images demonstrated increasing bone regeneration throughout time in both Nell-1 and BMP-2 protein-implanted defects as compared to control defects (Figure 4B). An interior view of the calvarial defects demonstrated the complete covering of the defect with new bone, without the entire volume filling as demonstrated by the absence of a flush interior border. Contralateral control defects had a higher percentage of bone regeneration than documented in critical size defects.[31] This phenomenon may be related to the presence of the PLGA scaffold or protein diffusion from the experimental defect. We compared defects with and without PLGA scaffolds up to 3 months after surgery and did not observe significant differences in healing (data not shown). Also, we did not observe differences in healing of control defects between control/control implants and control/experimental implants.

Histological Analysis of Bone Regeneration

MicroCT findings were confirmed with histological analysis of new bone formation in both Nell-1- and BMP-2-stimulated defects as detected with H&E staining (data not shown) and trichrome staining (Figure 5A). Trichrome staining revealed the presence of calcified bone as well as osteoid within the regenerate. Control samples revealed mostly fibrous tissue with minimal bone formation throughout 4 weeks. However, Nell-1- and BMP-2-implanted defects demonstrated initial osteoid formation, followed by mature bone and complete bony bridging by 4 weeks. Interestingly, new bone formed along the exocranial surface and from the osteogenic fronts. Nell-1 and BMP-2 protein-implanted defects demonstrated similar amounts of mature bone after 4 weeks of healing. Furthermore, the presence of osteoclasts through calcitonin receptor (CTR) staining was investigated to account for any possible differences in bone remodeling (Figure 5B). Although both Nell-1- and BMP-2-implanted defects demonstrated the presence of osteoclasts at week 4, control samples did not reveal a significant osteoclast presence. This finding confirms that bone remodeling contributed to differences in bone regeneration between control and Nell-1- or BMP-2-implanted samples.

Because the previous *in vitro* data demonstrated that Nell-1 stimulation significantly down-regulated the expression of OSX, we further investigated Nell-1's ability to regulate OSX production *in vivo*. Both control and BMP-2-implanted defects demonstrated OSX staining within the defect after 1 week of healing (Figure 5C), but with significantly reduced staining after 2 or 4 weeks (data not shown). The OSX-positive cells were predominantly located next to the osteogenic front, where one would expect to find preosteoblasts migrating from the bone into the defect. Additional positive staining for OSX could be found scattered throughout the area of the defect. Interestingly, minimal OSX staining was found within Nell-1-implanted defects throughout all time points, despite an active bone formation process. These findings indicated that Nell-1 stimulation resulted in a reduced number of OSX-producing cells, possibly because of an increased progression of differentiation.

Other factors within the microenvironment, such as BMP-7, can contribute to bone formation.[42] BMP-7 production was up-regulated in Nell-1- and BMP-2-implanted samples as compared to control (Figure 5D). The results correlate with previously published data demonstrating that Nell-1 up-regulates BMP-7 expression *in vitro*.[7] Finally, both Nell-1 and BMP-2 increased the production of protein associated with matrix formation (BSP; Figure 5E) and mineralization (OCN; Figure 5F). BSP production is characteristically found in the formative areas of new bone.[43] Thus, we investigated BSP production as it related to new bone formation in calvarial defects implanted with Nell-1 or BMP-2 protein as compared to control. BSP production first became apparent at week 2 (data not shown) as osteoid and bone were being formed and increased to maximum levels through week 4, indicating an ongoing process of bone formation within the defect. BSP production lined newly formed osteoid and bone, and was present within the rest of the regenerate at week 4 within defects implanted with Nell-1 or BMP-2. In contrast, control samples demonstrated only background staining, indicating significantly lower BSP production in these samples, possibly contributing to the absence of bone. Mature bone did not stain positive for BSP as indicated in previous publications.[43] The continued expression of BSP in Nell-1- and BMP-2-implanted samples at week 4 agrees with the live microCT data (Figure 4, A and B) that show significant continued bone formation in these samples through 12 weeks, whereas control samples failed to form bone. Additionally, the production of OCN, a protein associated with mineralization, was increased in Nell-1- and BMP-2-implanted samples as compared to the fibrous tissue of control sam-

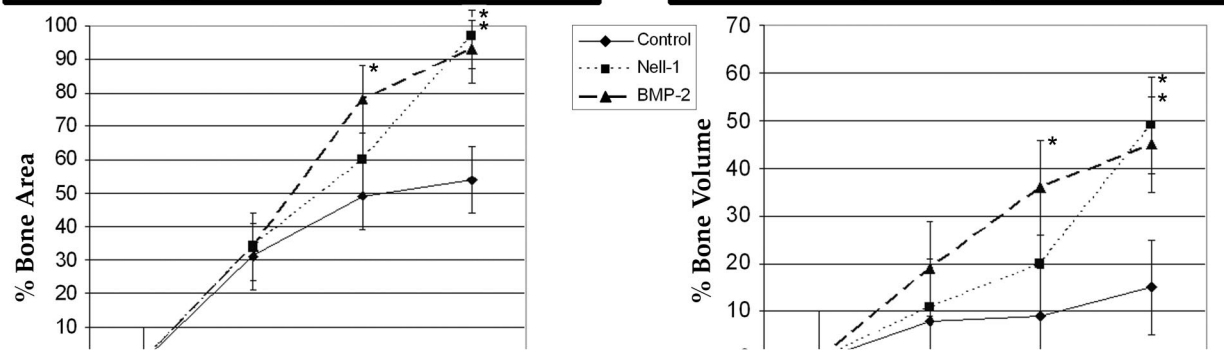
A

% Area

	Control	Nell-1	BMP-2
Week 1	31±23	34±30	34±26
Week 2	49±14	60±11	78±15*
Week 4	54±16	97±3*	93±8*

% Volume

	Control	Nell-1	BMP-2
Week 1	8±5	11±6	19±12
Week 2	9±8	20±13	36±23*
Week 4	15±10	49±11*	45±12*



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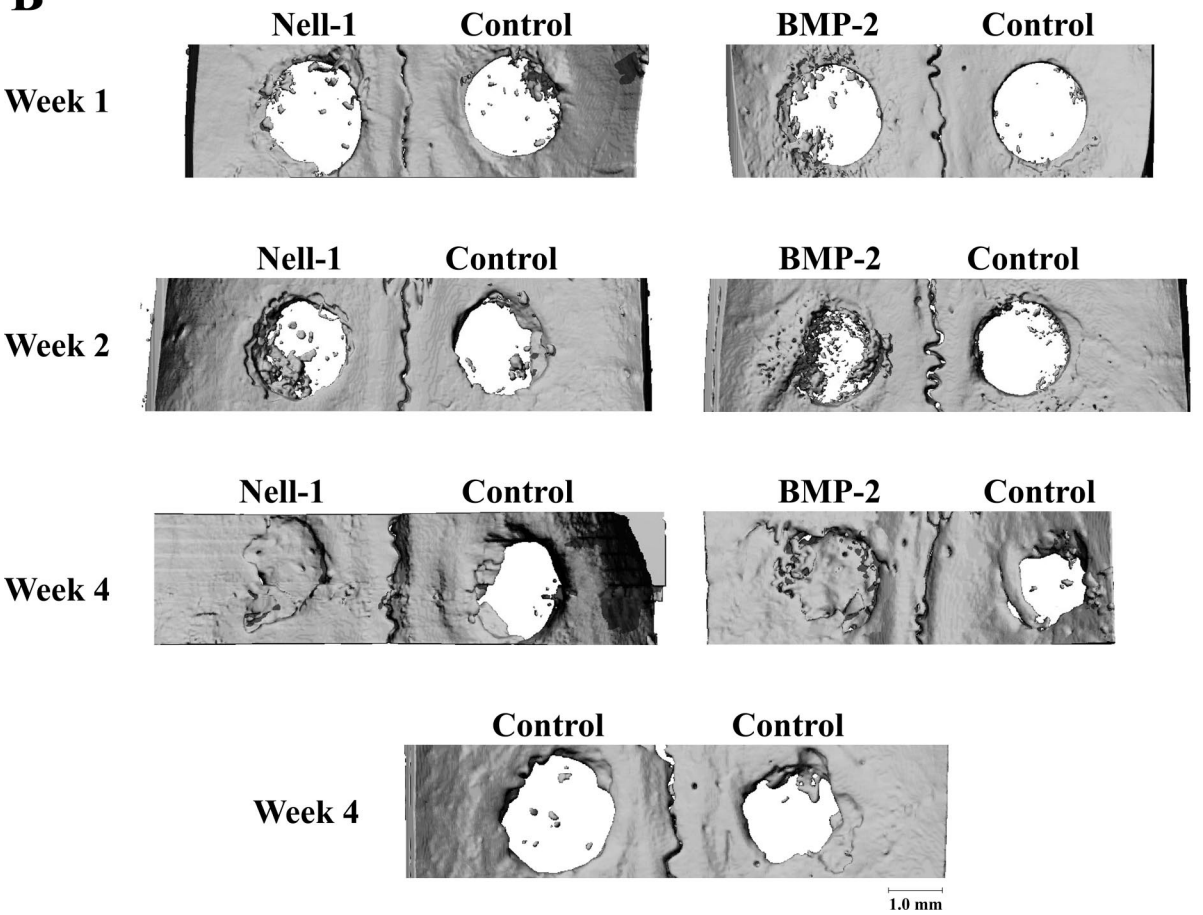

Figure 4. High-resolution microCT and quantification of bone regeneration. **A:** Quantification of percent area and volume bone regeneration in calvarial defects at weeks 1, 2, and 4. *Significant differences from control are noted; $P < 0.05$. **B:** Three-dimensional reconstruction of representative calvaria with Nell-1/control or BMP-2/control implants at each time point.

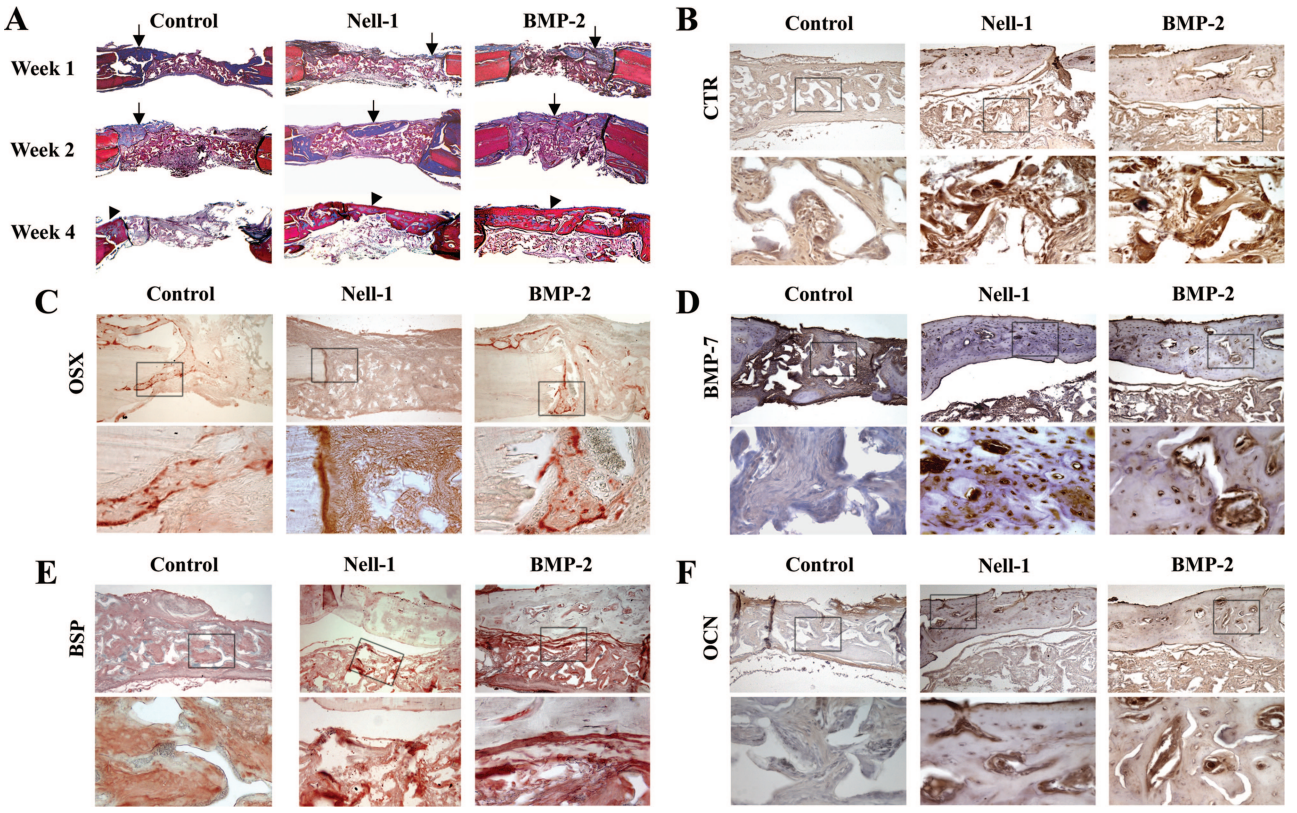

Figure 5. Histological analysis of bone regeneration in calvarial defects. **A:** Trichrome staining of calvarial defects demonstrated the presence of osteoid (blue) and mature bone (red) within defects. Increased mature bone formation was noted throughout time in Nell-1- and BMP-2-implanted defects, as compared to control defects. **Arrows** point to blue osteoid and **arrowheads** point to red mature bone. Immunohistochemistry on calvarial defect sections for CTR at week 4 (**B**), OSX at week 1 (**C**), BMP-7 at week 4 (**D**), BSP at week 4 (**E**), and OCN at week 4 (**F**). Areas within the back boxes are magnified below. Background staining (light brown) can be contrasted to positive staining (red or dark brown). Note the positive OSX staining located at the osteogenic fronts on control and BMP-2-implanted defects, but not Nell-1-implanted defects. In addition, positive CTR, BMP-7, BSP, and OCN staining is located within Nell-1- and BMP-2-implanted samples, as compared to control. BSP-producing cells surround the remaining scaffold and lay down bone matrix. As previously reported, mature bone did not stain for BSP. Original magnifications: ×40 (**A**); ×100 (**B–F**, top); ×400 (**B–F**, bottom).

ples. Taken together, the data suggest that Nell-1 and BMP-2 functioned similarly to stimulate increased osteogenic differentiation through increased production in BMP-7, BSP, and OCN but differed in their actions on osteogenic commitment with Nell-1 reducing the number of OSX-positive cells.

Discussion

Nell-1 is associated with premature suture closure in human craniosynostosis and induction of osteoblast differentiation.[7,13] Although Nell-1 knockout mice have not yet been generated, previous results using a Nell-1 antisense virus demonstrated decreased osteogenic differentiation.[7] Additionally, in a transgenic mouse model, the mutation of *N*-ethyl-*N*-nitrosourea-induced alleles, including Nell-1, resulted in cranial and other skeletal defects.[11] Although the exact role of Nell-1 is unknown, Nell-1 is suggested to play a key role as a regulator of craniofacial skeletal morphogenesis, specifically in committed osteoblastic differentiation. The objective of this research was to study the potential clinical application of Nell-1 in craniofacial skeletal defects. The current data demonstrate that Nell-1 is a stimulator of osteoblast differentiation and bone formation in normal postnatal development and in adult calvarial defects in rodents.

Nell-1 Induces Continuous Calvarial Bone Growth during Cranial Development

Previous studies have demonstrated that ~20 to 25% of newborn Nell-1 transgenic mice have severe phenotypes with the most noted affliction being calvarial overgrowth or overlap and the most severe affliction being exencephaly.[7,13] However, for the remaining Nell-1 transgenic mice, calvarial overgrowth discontinued after birth. This observation raised the question of whether Nell-1 is truly a local osteogenic factor or if the underlying environment such as the dura mater may play a role to inhibit the sutural calvarial bone to grow postnatally.[23] The current data confirmed that continued overexpression of Nell-1 in transgenic littermates in the absence of the dura mater recapitulated the calvarial suture overgrowth displayed in severely afflicted newborns. These data suggest that although Nell-1 is a potent osteoinductive molecule, adjacent tissues such as the dura mater may express molecules to modulate Nell-1 expression or action. Postnatally, the dura mater may inhibit Nell-1's osteoinductivity

to prevent sutural closure. This type of suture/dura mater interaction was demonstrated in the interaction between noggin and the TGF-β family.[44] The N-terminal thrombospondin-1 (TSP-1) domain and the cysteine-rich domains in Nell-1 suggest the ability of other proteins to bind to and modulate Nell-1's activities.[45,46]

The Interaction of Nell-1 with Growth Factors and Transcription Factors in Osteogenic Pathways

The previous data, including the directional transcriptional regulation of *Nell-1* by Cbfa1/Runx2,[15] prompted the current dissection of the role of Nell-1 in molecular pathways of osteoblast differentiation involving Cbfa1/Runx2. These known osteogenic pathways include those regulated by the TGF-β and FGF superfamilies.[31,47] TGF-β1 and BMP-2 are both members of the TGF-β superfamily and have been shown to induce osteogenic differentiation directly and indirectly through effects on Cbfa1/Runx2.[30] BMP-2 induces cellular chemotaxis, proliferation, and osteogenic differentiation of both osseous and nonosseous mesenchymal cells,[48,49] whereas TGF-β1 affects osteogenic differentiation only in osseous cells,[30] similarly to Nell-1.[7] In addition, Cbfa1/Runx2 activation and phosphorylation is induced by the FGF-2 pathway.[6] The transcription factor Cbfa1/Runx2 controls the progression of osteogenic differentiation and is often referred to as the master regulator of mesenchymal cell fate, through temporal activation and/or repression of cell growth and gene expression regulation.[50] Furthermore, the actions of Cbfa1/Runx2 are dose-dependent in craniofacial bone (neural crest origin), but not in long bone formation (nonneural crest cell origin).[51] Cbfa1/Runx2 has been reported to be expressed at higher levels within the craniofacial region as compared to other skeletal regions,[51] coordinately with Nell-1.[7] The hierarchy of other growth factors can be related to the regulation of Cbfa1/Runx2 within the osteogenic tree. Nell-1, on the other hand, has three Cbfa1/Runx2 binding sites [osteoblast-specific *cis*-acting element 2 (OSE2)] within its promoter and signals downstream of Cbfa1/Runx2.[14] *Nell-1* transcription was increased on TGF-β1 and FGF-2 stimulation, presumably through Cbfa1/Runx2, but not BMP-2, stimulation. Thus, BMP-2 did not regulate *Nell-1* expression and is not directly upstream from *Nell-1* gene expression in this system. Although TGF-β1 and BMP-2 belong to the same superfamily and both signal through Cbfa1/Runx2, only TGF-β1 was able to induce *Nell-1* transcription at 24 and 48 hours. Interestingly, unlike TGF-β, FGF, and Nell-1, BMP-2 has not been reported to induce cranial suture closure or overlap *in vivo* or *ex vivo*.[52]

We further investigated Nell-1's regulation of osteogenic gene transcription. Our previous work using microarray technologies has demonstrated that MC3T3-E1 cells infected with *AdNell-1* induced more than a twofold up-regulation of *OPN* and *OCN* gene expression, which are important markers of later osteoblastic differentiation.[7] This data in combination with the downstream regulation of Nell-1 lead us to hypothesize that Nell-1 was primarily involved in the later stages of osteoblastic differentiation. As predicted, the current results using MC3T3-E1 osteoblasts revealed that Nell-1 decreased the expression of early up-regulated makers of differentiation (ALP) but increased intermediate and later up-regulated makers (OPN and OCN). These results related both similarities and differences to TGF-β1-, FGF-2-, and BMP-2-induced osteogenic gene expression. In MC3T3-E1 cells, TGF-β1 decreases the expression of both early and later up-regulated markers of differentiation (ALP[53] and OCN[54]) and is primarily associated with matrix production. FGF-2, on the other hand, acts similar to Nell-1, by reducing ALP expression[55] and increasing OCN expression.[56] On the contrary, BMP-2 increases the expression of markers of early (ALP) and late (OCN) differentiation.[57] Furthermore, TGF-β1, and FGF-2 signal through multiple interacting pathways leading to increased PKC production and activity.[22,58] BMP-2, on the other hand, has been reported to stimulate osteogenic differentiation through PKC-dependent and -independent pathways.[59,60] Nell-1 interacts with specific PKC isoforms including PKCδ, βI, and ζ, but not PKCα, γ, and ε,[12] to exerts its signaling function in a still unidentified manner. Taken together, both FGF-2 and Nell-1 involve Cbfa1/Runx2 and PKC during signaling, are directly associated with CS, and stimulate similar osteoblast marker patterns in our experimental conditions. These data suggest that Nell-1 may stimulate osteogenic differentiation through a similar pathway as or participate in FGF-2 signaling pathways, while being more distinct from or indirectly involved in BMP-2 signaling pathways.

Recently, another transcription factor, OSX, has been implicated as an important regulator of osteogenesis. OSX signals downstream of Cbfa1/Runx2 in osteoblast differentiation.[36] *OSX* is expressed in osteoblasts of all endochondral and intramembranous bones, and knockout mice display an absence of endochondral and intramembranous bone formation, although chondrocyte organization and hypertrophy are normal.[36] *OSX* transcription is found transiently in differentiating chondroblasts but not in condensed chrondrogenic mesenchyme, suggesting that OSX functions in mesenchymal differentiation to distinguish the osteogenic pathway from the chrondrogenic.[61] BMP-2 treatment induces the expression of *OSX* through Cbfa1/Runx2-dependent and -independent pathways mediated by Dlx5,[61,62] whereas TGF-β1 and FGF-2 stimulations do not result in *OSX* transcription.[61] The current data report that whereas BMP-2 greatly increases the expression of *OSX*,[61,62] Nell-1 led to an initial dramatic decrease in *OSX* expression, with a slight elevation throughout time corresponding to decreased Nell-1 levels. Thus, these results further suggest that Nell-1 likely does not signal directly downstream from BMP-2. Taken together, Nell-1 may decrease the expression of early regulators of osteogenesis (OSX), possibly acting as a molecule to transition osteoblasts out of a proliferative holding position and into a differentiated phase (Figure 6).

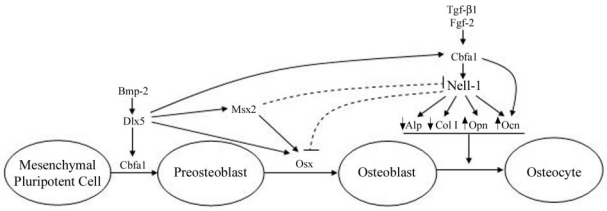

Figure 6. Hypothetical model diagramming Nell-1's regulation of osteogenic differentiation. Dashed line represents hypothetical modulation. Note that Nell-1 stimulates down-regulation of ALP and Col I but up-regulation of OPN and OCN during bone formation.

Nell-1 Induces Bone Formation and Regeneration in Calvarial Defects

Because of Nell-1's ability to affect and accelerate the natural progression of bone formation within cranial sutures, we further investigated the ability of Nell-1 to induce bone regeneration within calvarial defects. The calvarial defect animal model is well established for studying bone regeneration to test adjuncts to autogenous bone grafting, allografts, and alloplasts, and adenoviral delivery of growth factors.[63,64] As the microCT and histological data demonstrated, Nell-1 delivery induced significant bone formation as compared to control samples, which increased throughout a 12-week time period. A closer examination of the progressive bone formation through continuous live imaging, revealed the extension throughout time of the osteogenic fronts, which met to close the defect, suggesting the migration of osteoblasts or preosteoblasts from the adjacent uninjured bone, rather than periosteum or dura mater. The pattern of healing suggests that cellular migration into the defect was the limiting factor in healing and suggests a role for complementary cell therapy in future work.

Although new bone appeared woven during early stages, the bone matured and bone marrow pockets were apparent. The pattern of new bone formation indicated a protective healing strategy geared toward coverage of the brain before total volume regeneration. Most interestingly, Nell-1 induced equivalent levels of bone regeneration as BMP-2, one of the most potent known inducers of bone formation.[65,66]

Previous publications indicate that Nell-1-induced bone formation is a result of accelerated osteogenic differentiation[7]; however, this mechanism is not well understood. Regulation of osteogenesis and mineralization provides clues to the progression of osteogenic differentiation. The differential regulation of OSX *in vivo* by Nell-1 and BMP-2 provided an important clue as to the molecular mechanism of bone induction. Nell-1 implantation reduced the number of OSX-producing cells, suggesting a rapid differentiation of already committed osteoblasts within the defect to become bone-producing cells. Conversely, BMP-2 implantation recruited preosteoblasts and/or multipotent cells from the surrounding tissues to infiltrate the defect and differentiate into bone-producing cells. This differential initiation of bone production is possibly the fundamental difference between Nell-1- and BMP-2-induced bone formation and the reason why Nell-1 is more osteoblast-specific and less likely to produce unintended side effects. Additionally, both Nell-1 and BMP-2 were able to increase the production of BMP-7, BSP, and OCN. BMP-7 stimulates osteogenic differentiation,[67] whereas BSP is a protein that regulates mineralization and is a nucleator of hydroxyapatite.[43] BSP has a strong affinity to collagen fibrils[68] and mediates attachment of cells through its RGD sequences.[69] Importantly, BSP is strongly expressed at the formative surfaces during repair of calvarial defects[43] by inducing differentiation of osteoblast-like cells from undifferentiated mesenchymal cells.[70] The noted up-regulation of BSP in defects implanted with Nell-1 or BMP-2 may account for increased collagen formation, cellular attachment, and the greater bone formation within these defects as compared to control. Importantly, the elevated production of BSP through 4 weeks suggested an active bone-remodeling process. Finally, OCN is tightly regulated to bone mineralization through binding to hydroxyapatite as well as to recruitment of osteoblasts.[71] Although Nell-1 was first reported to be found within the cytoplasm of neuronal cells in the brain,[12] further research has revealed the extracellular actions of Nell-1 on osteochondral cells.[7,13,72] The current work further confirms the extracellular actions of Nell-1 because the application of Nell-1 protein within calvarial defects, significantly increased bone regeneration. These important findings reveal the therapeutic potential of Nell-1 protein and eliminating the need for *Nell-1*-mediated gene therapy.

Summary

Although Nell-1 and BMP-2 formed equivalent amounts of bone within calvarial defects, they each induced a differential pattern of gene expression, protein production, and initiation of bone formation. Cellular responses to Nell-1, through Cbfa1/Runx2-induced transcription, are likely limited to committed osteochondral differentiation.[13,72] Our results verify these findings because Nell-1 repressed the expression of *OSX* and *ALP*, a regulator and a marker, respectively, initially expressed in early osteogenic differentiation, and increased expression of *OPN* and *OCN*, which are associated with intermediate and later stage of osteogenic differentiation. The current study in coordination with previous data demonstrates that Nell-1 functions diversely from BMP-2 in this system. Nell-1 was regulated by FGF-2, TGF-β1, and Cbfa1/Runx2, indicating a possible crosstalk between these important pathways in osteoblast signaling. Although the data has begun to clarify the role of Nell-1 in osteoblast differentiation, further studies will continue to elucidate the mechanism of Nell-1 signaling.

Research into the etiologies of craniofacial deformities has identified many important molecular pathways to direct targeted therapy. However, a significant gap exists in our knowledge for improving predictability and long-term success in treating patients with craniofacial defects. Our current gold standard treatment of extensive

autogenous bone grafting often leaves poor esthetics, compromised function, and associated donor site morbidity.[73] The potential application of *Nell-1* to induce osteogenic differentiation is very valuable in translational research relating to bone healing and regeneration because of its osteoinductive properties, osteochondral specificity, and craniofacial localization. Thus, Nell-1 may be uniquely suited for bone regeneration within the craniofacial complex.

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