Craniosynostosis in transgenic mice overexpressing Nell-1

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Abstract

Previously, we reported NELL-1 as a novel molecule overexpressed during premature cranial suture closure in patients with craniosynostosis (CS), one of the most common congenital craniofacial deformities. Here we describe the creation and analysis of transgenic mice overexpressing Nell-1. Nell-1 transgenic animals exhibited CS-like phenotypes that ranged from simple to compound synostoses. Histologically, the osteogenic fronts of abnormally closing/closed sutures in these animals revealed calvarial overgrowth and overlap along with increased osteoblast differentiation and reduced cell proliferation. Furthermore, anomalies were restricted to calvarial bone, despite generalized, non-tissue-specific overexpression of Nell-1. In vitro, Nell-1 overexpression accelerated calvarial osteoblast differentiation and mineralization under normal culture conditions. Moreover, Nell-1 overexpression in osteoblasts was sufficient to promote alkaline phosphatase expression and micronodule formation. Conversely, downregulation of Nell-1 inhibited osteoblast differentiation in vitro. In summary, Nell-1 overexpression induced calvarial overgrowth resulting in premature suture closure in a rodent model. Nell-1, therefore, has a novel role in CS development, perhaps as part of a complex chain of events resulting in premature suture closure. On a cellular level, Nell-1 expression may modulate and be both sufficient and required for osteoblast differentiation.

Introduction

Craniosynostosis (CS), the premature closure of cranial sutures, affects 1 in 3,000 infants and therefore is one of the most common human congenital craniofacial deformities (1). Premature suture closure, which results in cranial dysmorphism, can be either familial or sporadic in origin (1). Neither gender nor ethnicity can be used to predict which infants will be affected. Although genetic linkage analyses of CS-related syndromes have provided a wealth of new information about the molecular control of suture formation, the biology of local suture closure, especially in nonsyndromic, nonfamilial CS, is still largely unknown.

Presently, more than 85 human mutations, which produce various familial CS syndromes, have been localized to the FGF receptor genes FGFR1, FGFR2, and FGFR3. All are “gain-of-function” mutations that result in increased receptor activity (1). No human CS syndromes have been linked to the FGF ligands; however, several animal models of CS have been associated with FGF overexpression (2, 3). The only described MSX2 mutation associated with CS (4) also results in increased MSX2 activity (5–7). While these candidate genes are known to play important roles in osteoblast proliferation and differentiation, they also have more generalized roles during embryogenesis. Thus, it is not surprising that transgenic mouse models with mutations in these genes often manifest extracranial abnormalities not observed in the majority of patients with CS (1, 2, 8).

Premature suture closure in human CS can be divided into two possibly distinct processes: calvarial overgrowth and bony fusion. While calvarial overgrowth may be essential to bringing the two opposing osteogenic fronts into proximity in order to induce bony fusion, it does not necessarily follow that calvarial overgrowth or overlap alone will result in bony fusion. Thus, the study of premature suture closure mechanisms must include study of both abnormal suture overgrowth/overlap and bony fusion (6).
Recently, FGF2 and FGFR1 have been implicated in premature cranial suture fusion via CBFA1-mediated pathways (8). Missense mutation of CBFA1 is linked to cleidocranial dysplasia, manifested as delayed suture closure (9). Therefore, examination of Cbfa1 (Runx2), a downstream target of Fgfr1 that is essential for bone formation, may be key to understanding the signaling cascade in CS. In addition, Msx2, a member of the highly conserved Msx homeobox gene family with pleiotropic effects in development, has been implicated in an animal model of CS (5, 6). Specifically, increased osteogenic cell proliferation has been proposed as a mechanism for premature suture closure in Msx2-overexpressing transgenic mice, which exhibit suture overgrowth/overlap without suture fusion.

To elucidate the molecular pathway for suture closure, we previously used differential display to identify genes that were specifically upregulated within abnormally fused sutures in patients with nonfamilial, nonsyndromic CS. We isolated and characterized NELL-1, which is a Nel-like, type 1 molecule (a protein strongly expressed in neural tissue, encoding an EGF-like domain) (10–12). Nell-1 is a secreted protein. Structurally, Nell-1 encodes a secretory signal peptide sequence, an NH2-terminal thrombospondin-1-like module, five von Willebrand factor–like repeats with six cysteine residues, and six EGF-like domains. Nell-1 is also highly conserved across species. For example, 93% amino acid sequence homology exists between rat Nell-1 and human NELL-1.

Nell-1 encodes a polypeptide with a molecular weight of 90 kDa. When overexpressed in COS cells, the glycosylated form is N-linked to a 50-kDa carbohydrate moiety in eukaryotic cells to generate the 140-kDa form found in the cytoplasm. This 140-kDa protein is further processed to a 130-kDa protein. The Nell-1 protein is secreted as a trimeric form with a high molecular weight (approximately 400 kDa) (13, 14).

Initial studies have suggested that NELL-1 is preferentially expressed in the craniofacial region of calvarial tissues (2–4). Premature suture closure in CS patients is remarkable for the degree of NELL-1 overexpression by osteoblast-like cells in osteogenic areas (12). Although NELL-1 overexpression and premature suture closure may be coincidental findings, our data suggest that NELL-1 may be a local regulatory factor in cranial suture closure.

In this study, we further verified that Nell-1 has a role in CS. We created a transgenic mouse model exhibiting generalized Nell-1 overexpression. Nell-1 transgenic animals share many of the same features as humans with CS. They demonstrate calvarial overgrowth/overlap and premature suture closure. Infection of osteoblasts with Nell-1 adenoviral constructs showed that Nell-1 promotes and accelerates differentiation in osteoblast lineage cells. In addition, Nell-1 downregulation inhibited osteoblast differentiation. Nell-1, therefore, represents a candidate gene for producing cranial suture closure and provides new insights in the study of CS and craniofacial development.

Methods

Preparation of transgenic mice overexpressing Nell-1. Rat Nell-1 cDNA was subcloned from pTM-70 (13, 14) into pCDNA1.1 (Invitrogen, Carlsbad, California, USA), which uses a CMV promoter and an SV40 polyadenylation site. The recombinant plasmid was first transfected into MC3T3 cells (a mouse calvarial cell line) to verify proper protein expression (data not shown). The 4.76-kb DNA fragment containing the CMV promoter, Nell-1 cDNA, and the SV40 polyadenylation site was then used for microinjection of oocytes. B6C3 mice were used to generate transgenic mice using standard protocols (15). The founders were mated with their nontransgenic littermates to set up transgenic lines.

Analysis of transgene copy number. Transgene copy numbers were estimated by PCR and Southern blot analysis. The PCR protocol of establishing transgene copy number was obtained at http://www.med.umich.edu/tamc/spike.html (16). The mass of transgene DNA per 5 μg genomic DNA was calculated as N bp transgene DNA/3 × 109 genomic DNA, based on the assumption that the haploid content of a mammalian genome is 3 × 109 bp and that it takes 10 μg DNA to spike. The size of the insert is 4.76 kb, and the one-copy standard is 7.933 pg per 10 μg genomic DNA. Thirty cycles of PCR were performed and products were separated on electrophoresis gels with ethidium bromide. The intensities were calculated using Eagle Eye II (Stratagene, La Jolla, California, USA).

Immunohistochemistry. Detailed preparation of Nell-1 antibody has been documented by Kuroda et al. (13, 14). The antibody recognizes the COOH-terminal region of Nell-1 (CSDVLECIIEN). The specificity of the antibody was confirmed by Western blot using protein extracted from Nell-1–transfected NIH3T3 cells. A standard avidin-biotin complex/immunoperoxidase protocol (Vector Elite Kit; Vector Laboratories Inc., Burlingame, California, USA) was used with 1:100 Nell-1 antibody dilution. Diaminobenzidine peroxidase substrate and 3-aminio-9-ethylcarbazole were used for visualization, and sections were counterstained with hematoxylin.
Magnetic resonance imaging. Magnetic resonance imaging (MRI) was performed on formalin-preserved specimens using a Bruker Biospec MR imager (Bruker BioSpin GmbH, Rheinstetten, Germany) with a 7.0-T, 18-cm clear-bore magnet equipped with a microimaging gradient set and a 35-mm internal diameter birdcage radiofrequency coil. Transaxial and sagittal images of the brain and calvarium were obtained using a gradient echo filtered imaging steady-state pulse sequence with the following parameters: TR/TE, 229.3/64.1 ms; flip angle, 30°; field of view, 2.3 cm; matrix, 256 × 256; slice thickness, 1 mm; and number of excitations, 8. In-plane spatial resolution was approximately 90 μm.

Microcomputerized tomography scan. All the data were collected at 30 kVp and 750 mA. The data was reconstructed using the cone-beam algorithm supplied with the MicroCat scanner (Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA). The matrix was 256 × 256 × 256, yielding an isotropic resolution of 140 μm. The quantitative procedures involve the placement of bone phantoms (long rods in the images) containing 0, 50, 250, and 750 mg/cc hydroxyapatite. Visualization of the data was performed using MetaMorph (two dimensional) (Universal Imaging Corp., West Chester, Pennsylvania, USA) and Amira (three dimensional) (Indeed – Visual Concepts GmbH, Berlin, Germany).

In vivo proliferation analysis. Newborn mice were injected with BrdU at 100 μg/g. Animals were sacrificed 2 hours after injection. The animals were fixed and immunostained with BrdU antibodies (Sigma-Aldrich, St. Louis, Missouri, USA). Calvarial sutures, brain, and tibiae from transgenic animals and their normal littermates were compared.

Recombinant defective adenovirus vectors harboring Nell-1 (AdNell-1) and antisense Nell-1 (AdAntiNell-1). Rat Nell-1 cDNA was inserted bidirectionally between the human CMV IE1 promoter and the SV40 splice/polyadenylation site flanked by nucleotide sequences from 1 to 454 and from 3,334 to 6,231 of the Ad5 virus. The resulting plasmid, pAdCMV-Nell-1, transcribes Nell-1 leftward relative to the standard Ad5 map. The recombinant adenovirus (Ad) (AdNell-1) were isolated by cotransfecting 293 cells with pAdCMV-Nell-1 and pJM17 (Microbix Biosystems Inc., Toronto, Canada), resulting in vectors defective in the E1-A viral gene. Clones of recombinant virus were plaque purified and confirmed by Southern blot analysis. Both AdNell-1 and AdLacZ were grown to a high titer and purified once through a CsCl cushion and again on a continuous CsCl gradient. The resulting stocks were 5 × 10⁸ pfu/ml as assayed by plaque formation on 293 cells. Northern and Western blots were performed to assure the incorporation and expression of the Nell-1 gene and its protein product.

Rat calvarial primary cell cultures (FRCCs). The isolation of osteogenic cells from embryonic day 18 (E18) rat calvaria was performed as previously described (12). The cells collected from digestions four, five, and six were pooled and plated at 2.5 × 10⁴/cm². Cells within passage two were used.

Adenoviral infection of osteoblasts. In order to observe the effects of overexpressing Nell-1, osteoblasts from different lineages were grown to 80% confluence in six-well plates. The media was aspirated and an infective dose (20 pfu/cell in 1 ml serum-free medium) was added to the cultures. Five sets of AdNell-1, AdAntiNell-1, and control Ad carrying β-Galactosidase (Adβ-Gal) were used. On days 12, 15, and 21 after infection, von Kossa staining was performed. The percentage of area mineralized was analyzed using the Image-Pro Plus system (Media Cybernetics, Silver Spring, Maryland, USA). Comparisons between mice were made using the Student t test.

In order to observe the effects of downregulating Nell-1, AdAntiNell-1 was added to fetal rat calvarial cell (FRCC) cultures as described above.

Microarray analysis. Microarrays were performed using RNA from AdNell-1– and Adβ-Gal–infected MC3T3 cells at 6, 9, and 12 days after infection. I. Nishimura and the University of California Los Angeles Microarrays Core Facility staff have developed bone-related microarrays. The microarrays contain over 37 genes with more than ten internal control genes. Confirmed markers include the following: bone matrix proteins (osteopontin, osteonectin, osteocalcin, bone sialoprotein); receptors (α2-integrin, vitamin D receptor, parathyroid receptor, estrogen receptor); osteoblastic markers (alkaline phosphatase, Cbfa1); and adhesive proteins (fibronectin, chondroitin sulfate proteoglycan 1, decorin, tenasin, syndecan, laminin); metalloproteinases (matrix metallopro-teinasises 1 and 2); growth factors (Bmp2, Bmp7); fibrillar collagens (collagens 1A1, 1A2, 3A1, 5A2, and 11A1); other collagens (collagens 4A1, 6A1, 7A1, 10A1, and 15A1); and fibril-associated collagen with interrupted triple helices (FACIIs) (collagens 9A1, 9A2, 12, 14, 16, and 19).

RNA (30 μg total RNA for Cy3 and 60 μg for Cy5) was labeled with random hexamer primers and Cy3- or Cy5-dUTP. The reverse transcriptase–labeled probes were hybridized onto the arrays. Multiple laser scans were performed with a 418 Array Scanner (Affymetrix Inc., Santa Clara, California, USA) to provide mean readouts and standard deviations to verify the reproducibility of the measurements. An average of all the internal controls was calculated and used to normalize hybridization intensities using the IPLab version 3.2 MicroArray suite (Scanealytics Inc., Fairfax, Virginia, USA). The correlation of all
Results

Construction of CMV promoter/Nell-1 transgenic mice. To investigate the effects of generalized Nell-1 overexpression in vivo, transgenic mice in which Nell-1 is expressed under the control of the CMV promoter were produced. Copy number was confirmed by Southern blot and PCR (Figure 1a). RNA analysis (Figure 1b) and immunohistochemistry (data not shown) further confirmed expression of Nell-1 in founders. Nell-1–overexpressing founders were crossed with nontransgenic littermates, and comprehensive analyses were conducted on F₂ progeny. Because most human CS phenotypes are readily apparent in newborns, 42 newborn mice, representing six litters from two lines, were examined. The morphology of these mice was assessed for developmental anomalies, including suture closure. The mice were subsequently genotyped. Suture patency was determined by the absence (indicating suture closure) or the presence (indicating suture patency) of visible blood vessels underneath the suture. Suture closure was further confirmed under a dissecting microscope. Two of the six litters examined, representing 20 progeny, did not yield any newborns with obvious craniofacial defects and were progeny from these litters were not examined further. Progeny with craniofacial defects were recovered in each of the four remaining litters. The progeny of these four litters (22 mice) were analyzed further. A limitation of this rapid screening method is that mild CS with only focal points of suture closure may not be detected, and therefore Nell-1 overexpression might appear to have lower penetrance.

Figure 1

Nell-1 transgenic mice compared with nontransgenic littermates. (a) Transgene copy number. The founders (FA and FB) and their progeny (TF₂A1, TF₂A2, and TF₂B1) have copy numbers between 50 and 100. TF₂A1 and TF₂A2 are from the founder A line. TF₂B1, TF₂B2, and TF₂B3 are from the founder B line. (b) RT-PCR analyses of Nell-1 RNA expression in both founders. C, control Nell-1 plasmid; M, muscle; H, heart; B, bone; K, kidney; L, liver. (c) Whole body (without head) RNA of newborn progeny. TF₂A1 and TF₂A2 express different levels of Nell-1. TF₂B1 expresses Nell-1 weakly, while TF₂B2 and TF₂B3 have no Nell-1 expression. (d) Left panels, immunolocalization of Nell-1 protein in newborn NF₂ epithelium, muscle, and calvarial bone. There is no detectable Nell-1 expression (brown staining indicates the presence of Nell-1) except some staining in the calvarial bone. Right panels, immunolocalization of Nell-1 protein in TF₂A2 epithelium, muscle, and calvarial bone. Abundant Nell-1 expression is present throughout all soft tissue layers as well as in bone. Bar represents 50 μm.

Thirteen (60%) of the 22 newborn progeny were transgenic, with gene copy numbers similar to the founder Nell-1 mice (prediction is 50%). Nell-1 RNA levels of the 13 Nell-1 DNA–positive transgenic F₂ (TF₂) mice were examined. Eight (62%) were positive for Nell-1 RNA expression. However, the level of expression varied (Figure 1c). The reason for low or nearly absent Nell-1 expression in some TF₂ mice despite their high transgene copy numbers is not clear, but epigenetic effects such as heterochromatin formation around the inserts may play a significant role in the high variability of transgene expression (17). RNA levels also differed in different tissues isolated from the same litter. Liu et al. also made this observation of variegation when they overexpressed Msx2 using a CMV promoter (5, 6). Therefore transgenic Nell-1 transcription may not necessarily correlate with osteoblastic markers as a group was calculated and compared between the AdNell-1–infected cells and the Adβ-Gal–infected control cells.

RT-PCR. DNase-treated total RNA was used. After initial verification of gene fragment expression through high-cycle PCR, another low-cycle PCR was performed to quantify relative gene expression (12). For each candidate molecule, we determined the cycle number most likely to fall within the linear amplification range by successively reducing the number of cycles (range, 15–35 cycles). Electrophoreses were performed and hybridized with sequence-specific probes labeled wit P32. A PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA) was used to measure the intensities. For each sample, the densitometry value was divided by the Gapdh value (performed at 20 cycles) and normalized. Primer sequences were as follows. Msx2 forward, 5′-CCTCGGTCAAGTGGAAAAATC-3; reverse, 5′-TGGACAGGACTTTTCTGGG-3; probe, 5′-GAGCCAGCGTGCAGAGGAG-3′ (annealing temperature, 68°C). Cbfa1: forward, 5′-CTGTGGCGTCTACTACAAGGTG-3; reverse, 5′-GGATTCTGGGAATGACACAG-3′; probe, 5′-CCTACTCATGTTGGAGGAGTCCTG-3′ (annealing temperature, 66°C). Osteocalcin: forward, 5′-ATGAGGACCTCTCTCTGGCT-3; reverse, 5′-GT-GGTGCCATAGATGCCTTGTG-3′; probe CATGTCAGGAGGAGCA-3′ (annealing temperature, 66°C). Osteopontin: forward, 5′-AGCAGAAATCTAAGTGC-3′; reverse, 5′-GATTATAGTGACAGACAG-3′; probe 5′-GCCCTGAGCTTAGTTCGTTG-3′ (annealing temperature, 66°C). Nell-1: (12).

Flow cytometry analysis. Cells were seeded on 60-mm plates at 5 × 10⁵ cells/plate. Cells were harvested at 24, 36, 48, and 72 hours after infection with AdNell-1 and Adβ-Gal. One million cells were used for flow cytometry, and this procedure was repeated three times. Hypotonic DNA staining buffer containing propidium iodide was added to the cells for flow cytometry.
To determine whether Nell-1 overexpression in our transgenic model was physiologically relevant, we compared Nell-1 RNA expression levels from the whole heads of three TF₂ progeny with mild CS phenotypes to levels in nontransgenic normal littermates (NF₂ mice). TF₂ mice displayed up to fourfold-increased Nell-1 expression (data not shown). This was comparable to levels of NELL-1 overexpression in human CS patients in whom two- to fourfold increases have been observed [2]. This suggests that Nell-1 overexpression levels in our model were clinically relevant rather than superphysiologic.

Phenotypic analyses of Nell-1 transgenic mice. Three of the eight Nell-1 RNA-positive TF₂ mice demonstrated severe craniofacial anomalies and died shortly after birth (see Figure 2, a–c, and Figure 4). These mice also demonstrated detectable Nell-1 transgene expression in their total body mRNA (Figure 1c) that was verified by Nell-1 immunostaining of skin, liver, and calvaria (Figure 1d).

Morphological examination of one of the most severely affected TF₂ mice revealed a large protuberance in the paramedial parietal area with completely closed sagittal and posterior-frontal (PF) sutures and partially closed coronal sutures (Figure 2, a–c). Clinically, this is similar to craniofasciocerebral dysplasia, a form of human CS with premature sagittal, metopic, and coronal suture closure with secondary frontal bone bossing and paramedial encephalocele (Figure 2d) [1]. Brain MRI of this TF₂ mouse revealed significantly reduced ventricle size and increased parenchymal edema, both of which are suggestive of increased intracranial pressures (Figure 2e). Continued brain growth in the face of premature suture closure also generates increased intracranial pressures in humans with untreated CS. Microcomputerized tomography (MCT) scan and MRI analysis also demonstrated structural abnormalities in the cranium of this TF₂ mouse (Figure 2, f and g).

Histological examination of Nell-1 phenotype-positive TF₂ mice revealed distinct differences from NF₂ littermates. As in human CS, TF₂ mice displayed prematurely closing sutures seen histologically as thickened, disorganized ridges of calvarial ridges with closing/overlapping osteogenic fronts (Figure 3, a and b). Whole-mount skeletal staining did not show any observable extracranial skeletal anomalies. Hematoxylin and eosin and trtartrate-resistant acid phosphatase staining of palatal and midmendible sutures, vertebrae, and long bones did not reveal any abnormal histology or increase in osteoclast number. Therefore, the effects of Nell-1 expression appear to be confined to the calvaria. Despite pan-tissue Nell-1 expression due to the use of the CMV promoter, TF₂ mice exhibited cranial-specific anomalies that primarily affected calvarial suture patency and closure. Immunohistochemistry showed increased in vivo expression of osteoblastic differentiation markers (Figure 3, c and d).
represents alkaline phosphatase staining of a micronodule. (controls. (effects are osteoblast-specific. infected controls did (Figure ad osteogenesis is the cardinal feature of TF overexpression may alter normal calvarial osteoblast cell cycling and differentiation pathways to promote premature osteogenesis. To examine TF2 embryologic development during gestation, two litters of E15 TF2 progeny were sacrificed. Nonviable littermates with exencephaly-like phenotypes were observed in two of 19 embryos. Interestingly, Liu et al. reported a similar finding of exencephaly for Msx2-overexpressing mice (6). The etiology for this phenotype is not clear. This result may also help to explain the observed low incidence of severely affected TF2 progeny among newborn mice.

Overexpression of Nell-1 in vitro accelerates osteoblast differentiation. Dysregulated bone formation has been proposed as a possible mechanism for calvarial overgrowth/overlap and premature suture closure (18). Because abnormal suture site osteogenesis is the cardinal feature of Nell-1 TF2 mice exhibiting premature suture closure, we hypothesized that Nell-1 overexpression may alter normal calvarial osteoblast cell cycling and differentiation pathways to promote premature osteogenesis.

To test our hypothesis, we first examined the effect of Nell-1 on mineralization, a hallmark of osteoblast differentiation in vitro. Primary FRCC and MC3T3 (a mouse calvarial osteoblast-like cell line) cultures were infected with AdNell-1 at 20 pfu/cell in the presence of ascorbic acid. Ascorbic acid is essential for the induction and terminal differentiation/mineralization of osteoblasts (19). AdNell-1–infected FRCC and MC3T3 cultures mineralized more rapidly and profusely (more than sixfold) than Adβ-Gal–infected controls did (Figure 5, a and b). In contrast, AdNell-1 infection did not elicit any mineralization response in NIH3T3, adult, or fetal rat primary fibroblast cells (data not shown). These data suggest that Nell-1 accelerates osteoblast mineralization and that the effects are osteoblast-specific.
infected cells are represented on the x axis. HKGs $^2$ represents the correlation of housekeeping genes (filled squares) between the two samples. ECMS $^2$ represents the correlation of candidate gene expression (open squares) between the two samples. A photograph of the microarray reading is attached in the upper left corner of each diagram. A twofold or greater upregulation is represented in red, while a twofold or greater downregulation is represented in green (g) Table summarizing genes with a difference in expression that is twofold higher or lower after AdNell-1 infection. The ratio is calculated as Nell-1/β-Gal. Col: collagen.

Our previous in vivo BrdU results demonstrated significantly reduced cell proliferation along the osteogenic front in Tβ mice. To determine whether Nell-1 overexpression in vitro also affects cell cycling, AdNell-1–infected MC3T3 cells (and Adβ-Gal controls, with and without ascorbic acid treatment and with and without 24 hours of serum starvation) were examined by flow cytometry at 24 and 48 hours after infection. No statistically significant changes were observed in populations in different phases of the cell cycle (two-tailed Student t test, P > 0.05). The fact that MC3T3 cells did not demonstrate decreased proliferation after Nell-1 transfection may reflect inherent differences between in vivo and in vitro osteoblast cells or the influence of the extracellular milieu and stage of cellular differentiation.

Normal in vitro osteoblast differentiation is heralded by nodule formation (osteoblast cell aggregates) followed by mineralization. This differentiation program requires ascorbic acid. Interestingly, AdNell-1–infected MC3T3 cells, when cultured without ascorbic acid, also formed nodules expressing alkaline phosphatase beginning on day 3 after infection; control Adβ-Gal–infected cells did not. Nell-1–induced nodules in the absence of ascorbic acid, however, were smaller (≤ 20 cells per nodule, detectable at 100× magnification), and did not reveal mineralization with von Kossa staining (Figure 5c). Moreover, late differentiation markers such as osteopontin were not expressed in these “micronodules.” The formation of micronodules by AdNell-1–infected osteoblasts in the absence of ascorbic acid suggests that Nell-1 alone may influence cell-cell adhesion but is not sufficient to induce full osteoblast differentiation.

To prove that Nell-1 enhances osteoblast differentiation, RNA from AdNell-1–infected MC3T3 cells, cultured under normal conditions with ascorbic acid, were subjected to microarray analyses of various bone-specific markers at 6, 9, and 12 days after infection (Figure 5, d–f). The purpose of the microarray was to determine whether AdNell-1–infected and control Adβ-Gal–infected cells demonstrated distinct differences in overall osteoblast differentiation marker expression patterns using regression analysis. By day 12, the expression pattern of osteoblast differentiation markers was distinctly different between AdNell-1–infected cells and Adβ-Gal–infected cells ($^2 = 0.334$). Microarray analyses used in this experiment were not meant to quantitate the expression of individual genes. Individual gene expression patterns should be interpreted with caution, e.g., genes with two or more fold up or downregulation should then be analyzed. Results should also be confirmed with RT-PCR or RNA analyses. Late differentiation markers, such as Bmp7, osteopontin, and osteocalcin, were upregulated more than twofold in AdNell-1–infected cells, while earlier markers, such as type I collagen and osteonectin, were downregulated more than twofold (Figure 5g). This suggests that Nell-1 promotes osteoblast differentiation. Osteocalcin and osteopontin RNA upregulation were verified by RNA electrophoresis (see Figure 6, c and d). Neither microarray nor reduced-cycle RT-PCR analyses demonstrated any significant changes in expression of Cbfa1, Tgfβ1, -β2, and -β3, or Tgfβ types -I, -II, and -III receptors, Fgfr1, or Fgfr2 in AdNell-1–infected MC3T3 cells (data not shown). This suggests that Nell-1 may operate downstream of these candidate genes or may affect distinctly different pathways.

> ![Figure 6](image_url)

**Figure 6**: Effect of Nell-1 downregulation on alkaline phosphatase expression and bone marker expression. (a) Western blot analysis of Nell-1 protein expression in rat FRCCs infected with 20 pfu/cell AdAntiNell-1 or Adβ-Gal control. Downregulation of approximately 60% is observed. (b) Alkaline phosphatase staining (in red) of FRCCs. AdAntiNell-1–infected cells have significantly less staining than do control and AdNell-1–infected cells. (c) Northern analyses of FRCCs on days 3, 6, 9, and 12 after infection. AdAntiNell-1–infected cells have significantly less osteocalcin and osteopontin expression. (d) Expression of osteocalcin (OC) and osteopontin (OP) measured by PhosphorImager and normalized by GAPDH.

Downregulation of Nell-1 in vitro delays osteoblast differentiation. To further address the physiologic function of Nell-1 in osteoblast differentiation, we tested the effect of downregulating the Nell-1 protein through adenoviral antisense Nell-1 infection in osteoblasts. FRCC cultures were infected with AdAntiNell-1 at 20 pfu/cell in the presence of ascorbic acid. AdAntNell-1 downregulated Nell-1 protein expression to 40% of its normal expression level (Figure 6a). FRCC cultures expressed significantly less alkaline phosphatase than did Adβ-Gal–infected controls or AdNell-1–infected cells (Figure 6b). Osteocalcin and osteopontin RNA expression was also downregulated in AdAntiNell-1 cells (Figure 6, c and d). The ratio of osteocalcin in AdAntiNell-1–infected cells to osteocalcin in Adβ-Gal controls was less than 1:4 on day 9 and 1:2 on day 12 by Northern analysis. The ratio of osteopontin in AdAntiNell-1–infected cells to that in Adβ-Gal controls was less than 1:5 on days 6 and 9, and less than 2:5 on
NELL-1 is a relatively newly discovered molecule with unknown function. Because of the observed transient upregulation of NELL-1 during premature suture closure in CS patients (12), we simulated NELL-1 overexpression in a mouse model in order to investigate novel potential functions of Nell-1 in craniofacial development and pathology. We observed early suture closure and increased osteoblast differentiation in Nell-1 transgenic mice. Therefore, Nell-1 is likely a candidate for the control of local suture closure, and the overexpression of Nell-1 may play an important role in the mechanism of premature suture closure in CS. Based on our overexpression and knockdown in vitro data, Nell-1 most likely influences osteoblast differentiation. However, the molecular mechanism is unknown.

Effect of Nell-1 on osteoblast differentiation. Normal osteoblasts cultured without ascorbic acid do not differentiate. Osteoblasts overexpressing Nell-1, on the other hand, form micronodules and express alkaline phosphatase in the absence of ascorbic acid. This suggests that Nell-1 alone is sufficient to induce some degree of osteoblast differentiation.

In addition, RNA microarray analyses of Nell-1 overexpression in osteoblasts cultured under normal conditions (i.e., with ascorbic acid) demonstrated upregulation of late differentiation markers at day 12 after transfection. AdNell-1-transfected osteoblasts also exhibited increased mineralization beginning on day 12 after transfection. These data indicate that Nell-1 may accelerate the rate of calvarial osteoblast differentiation and mineralization.

Nell-1 overexpression may not reflect the true physiological function of Nell-1, but rather the effect of Nell-1 overexpression on other thrombospondin-like molecules. Downregulation of Nell-1 clearly inhibited osteoblast differentiation. Nell-1 is therefore likely to be both sufficient and required for osteoblast differentiation in vitro. However, Nell-1 null mice need to be produced in order to justify this conclusion in vivo.

Nell-1’s relation to currently known CS models. Nell-1 overexpression produces craniofacial abnormalities similar to those
resulting from Msx2 overexpression in vivo. Both mouse models exhibit suture overgrowth and an increased incidence of exencephaly. However, the cellular functions of these two genes appear to be distinctly different; continuous Msx2 overexpression induces proliferation and inhibits differentiation, while Nell-1 enhances differentiation. Mice with a Pro\textsuperscript{250} → Arg mutation in Fgfr1, which induces Cbfa1 overexpression, have distinctly different phenotypes from mice overexpressing Nell-1 because calvarial fusion occurs much later (postnatal days 16–21) and gross suture overlap does not occur (6) in the mice with the Pro\textsuperscript{250} mutation. However, Cbfa1 has a similar cellular function to Nell-1 in vitro; both induce osteoblast differentiation with upregulation of bone marker genes. Nell-1 expression is modulated by Msx2 and Cbfa1. Cbfa1 transfection of FRCCs upregulated Nell-1 expression within 24 hours, while Msx2 transfection and Cbfa1/Msx2 cotransfection downregulated Nell-1 expression (unpublished observations). While all these candidate genes are important to the understanding of CS, Msx2 may be important in the earlier stages of CS (5, 6), while Fgfr1/Cbfa1 may play a role in the later stages of suture closure. Future investigation of the Nell-1 promoter, which contains conserved Cbfa1 and Msx binding sequences, may provide further understanding of their interactions (Figure 7). These observations underscore the complexity of the dynamic genetic and environmental interactions in craniofacial growth and development.

In conclusion, we have created an animal model of human nonsyndromic CS by overexpressing Nell-1. Unlike other available CS models involving mutations in FGFRs or homeobox genes (1, 2, 8), our animal model exhibited anomalies that were localized to the craniofacial skeleton. We hypothesize that Nell-1 is sufficient, and probably required, to promote and accelerate calvarial osteoblast differentiation and bone formation. Mechanistically, Nell-1 overexpression induces intramembranous bone formation in cranial sutures and may lead to calvarial overgrowth/overlap and subsequent premature suture closure.

Although Nell-1 has not yet been identified as a cause of CS in human genetic studies, the data strongly suggest that Nell-1 is part of a complex chain of events resulting in premature suture closure (1). The resemblance of Nell-1 transgenic mice to humans with nonsyndromic CS and Nell-1’s association with known CS candidate genes provides new insights for CS research. Further investigation of the regulation and mechanism of Nell-1 in suture closure and bone formation can potentially accelerate our understanding of the cascade of events leading to premature suture closure in CS.

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Footnotes

Conflict of interest: No conflict of interest has been declared.

Nonstandard abbreviations used: craniosynostosis (CS); FGF receptor (FGFR); cytomegalovirus, (CMV); magnetic resonance imaging (MRI); adenovirus (Ad); embryonic day 18 (E18); β-Galactosidase (β-Gal); fetal rat calvarial cell (FRCC); transgenic F\textsubscript{2} (TF\textsubscript{2}); normal F\textsubscript{2} (NF\textsubscript{2}); posterior-frontal (PF); microcomputerized tomography (MCT).

References

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Previously, we reported NELL-1 as a novel molecule overexpressed during premature cranial suture closure in patients with craniosynostosis (CS), one of the most common congenital craniofacial deformities. Here we describe the creation and analysis of transgenic mice overexpressing Nell-1. Nell-1 transgenic animals exhibited CS-like phenotypes that ranged from simple to compound synostoses. Histologically, the osteogenic fronts of abnormally closing/closed sutures in these animals revealed calvarial overgrowth and overlap along with increased osteoblast differentiation and reduced cell proliferation. Nell-1 transgenic animals exhibited CS-like phenotypes that ranged from simple to compound synostoses. Histologically, the osteogenic fronts of abnormally closing/closed sutures in these animals revealed calvarial overgrowth and overlap along with increased osteoblast differentiation and reduced cell proliferation. Furthermore, anomalies were restricted to calvarial bone, despite generalized, non-tissue-specific overexpression of Nell-1. Zhang X, Kuroda S, Carpenter D, Nishimura I, Soo C, Moats R et al. Craniosynostosis in transgenic mice overexpressing Nell-1. Journal of Clinical Investigation. 2002 Sep;110(6):861-870. https://doi.org/10.1172/JCI200215375. Human Nell-1 was first cloned from a fetal brain cDNA library in 1996 without any known functions. Later its overexpression was first associated with human craniosynostosis (CS), and functionally validated in a Nell-1 overexpression transgenic mouse model in 1999 and 2002, respectively at Dr. Ting’s lab. Subsequently, a series of substantial studies on Nell-1’s osteogenic function and the underlying molecular mechanisms were conducted to pave a solid foundation for its translational study, and helped obtain millions of dollars’ research fund from NIH and other funding agents.