Nell-1, a Key Functional Mediator of Runx2, Partially Rescues Calvarial Defects in Runx2\textsuperscript{+/−} Mice

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ABSTRACT
Mesenchymal stem cell commitment to an osteoprogenitor lineage requires the activity of Runx2, a molecule implicated in the etiopathology of multiple congenital craniofacial anomalies. Through promoter analyses, we have recently identified a new direct transcriptional target of Runx2, Nell-1, a craniosynostosis (CS)–associated molecule with potent osteogenic properties. This study investigated the mechanistic and functional relationship between Nell-1 and Runx2 in regulating osteoblast differentiation. The results showed that spatiotemporal distribution and expression levels of Nell-1 correlated closely with those of endogenous Runx2 during craniofacial development. Phenotypically, cross-mating Nell-1 overexpression transgenic (CMV-Nell-1) mice with Runx2 haploinsufficient (Runx2\textsuperscript{+/−}) mice partially rescued the calvarial defects in the cleidocranial dysplasia (CCD)–like phenotype of Runx2\textsuperscript{+/−} mice, whereas Nell-1 protein induced mineralization and bone formation in Runx2\textsuperscript{+/−} but not Runx2\textsuperscript{−/−} calvarial explants. Runx2-mediated osteoblastic gene expression and/or mineralization was severely reduced by Nell-1 siRNA oligos transfection into Runx2\textsuperscript{+/−} newborn mouse calvarial cells (NMCCs) or in N-ethyl-N-nitrosourea (ENU)–induced Nell-1\textsuperscript{−/−} NMCCs. Meanwhile, Nell-1 overexpression partially rescued osteoblastic gene expression but not mineralization in Runx2 null (Runx2\textsuperscript{−/−}) NMCCs. Mechanistically, irrespective of Runx2 genotype, Nell-1 signaling activates ERK1/2 and JNK1 mitogen-activated protein kinase (MAPK) pathways in NMCCs and enhances Runx2 phosphorylation and activity when Runx2 is present. Collectively, these data demonstrate that Nell-1 is a critical downstream Runx2 functional mediator insofar as Runx2-regulated Nell-1 promotes osteoblastic differentiation through, in part, activation of MAPK and enhanced phosphorylation of Runx2, and Runx2 activity is significantly reduced when Nell-1 is blocked or absent. © 2011 American Society for Bone and Mineral Research.

KEY WORDS: NELL-1; RUNX2; TRANSGENIC ANIMAL; CRANIOFACIAL DEVELOPMENT; CLEIDOCRANIAL DYSPLASIA

Introduction
Commitment of undifferentiated mesenchymal stem cells to an osteoprogenitor lineage is first marked by expression of runt-related transcription factor 2 (Runx2; also known as Pebp2xA/Cbfa1/Aml3).\textsuperscript{(1,2)} Runx2 is essential for osteoblast formation and function because it is expressed by all osteoblasts irrespective of embryonic origin or mode of ossification.\textsuperscript{(3)} Runx2 null mutant mice completely lack mineralized bone formation, whereas heterozygous Runx2 loss-of-function mice manifest a phenotype similar to cleidocranial dysplasia (CCD) in humans, consisting of clavicular hypoplasia, delayed development and ossification of cranial bones causing open anterior and posterior fontanelles, smaller parietal and interparietal cranial bones, and multiple wormian bones (small bones in the sutures).\textsuperscript{(4,5)} Since Runx2 is a transcription factor, it undoubtedly exerts its critical osteogenic effects in part through downstream functional mediators.\textsuperscript{(6)} However, knockout models of many osteoblastic genes containing the consensus RUNX2 binding site osteoblast-specific binding elements 2 (OSE2), such as α1 type I collagen,\textsuperscript{(7)} bone sialoprotein,\textsuperscript{(8)} osteopontin,\textsuperscript{(9)} and osteocalcin\textsuperscript{(10)} in mice have not yielded significant defects similar to Runx2 deficiency.
Our data indicates that Nell-1 may be a key player in addition to Osx, another critical transcriptional factor for osteoblasts, in the Runx2 network regulating osteoblastic differentiation.(11–13)

NELL-1, a secreted protein strongly expressed in neural tissues and containing epidermal growth factor (EGF)-like domains (Nel-)like protein type 1, was detected originally to be upregulated in pathologically fusing and fused sutures in nonsyndromic unilateral coronal synostosis (UCS) patients. (14) Nell-1-overexpressing mice (CMV-Nell-1) exhibit craniosynostosis (CS)-like phenotypes that ranged from simple to compound synostoses. (12) Through promoter analyses, we have established NELL-1 as a direct target of RUNX2, the master gene of osteochondrogenic differentiation. (15,16) The restoration of Nell-1 mRNA expression after Runx2 transfection into Runx2-/- cells indirectly confirms the existence of functional OSE2 binding sites in mouse Nell-1 promoter and further supports in silico analysis findings of NELL-1 transcriptional regulation by RUNX2. (15) Furthermore, ENU-induced Nell-1-deficient mice display similar CCD-like calvarial phenotypes as Runx2-/- mice in addition to rib cage and vertebral abnormalities. (16) The fact that Runx2 directly promotes NELL-1 transcripts and ENU Nell-1-deficient mice exhibit a similar CCD-like phenotype as Runx2-/- mice suggests that NELL-1 may mediate a significant subset of downstream RUNX2 functions during osteoblastic differentiation in vivo.

The Nell-1 molecule itself is highly conserved across species. Rat and human Nell-1 proteins share a 93% predicted amino acid homology (17) and contain several conserved motifs. (17) More important, Nell-1 has revealed its osteoinductive potency by promoting bone regeneration in multiple animal models. (18) The Nell-1 molecule itself is highly conserved across species. (18) Nell-1 is strongly expressed in neural tissues and contains epidermal growth factor (EGF)-like domains (Nel-)like protein type 1, was detected originally to be upregulated in pathologically fusing and fused sutures in nonsyndromic unilateral coronal synostosis (UCS) patients. (14) Nell-1-overexpressing mice (CMV-Nell-1) exhibit craniosynostosis (CS)-like phenotypes that ranged from simple to compound synostoses. (12) Through promoter analyses, we have established NELL-1 as a direct target of RUNX2, the master gene of osteochondrogenic differentiation. (15,16) The restoration of Nell-1 mRNA expression after Runx2 transfection into Runx2-/- cells indirectly confirms the existence of functional OSE2 binding sites in mouse Nell-1 promoter and further supports in silico analysis findings of NELL-1 transcriptional regulation by RUNX2. (15) Furthermore, ENU-induced Nell-1-deficient mice display similar CCD-like calvarial phenotypes as Runx2-/- mice in addition to rib cage and vertebral abnormalities. (16) The fact that Runx2 directly promotes NELL-1 transcripts and ENU Nell-1-deficient mice exhibit a similar CCD-like phenotype as Runx2-/- mice suggests that NELL-1 may mediate a significant subset of downstream RUNX2 functions during osteoblastic differentiation in vivo.

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### Materials and Methods

**Generation of Runx2-deficient plus Nell-1 transgenic mice**

Runx2 heterozygous deficient mice (Runx2-+/-) (4) were mated with Nell-1-overexpressing mice (CMV-Nell-1) (12) to generate Runx2-/-/CMV-Nell-1 mice and Runx2-/-/CMV-Nell-1 mice. Mouse genotypes were determined by PCR, and expression levels of Nell-1 and Runx2 were monitored using RT-PCR and were further verified by immunohistochemistry. Mouse embryos were collected from mating among wild-type mice with vaginal plugs defined as E0.5 days postcoitum (dpc). Table 1 lists the total number of animals used for skeletal staining, micro-computed tomography (μCT), and histology. 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.

**Ex vivo calvarial organ culture**

Calvarial vaults of newborn Runx2-/- mice were harvested and placed in serum-free B Gib (Biggers, Gwatkin, Judah) medium with l-glutamine and supplemented with 100 unit/mL of penicillin, 100 μg/mL of streptomycin, 2.5 μg/mL of amphotericin B, 100 μg/mL of l-ascorbic acid, and 10 mM glycerophosphate. Recombinant Nell-1 (rNell-1) protein (Katayama Chemical, Ltd., Osaka, Japan) at 100 ng/mL was added beginning on day 1 plus rNell-1 (n = 8) or minus rNell-1 (n = 5). On day 4, calcein was added to the culture medium at 2 μg/mL, and the explants were maintained for a total of 9 days before harvesting for gross and histologic analysis of tissue ossification. The calcein deposition on explants was observed with an Olympus SZX12 fluorescent microscope (Melville, NY, USA), and the relative intensity of green fluorescence representing the degree of mineralization on whole explanted calvaria as well as defined coronal and sagittal suture areas was quantified using Image Pro Plus (Bethesda, MD, USA). The methyl methacrylate–embedded sections were analyzed under an Olympus BX51 fluorescent microscope.

**Skeletal and histologic analysis**

Newborn mice with the genotypes described in Table 1 were euthanized with an overdose of phenobarbital, skinned and eviscerated, and then fixed in 95% ethanol for 24 hours at room temperature. Standard skeletal staining was performed using

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**Table 1. Summary of Animals Used for Detailed Analysis**

| Genotype          | No. used for skeletal analyses | No. used for μCT | No. used for histology | No. of animals “rescued” from Runx2 deficiency |
|-------------------|-------------------------------|------------------|------------------------|-----------------------------------------------|
| Runx2+/-/         |                               |                  |                        |                                               |
| CMV-Nell-1        | 12                            | 4                | 3                      | 5 Not applicable                              |
| WT                | 15                            | 2                | 3                      | 10 Not applicable                             |
| Runx2+/-/         |                               |                  |                        |                                               |
| CMV-Nell-1        | 27                            | 15               | 7                      | 5 16 of 22                                    |
| WT                | 35                            | 20               | 5                      | 10 Not applicable                             |
| Runx2-/-/         |                               |                  |                        |                                               |
| CMV-Nell-1        | 8                             | 3                | 1                      | 4 0 of 4                                      |
| WT                | 9                             | 5                | 1                      | 3 Not applicable                              |
| Total             | 106                           | 49               | 20                     | 37                                            |

“Determination of “rescue” status was based on skeletal staining and μCT analysis.
alcan blue for negatively charged proteoglycans and alizarin red for calcium to provide gross distinction between cartilage and mineralized tissue, respectively. For histology, tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Skeletal and histologic images were acquired using a MicroFire digital camera with PictureFrame software (Optronics, Goleta, CA, USA) attached to Olympus SZX12 and BX51 microscopes.

High-resolution μCT analysis

High-resolution μCT using 9- to 20-μm resolution technology from μCT40 (Scanco USA, Wayne, PA, USA) was performed as described previously. μCT data were collected at 50 kVp and 160 μA and reconstructed using the cone-beam algorithm supplied with the μCT scanner by Scanco. A threshold of 130 for 3D reconstruction of newborn mouse heads was determined empirically by evaluating skeletal image of newborn wild-type mouse heads with serial thresholds to choose the one where no soft tissue was detected. In addition, CT-based morphometric analyses were performed on the size of the anterior fontanel, the closest distance of the sagittal suture, and the average thickness of parietal bone plates on the same plane in each group including Runx2+/− (n = 3), Runx2+/+ (n = 5), and Runx2+/-/CMV-Nell-1 (n = 7) newborn mice. Data are presented as the mean ± SD and analyzed with a two-tailed Student’s t test, with p ≤ 0.05 considered significant.

Immunohistochemistry

Whole-head samples from E14.5, E16.5, E18.5, and newborn mice were fixed in 4% paraformaldehyde and then processed for paraffin embedding. Paraffin-embedded sections were used for immunohistochemistry employing a previously described protocol. Polyclonal rabbit IgG of anti-mouse Nell-1 antibodies were synthesized against an 11-amino-acid peptide, and the specificity of the affinity-purified antibody was further confirmed by PCR or RT-PCR and limited to three passages, and the genotype of isolated NMCCs was further confirmed by PCR or RT-PCR and immunocytochemistry. NMCCs at third passage were used in all experiments.

Isolation and culture of NMCCs

Isolation of Runx2−/− or ENU-induced Nell-1-deficient new-born mouse calvarial cells (NMCCs; n = 5) as well as their corresponding wild-type (n = 10) or heterozygous NMCCs (n = 10) was conducted within 2 hours of delivery of mice, as reported previously. The NMCCs were cultured in α minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY, USA), 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Cell expansion was limited to three passages, and the genotype of isolated NMCCs was further confirmed by PCR or RT-PCR and immunocytochemistry. NMCCs at third passage were used in all experiments.

Real-time RT-PCR

TaQMan primer probe sets for Alp, Ocn, Nell-1, Runx2, and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were purchased (Applied Biosystems, Inc., Foster City, CA, USA) and analyzed using an ABI Prism 7300 real-time reverse-transcriptase (RT) PCR system (Applied Biosystems) as described previously. Relative gene expression profiles were calculated using the comparative quantification formula as 2−ΔΔCt based on the evaluation of similar dynamic ranges for RT-PCR efficiency of both Gapdh and the target genes. All data are representative of three experimental sets of cells or three mice tissue specimens with duplicate PCR running and are presented as the fold difference. In addition, the Northern blot hybridization and reduced-cycle RT-PCR also were used for the detection of endogenous levels of Runx2, Nell-1, Ocn, Oso, Dlx5, and Msx2 in whole-head tissues of mice with different genotypes, as described previously. PCR primer sequences are available on request.

Western blot detection of MAPK pathways activation

NMCCs were synchronized for 18 hours in 0.1% fetal bovine serum (FBS) growth medium before being stimulated with 100 ng/mL of rNell-1 for the indicated times. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with inhibitors of proteinase and phosphatase (Invitrogen) and underwent Western blot analysis with antibodies against total and phosphorylated ERK, JNK and p38 (Santa Cruz Biotechnology) using previously reported protocols.

Immunoprecipitation for Runx2 phosphorylation study

NMCCs were synchronized as described earlier and preincubated for 1 hour with three MAPK inhibitors at 25 μM SPh00125 for JNK, 50 μM PD98059 for ERK, and 10 μM SB203580 for p38 or equal volume of DMSO vehicle in 0.1% FBS growth medium before being stimulated with 100 ng/mL of rNell-1 for 1 hour. Immunoprecipitation was done by adding 2 μg of anti-Runx2 (Abcam, Cambridge, MA, USA) to 500 μL of cell lysate and incubating for 1 hour at 4°C, followed by mixing with 50 μL of protein G sepharose. The immunoprecipitation products were probed with anti-Runx2 and anti-pSer (Santa Cruz Biotechnology) for detecting Runx2 phosphorylation status.

Reporter assay for transactivation analysis

The reporter assay was done by cotransfection of plasmids of 6OSF2 plus Renilla control with either Runx2 expression plasmid pcDNA-Runx2 or empty-vector pcDNA3.1 into Runx2+/- and Runx2−/− NMCCs using Lipofectamine 2000 (Invitrogen). Then 100 ng/mL or higher at 800 and 1600 ng/mL of rNell-1 and equal volumes of PBS as control were added to medium 24 hours after transfection, and the medium was incubated for another 24 hours before collecting cell lysate for luciferase assay using a dual luciferase detection kit (Promega, San Luis Obispo, CA, USA) as described previously.

Adenoviral transduction with NMCCs

NMCCs at 80% confluence were transduced with AdNell-1, AdLacZ, or AdRunx2 at a multiplicity of infection (MOI) of 50
plaque-forming units (pfu) per cell in α-MEM, as described previously.\textsuperscript{13} Alkaline phosphatase activity and the expression of Nell-1, Alp, Ocn, and Omp and/or mineralization were analyzed at the indicated time points.

Transfection of Nell-1 siRNA into NMCCs

Mouse Nell-1 siRNA oligos were designed and synthesized by Qiagen with the HiPerformance Design Algorithm based on the sequences of mouse Nell-1 (Gene Accession No. AK046127). The specific target sequence was as follows: CAGGTGTGGATTCTGAGAAGAGAA. RNAiffect reagent and unrelated negative control siRNA (Qiagen) were used for the transfection of siRNA into NMCCs according to the manufacturer’s instructions. Briefly, NMCCs were seeded at 5 × 10\(^4\) cells/cm\(^2\) into 24-well plates and allowed to reach 80% confluence the following day for siRNA transfection. Then 50 ng of siRNA oligos per well was mixed with the Perfect reagent at a ratio of 1:3 and incubated for 10 minutes at room temperature. The blocking efficacy for the expression of mouse Nell-1 mRNA and protein was measured at 48 and 72 hours after transfection with RT-PCR and Western blot analysis with Nell-1-specific antibody.\textsuperscript{13} The transfection efficiency and blocking specificity of Nell-1 siRNA oligos into these cells was monitored by introducing unrelated siRNA oligos labeled with fluorescein using the same strategy. For some groups, the transfection of mouse Nell-1 siRNA oligos was followed by the transduction of AdRunx2\textsuperscript{24} into the same cells 24 hours later. Seventy-two hours after seeding, cells were switched to differentiation medium containing 50 μg/mL of ascorbic acid and 10 mM glycerol phosphate. The Western blot for Nell-1, real-time PCR for Nell-1, Runx2, Alp, Ocn, and Omp mRNA, and von Kossa staining were carried out accordingly.

ALP and mineralization assays

Alkaline phosphatase (ALP) staining assay was carried out with the Leukocytes ALP Staining Kit (86R-1KT, Sigma, St Louis, MO, USA) as described previously.\textsuperscript{13} Quantitative ALP activity was assayed with cell lysate and ALP Buffer Solution (Sigma) and phosphatase substrate capsule (Sigma), as reported previously.\textsuperscript{20} All measurements were read in triplicate, and the ALP activity was normalized to corresponding protein quantifications before making relative fold determination against nontransduced control. The data are presented as the mean ± 5D with a r-test significance of p ≤ 0.05. The von Kossa and alizarin red staining (ARS)/quantification assays were performed as described previously.\textsuperscript{12,20}

Cell proliferation and apoptosis analysis

To determine nonspecific toxicity of MAPK inhibitors, NMCCs were seeded at 2 × 10\(^3\) cells/well in 96-well plates in the presence of MAPK inhibitors or DMSO vehicle for proliferation study by the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] method (Promega). The optical density (OD) values at 490 nm were plotted for relative comparison among different treatment groups. For apoptosis analysis, NMCCs were plated in 6-well culture dishes and treated with MAPK inhibitors at the same concentration used for the Runx2 phosphorylation study for 6 days in osteoblastic differentiation medium. The Annexin V and propidium iodide (PI) Kit (Pharmingen, San Diego, CA, USA) was used for staining cells, followed by flow cytometric analysis. The percentage of early and late apoptotic cells was calculated and presented.

Results

Spatiotemporal expression of Nell-1 and Runx2 during mouse craniofacial bone development

To determine the spatiotemporal expression pattern of Runx2 and Nell-1 during craniofacial bone formation, immunohistochemistry was performed on whole-head sections of E14.5, E16.5, and E18.5 mouse embryos. Consistent with its critical role in osteoblastogenesis, Runx2 expression was present in every axial, appendicular, and cranial skeletal anlage at E12.5,\textsuperscript{3} although mineralization of craniofacial bones occurs later, at E14.5. In E14.5 mice, significantly high levels of Nell-1 expression closely approximated and overlapped with Runx2 expression in temporal and parietal bone plates (Fig. 1A). Osteoblast cells along the temporal bone plates with typical nucleus staining for Runx2 (Fig. 1A, lower panel) also stained strongly for Nell-1 in the cytoplasm and nucleus (Fig. 1A, upper panel). In E16.5 mice, the spatial distribution of Nell-1 expression expanded to cover bone-forming areas of the cranial vault, base, and mandible (Fig. 1B). High-intensity Nell-1-positive cells localized primarily to ossifying membranous bone-forming regions in the cranial base as well as nonossifying regions such as the dura mater (Fig. 1B, upper panel). Interestingly, in E18.5 mice, Runx2 expression was relatively low in calvarial bone and dura mater when compared with Nell-1 expression (Fig. 1C). To complement the immunohistochemistry data, relative Runx2 and Nell-1 mRNA levels in isolated E14.5, E16.5, and E18.5 calvarial bones were determined by real-time RT-PCR. Nell-1 expression closely paralleled Runx2 mRNA expression with a significant decline in levels from E14.5 to E16.5 (Fig. 1D, upper panel). Overall, the craniofacial localization and expression pattern of Nell-1 followed that of Runx2. The close temporal and spatial overlap in Runx2 and Nell-1 expression at multiple embryonic stages suggests a possible regulatory relationship between Runx2 and Nell-1 during mouse craniofacial development.

To determine if the observed similarity in Runx2 and Nell-1 expression patterns in calvarial bones were also present in long bones, neonatal calvaria and long bone (femur and tibia) RNA were analyzed by real-time RT-PCR. Results demonstrated that Runx2 levels in newborn calvarial bone were higher than in newborn long bone. Relatively high Runx2 levels in calvaria were associated with high calvarial Nell-1 levels, whereas lower Runx2 levels in long bone were associated with lower Nell-1 levels (Fig. 1D, lower panel). These data demonstrated closely paralleled expression patterns for Nell-1 and Runx2 in both calvarial and long bone tissues, as well as significantly higher Nell-1 and Runx2 RNA levels in calvaria versus long bones during development.

Diminished expression of Nell-1 in craniofacial tissues with Runx2 deficiency

The preferential expression of Nell-1 and Runx2 in calvaria versus long bones and their spatiotemporal distribution pattern in
craniofacial bones led us to further investigate the relationship between Runx2 and Nell-1 in the context of Runx2 disruption. Whole-head tissues from wild-type (Runx2^{+/+}), Runx2^{+/−}, and Runx2^{−/−} newborn mice were examined for expression of Nell-1 and Ocn by Northern blot (Supplemental Fig. S1A). The highest expression of an approximately 3.5-kb single transcript of Nell-1 and an approximately 0.7-kb Ocn was readily detected in Runx2^{+/+} tissue, and only weak signals were observed in Runx2^{+/−}, whereas both Nell-1 and Ocn were undetectable in all Runx2^{−/−} tissues. In addition, the expression levels of Nell-1 and Ocn, as well as Runx2 and other genes critical in craniofacial development, including Osr, Dlx5, and Msx2, were verified and screened.
with reduced-cycle RT-PCR using the same RNAs from whole-head tissues used for Northern blot analysis (Fig. 2A). Results revealed that the levels of Runx2 expression in Runx2+/−, Runx2+/−, and Runx2−/− samples correlated very well with the expression levels of Nell-1, Ocn, and Osx, whereas the levels of Dlx5 and Msx2 were similar in both Runx2+/− and Runx2−/− samples. These results indicate that Nell-1, Ocn, and Osx gene transactivation is highly dependent on underlying Runx2 levels.

To specifically correlate Runx2 and Nell-1 expression in bone-forming tissues, the gene expression levels of Runx2, Nell-1, Ocn, Opn, and Alp were further analyzed with newborn mouse calvarial cells (NMCCs) (Fig. 2B) by real-time RT-PCR, and the protein level of Nell-1 in situ was detected in Runx2−/− mouse head (scale bar: 500 μm) in contrast to its WT littermates. Corresponding sagittal suture (upper, black border; scale bar: 100 μm), temporal bone (lower, red border; scale bar: 100 μm) and the mandible (green border; scale bar: 50 μm). Nell-1 protein was undetectable in the Runx2−/− mouse head (scale bar: 500 μm) in contrast to its WT littermates. Corresponding sagittal suture (upper, black border; scale bar: 100 μm), temporal bone (lower, red border; scale bar: 100 μm) and the mandible (green border; scale bar: 50 μm) areas show distinct absence of bone development and no Nell-1 staining. Nell-1 staining in Runx2−/− animals was present primarily in skin (G) and a few chondrocytes (H) (arrows) in Meckel’s cartilage (mC).
bones compared with wild-type mice (Fig. 2C–H). Collectively, the close correlation between Nell-1 and Runx2 expression indicates that Runx2 is an important in vivo regulator of Nell-1 expression and is consistent with our data demonstrating potentially functional Runx2 binding sites on the Nell-1 promoter.\(^\text{(15)}\)

Nell-1 partially rescues CCD-like calvarial defects in Runx2\(^{+/−}\) mice

Global Nell-1-overexpressing mice using a CMV promoter (CMV-Nell-1) exhibit phenotypes related to calvarial overgrowth and premature suture fusion without obvious extracranial abnormalities (despite verification of global Nell-1 transgene expression).\(^\text{(12)}\) To determine whether Nell-1 can functionally compensate for some aspects of Runx2 deficiency (eg, CCD phenotype), Runx2\(^{+/−}\) mice were mated to CMV-Nell-1 mice to generate Runx2\(^{+/−}\)/CMV-Nell-1 mice. A total of 106 newborn mice recovered from 14 litters were included in this study (Table 1). As expected, newborn wild-type mice showed dramatically different patterns of cranial bone skeletal staining compared with Runx2\(^{+/−}\) mice. Consistent with previous reports, the majority of Runx2\(^{+/−}\) mice exhibited developmental anomalies resembling the CCD phenotype described by Otto and colleagues (eg, hypoplastic clavicles, wide sutures, and delayed membranous bone ossification resulting in open anterior and posterior fontanelles as well as wide cranial sutures)\(^\text{(4)}\) (Fig. 3A, B).

Remarkably, 16 of the 22 Runx2\(^{+/−}\)/CMV-Nell-1 mice demonstrated ‘‘rescue’’ of calvarial bone defects associated with the CCD phenotype on skeletal or \(\mu\)CT analyses (Fig. 3C, D), whereas Nell-1 did not rescue bone formation in the Runx2 null mice (data not shown). Quantitative \(\mu\)CT data confirmed significant

![Fig. 3. Skeletal and histochemical analysis of rescued CCD Runx2\(^{+/−}\) newborn mice. (A) The skeletal staining (top) and \(\mu\)CT (bottom) of a wild-type mouse demonstrating typical mineralization borders (dotted light blue line) and the location of the anterior (red asterisk) calvarial fontanel. The coronal sutures (green arrows) also are highlighted. (B) Runx2\(^{+/−}\) animal demonstrating widely patent midline sutures and fontanel. Defective mineralization and bone formation are present in the poorly stained tissue (between yellow and light-blue dotted lines) lateral to the midline calvarial defect. A lucency also can be seen in the area of the coronal suture (green arrows, top and bottom pictures). (C) Runx2\(^{+/−}\)/CMV-Nell-1 animal demonstrating significantly increased calvarial bone formation relative to the Runx2\(^{+/−}\) animal on skeletal staining and \(\mu\)CT. Note the restoration of bony overlap at the coronal sutures (green arrows) and decreased anterior fontanel size (red asterisk). Scale bar: 1 mm for \(\mu\)CT images. (D) The red line is drawn through the location of parietal bones (top, upper left image) for cross-sectional analysis. Cross-sectional view with labels d\(1\) to d\(4\) (top, center image). The label d\(1\) represents sagittal suture width, whereas d\(2\) through d\(4\) are measurement points of parietal bone thickness starting from d\(2\) at 0.5-mm intervals. Quantitative analysis of the average width and thickness are depicted graphically (bottom charts, \(^*p < 0.05\); \(^**p < 0.05\); NS = no significance). (E–G) Immunohistochemistry of Nell-1, Opn, and Ocn as well as (H) Alp enzymatic histochemistry on calvarial bones. Runx2\(^{+/−}\)/CMV-Nell-1 tissues demonstrated markedly higher staining intensity (red color) for Nell-1, Opn, and Ocn (yellow arrows) relative to Runx2\(^{+/−}\) tissues, whereas Alp activity did not show significant differences. Scale bar: 50 \(\mu\)m.
reduction of sagittal suture widths in Runx2+/−/CMV-Nell-1 mice compared with Runx2+/− mice (p < .05; Fig. 3D). Furthermore, parietal bone thickness in Runx2+/−/CMV-Nell-1 mice was significantly increased relative to Runx2+/− mice (p < .01) and comparable with wild-type (Runx2+/+) bone thickness (Fig. 3D). In addition, the anterior fontanelle openings in Runx2+/−/CMV-Nell-1 mice were markedly smaller than in Runx2+/− mice by visualization (Fig. 3C). Preliminary analysis of calvaria from P21 mice by μCT also revealed narrower sagittal sutures and increased intensity of calvarial bones in Runx2+/−/CMV-Nell-1 mice compared with Runx2+/− mice (Supplemental Fig. S1B). There was no rescue of clavicular hypoplasia because no significant difference was observed in clavicle length or thickness between Runx2+/− and Runx2+/−/CMV-Nell-1 mice (data not shown).

Immunohistochemistry clearly demonstrated increased Nell-1, Opn, and Ocn in calvarial bone plates of the rescued Runx2+/−/CMV-Nell-1 mice (Fig. 3E–G) but not increased Alp (Fig. 3H). These data show that Nell-1 can functionally compensate for some aspects of Runx2 deficiency and suggest that Nell-1 is a key downstream mediator of Runx2’s effects on osteoblastic differentiation and bone formation.

Nell-1 enhanced mineralization in ex vivo calvarial explants from Runx2+/− mice

Since a global Nell-1 expression model partially rescued the CCD phenotype in Runx2+/− mice, to further confirm that the
observed partial rescue of the CCD phenotype in vivo is attributable to Nell-1 activity in osteoblastic cell lineages, calvarial explants from Runx2−/− and Runx2+/− newborn mice were cultured in the presence (Fig. 4A–C, G) or absence (Fig. 4D–F, H) of recombinant rNell-1 to create an environment without systemic influences. In general, Runx2−/− explants stimulated with rNell-1 contained (Fig. 4A–C) more mineralization (as detected by calcein incorporation fluorescence) than explants cultured in the absence of rNell-1 (Fig. 4D–F). Specifically, rNell-1 stimulated significantly increased calcein uptake at actively extending osteogenic fronts of parietal bone plates (Fig. 4A). Histologic sections of sagittal and coronal sutures revealed markedly increased mineralization accompanied by impeding sagittal suture fusion (Fig. 4B) and actual coronal suture fusion (Fig. 4C). Normally, wild-type mice sagittal and coronal sutures are expected to remain patent (25) rNell-1 treatment also increased the thickness and size of parietal bones when compared with PBS vehicle control (Fig. 4D–F). Meanwhile, the control calvarias exhibited minimal mineralization within the sagittal suture mesenchyme (Fig. 4E) and between the overlapping frontal and parietal bone plates comprising the coronal suture (Fig. 4F). Consistent with the lack of Nell-1 rescue of bone formation in Runx2−/− mice, Runx2−/− calvarial explants cultured with or without rNell-1 did not exhibit any significant differences in alizarin red staining (Fig. 4G, H). Furthermore, the mineralization intensity increased by 46% overall with the presence of rNell-1 compared with the vehicle group when whole explanted calvaria were measured (wEx). There was a slightly higher increase, at 63% and 55%, in defined sagittal and coronal suture (sS and cS) regions of the samples with rNell-1 treatment over vehicle control, respectively (Fig. 4I). Taken together, these ex vivo studies clearly demonstrated that Nell-1 was sufficient to promote mineralization and bone formation (eg, suture fusion) in Runx2−/− mice containing committed preosteoblast cells but insufficient to promote mineralization in Runx2+/− mice containing undifferentiated mesenchymal cells. These data indicate that Nell-1 is not sufficient to promote bone formation in the absence of Runx2 and that some level of Runx2-dependent factors or Runx2 itself is required for Nell-1 to promote full osteoblastic differentiation.

To investigate the underlying mechanisms of Nell-1 responsiveness in Runx2−/− backgrounds and lack thereof in Runx2+/− backgrounds, the activation of three major MAPK pathways, including ERK, JNK, and p38, and Runx2 phosphorylation status were investigated using Runx2−/− and/or Runx2+/− NMCCs. Currently, it is known that phosphorylation can modulate Runx2 activity, and increased Runx2 phosphorylation has been observed after rNell-1 stimulation of rat fetal calvarial cells (26,27). Our data indicated that rNell-1 stimulation markedly intensified phosphorylation of both ERK1/2 and JNK1 starting at 10 minutes in both Runx2−/− and Runx2+/− cells, but phosphorylation was higher and more sustained in Runx2−/− cells than in Runx2+/− cells (Fig. 4J). Interestingly, phosphorylated p38 (p-p38) levels were consistently high and did not change much on Nell-1 stimulation in either Runx2−/− or Runx2+/− cells (Fig. 4J). The Runx2−/− NMCCs exhibited similar ERK and JNK pathway phosphorylation patterns as that of Runx2+/− NMCCs in response to rNell-1 stimulation (Supplemental Fig. S1C and Fig. 4J). Meanwhile, rNell-1 increased phosphorylated Runx2 levels (WB; p-Ser) in Runx2+/− NMCCs (Fig. 4K). Furthermore, Nell-1 significantly increased the transactivating activity of Runx2 (Fig. 4L). Relative luciferase activity using the 6OSE2 reporter plasmid (6OSE) was significantly higher in Runx2+/− NMCCs treated with rNell-1 (versus PBS) when cotransfected with empty vector (pcDNA3.1) and in both Runx2+/− and Runx2−/− NMCCs when treated with rNell-1 (versus PBS) and cotransfected with Runx2 expression plasmid (pRunx2). Overall, a dose-dependent effect on 6OSE activity was observed with increasing rNell-1 application (Supplemental Fig. S1D). These data indicate that Nell-1 responsiveness in Runx2−/− but not Runx2−/− backgrounds may result from sustainable and strong activation of ERK and JNK pathways and enhanced phosphorylation of, in

Fig. 4. Nell-1 protein promotes Runx2+/− calvarial explant mineralization and activates MAPK pathways with NMCCs. (A) Runx2+/− calvarial explants with rNell-1 stimulation displayed intense calcein fluorescent labeling at the peripheral edges of parietal bones (P), coronal sutures (cS), and sagittal sutures (sS), indicating active mineralization. (B) Coronal section displaying fluorescent incorporation in a sagittal suture (sS) corresponding to the red line in panel A. rNell-1 induces significant mineralization in sagittal suture mesenchyme and osteogenic fronts of parietal bone (yellow arrows). (C) Coronal section displaying fluorescent incorporation in a coronal suture corresponding to the yellow line in panel A. Nell-1 induces marked new bone formation with resulting fusion of the overlapping frontal and parietal bone plates at the coronal suture (yellow arrow). (D) Runx2+/− calvarial explants without rNell-1 stimulation revealed reduced mineralization, as represented by green fluorescent staining throughout the entire calvarial vault. (E) Coronal section displaying fluorescent incorporation in a sagittal suture corresponding to the red line in panel D. Reduced sutural and osteogenic front mineralization were observed. (F) Coronal section displaying fluorescent incorporation in a sagittal suture corresponding to the yellow line in panel D. A well-separated gap between the overlapping frontal and parietal bones demonstrating parietal suture patency (yellow arrow). (G) Skeletal staining of Runx2−/− calvarial explants (with brain attached) with Nell-1 or (H) without rNell-1 did not show any alizarin red staining except for hyoid bone (Hb). Scale bars: 500 μm for A and D; 50 μm for B, C, E, and F; 1 mm for G and H. (I) Quantitative measurement of mineralization intensity using ImageProPlus on whole explanted calvaria (wEx) and the defined sagittal suture (sS) and coronal suture (cS) regions of the samples with rNell-1 treatment over vehicle control, respectively (Fig. 4I). Taken together, these ex vivo studies clearly demonstrated that Nell-1 was sufficient to promote mineralization and bone formation (eg, suture fusion) in Runx2−/− mice containing committed preosteoblast cells but insufficient to promote mineralization in Runx2+/− mice containing undifferentiated mesenchymal cells. These data indicate that
part, serine residues on Runx2 that results in increased Runx2 activity.

Nell-1 knockdown partially blocked Runx2-induced osteoblastic differentiation of NMCCs

Having established that Nell-1 is sufficient to promote mineralization and bone formation (eg, suture fusion) in partially deficient Runx2 states (eg, Runx2\(^{+/−}\)), the next step was to determine whether normal Runx2-induced osteoblastic differentiation (eg, Runx2\(^{+/+}\)) requires Nell-1. Wild-type (Runx2\(^{+/+}\)) NMCCs express endogenous Nell-1 at relatively low levels, but Nell-1 expression is rapidly induced on Runx2 stimulation. To establish whether Nell-1 blockade will inhibit Runx2-mediated osteogenic differentiation, wild-type NMCCs were pretransfected with Nell-1 siRNA (siRNA\(^{Nell-1}\)) or unrelated negative control siRNA 24 hours prior to AdRunx2 infection and compared with AdRunx2 transduction alone and no treatment controls.

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![Graphs and images](image-url)

Fig. 5. Nell-1 is critical for Runx2-induced osteoblastic differentiation in NMCCs. (A) Real-time RT-PCR Nell-1 expression (top bar graph) 48 hours after siRNA\(^{Nell-1}\) and unrelated negative control siRNA transfection and/or 24 hours after AdRunx2 transduction. AdRunx2 significantly increased Nell-1 transcripts in calvarial cells up to 9.5-fold, whereas siRNA\(^{Nell-1}\) transfection decreased the AdRunx2-mediated Nell-1 response by approximately 3-fold. No-treatment control revealed low basal Nell-1 expression. In agreement with the real-time RT-PCR Nell-1 expression data, siRNA\(^{Nell-1}\) transfection also blocked upregulation of Nell-1 protein by AdRunx2 72 hours after transduction (bottom image of Western blot). (B) Real-time RT-PCR (top bar graph) of relative Nell-1, Runx2, Ocn, and Alp levels 48 hours after siRNA transfection and/or 24 hours after AdRunx2 transduction. siRNA\(^{Nell-1}\) transfection significantly abrogated AdRunx2-associated upregulation of Nell-1, Ocn, and Ocn but not Alp (\(p < .05; **p < .01\)). Von Kossa staining (bottom images) of NMCCs on day 18 showed that siRNA\(^{Nell-1}\) transfection significantly inhibited mineralization of AdRunx2-transduced calvarial cells (whole-plate image at left and higher-magnification images at right of each panel). (C) AdRunx2 transduction promoted mineralization (ARS = alizarin red staining) in wild-type calvarial cells but not in Nell-1\(^{+/−}\) calvarial cells from ENU-induced Nell-1-deficient mice (whole plate image at left and higher-magnification image at bottom of each panel). (D) rNell-1 significantly enhanced Runx2 phosphorylation (WB: p-Ser) (top image, first two lanes). All three MAPK inhibitors partially blocked Nell-1-associated Runx2 phosphorylation and mineralization (ARS) in Runx2\(^{+/−}\) NMCCs (lower bar graph). \(p < .05\); NS = no significance.
(Fig. 5A, B). Real-time RT-PCR and Western blot analyses confirmed that only siRNA<sup>Nell-1</sup> significantly blocked Runx2-induced production of Nell-1 transcripts and protein. Quantitatively, Nell-1 mRNA was 9.5-fold higher in NMCs transduced with AdRunx2 compared with control NMCCs. Up to 70% of the upregulated Nell-1 mRNA was significantly knocked down using siRNA<sup>Nell-1</sup> treatment (Fig. 5A). Functionally, siRNA<sup>Nell-1</sup> treatment significantly decreased Opn and Ocn expression by 80% and 50%, respectively, without significant effects on Alp or Runx2 transcription (p > 0.05; Fig. 5B, upper panel). Meanwhile, NMCCs transduced with AdRunx2 revealed more prominent and thicker mineralized clusters, as detected by von Kossa staining, whereas siRNA<sup>Nell-1</sup> addition substantially inhibited mineralization in the AdRunx2-transduced NMCCs (Fig. 5B, lower panel). Consistent with the Alp mRNA data in Fig. 5B, Alp activity of NMCCs on days 3, 6, and 9 after transfection with Nell-1 siRNA with or without AdRunx2 revealed only minor alterations on days 6 and 9 (data not shown).

Notably, the transduction of AdRunx2 enhanced mineralization [as assessed by alizarin red staining (ARS)] on wild-type (ie, Nell-1<sup>+/−</sup>) calvarial cells but not on Nell-1<sup>−/−</sup> calvarial cells derived from ENU-induced Nell-1-deficient mouse (Fig. 5C), indicating that Nell-1 is required for Runx2’s function on terminal osteoblast differentiation and mineralization. In addition, Nell-1 significantly increased phosphorylated Runx2 (WB: p-Ser) in Runx2<sup>+/−</sup> and Runx2<sup>+/+</sup> NMCCs. Meanwhile, all three MAPK inhibitors (JNK, p38, and ERK) partially blocked Runx2 phosphorylation and decreased Nell-1-induced mineralization (Fig. 5D). However, the inhibitors of JNK (SP600125) and ERK (PD90589) also decreased cell proliferation (Supplemental Fig. S1E) and increased apoptosis when added alone (Supplemental Fig. S2) in NMCCs. Thus nonspecific toxic effects from the inhibitors also may have contributed to the observed suppression of Nell-1-induced mineralization.

Collectively, these data show that Nell-1 knockdown by siRNA<sup>Nell-1</sup> or intrinsic Nell-1 deficiency (Nell-1<sup>−/−</sup> from ENU-induced mutation) functionally compromises Runx2-induced Opn and Ocn expression and ECM mineralization—confirming that Nell-1 is a significant functional mediator of downstream Runx2 activity. In addition, the enhanced Runx2 phosphorylation through activation of MAPK pathways by Nell-1 is one of the mechanisms underlying the functional relationship between Runx2 and Nell-1.

Nell-1 partially restored osteoblastic differentiation in Runx2 null NMCCs

Although Nell-1 overexpression did not restore bone formation in the Runx2<sup>−/−</sup>/CMV-Nell-1 mice and rNell-1 did not mineralize Runx2<sup>−/−</sup> calvarial explants, it is of interest to determine whether Nell-1 can induce osteogenic differentiation in the absence of Runx2—because Nell-1 expression was not completely absent in Runx2-deficient animals (Fig. 2A, F–H). Osteoblastic marker gene expression, Alp activity, and protein were examined in Runx2<sup>−/−</sup> NMCCs transduced with AdNell-1 and AdLacZ. Surprisingly, Alp activity was significantly increased in AdNell-1-transduced Runx2<sup>−/−</sup> NMCCs on day 12 (Fig. 6A, B), and Alp transcripts were increased over 20-fold on day 9 (Fig. 6C). Notably, Opn, a slightly later marker of osteoblastic differentiation, also was increased fourfold on day 9, whereas Ocn remained unchanged (Fig. 6C). These data are distinctly different from those in Runx2<sup>+/−</sup> animals, where Opn, Ocn, but not Alp, were significantly upregulated with in vivo Nell-1 compensation (Fig. 3F–H). Overexpression of Nell-1, however, was insufficient to induce mineralization in Runx2<sup>−/−</sup> NMCCs even after 27 days of culture, whereas Nell-1-transduced Runx2<sup>−/−</sup> NMCCs were highly mineralized (Fig. 6D). Taken together, these results show that Nell-1 overexpression in Runx2<sup>−/−</sup> NMCCs can induce a certain level of osteogenic differentiation independent of Runx2 (as evidenced by initial Alp and then Opn expression), but Nell-1 alone is insufficient to promote terminal osteoblastic differentiation (ie, mineralization) in Runx2<sup>−/−</sup> NMCCs cells. This accounts for the inability of Nell-1 to rescue mineralized bone formation in Runx2<sup>−/−</sup> mice.

**Discussion**

Commitment of undifferentiated mesenchymal stem cells to an osteoprogenitor lineage is first marked by expression of Runx2<sup>1,2</sup>. Furthermore, most described osteinductive factors appear to converge mechanistically at Runx2 as well. Examples include bone morphogenetic proteins (BMP) 2 and 7, insulin-like growth factor (IGF-1), and transforming growth factor (TGF) β1, which are known to functionally modulate Runx2<sup>1,28–30</sup>. Meanwhile, fibroblast growth factor (FGF) 2 may increase Runx2 activity through a mitogen-activated protein kinase (MAPK) pathway, whereas parathyroid hormone (PTH) may increase Runx2 activity through protein kinase A and C (PKA and PKC) pathways.<sup>27,31</sup> In addition, Javed and colleagues have demonstrated recently that RUNX2 is a critical molecular end point for execution and completion of TGF-β/BMP signaling in osteoblasts.<sup>32</sup> Overall, Runx2 is essential for osteoblast formation and function because it is expressed by all osteoblasts irrespective of embryonic origin or mode of ossification.<sup>33</sup> However, the exact mechanism by which Runx2 exerts its activity on osteoblasts and chondrocytes is largely unknown. Except for osterix (Osx), another transcription factor that is absolutely required for osteoblast formation,<sup>1,13,33</sup> attempts thus far to identify critical downstream functional mediators of Runx2 in mineralization and bone formation have not been successful. We previously established that the human NELL-1 gene is directly regulated by Runx2<sup>13</sup> and that Nell-1-deficient mice exhibit severe axial and appendicular skeletal anomalies<sup>10</sup>—indicating that Runx2-regulated Nell-1 is required for normal skeletogenesis. We show here that Nell-1 is a critical downstream Runx2 functional mediator.

In this study, we verified on multiple levels that mechanistically, Runx2 exerts many of its effects on osteoblasts through Nell-1. Specifically, (1) temporally and spatially, Nell-1 expression correlated closely with endogenous Runx2 expression in calvarial and long bone tissues in wild-type and Runx2-deficient mice, (2) cross-mating CMV-Nell-1 mice with Runx2<sup>−/−</sup> mice partially rescued the CCD-like calvarial defects phenotype, (3) rNell-1 protein added to mineralization-defective Runx2<sup>−/−</sup> calvarial explants induced mineralization and bone formation at sagittal
Nell-1 protein increases ERK1/2 and JNK1 phosphorylation, which is followed by increased Runx2 phosphorylation and activity in a dose-dependent manner, (5) Runx2-mediated osteoblastic differentiation and mineralization was significantly reduced by transfection of Nell-1 siRNA to Runx2+/+ NMCCs and in ENU-mutated Nell-1+/−/− NMCCs, and (6) AdNell-1 partially rescued osteoblastic gene expression but not mineralization in newborn calvarial cells from Runx2+/−/− mice. Collectively, these data demonstrate that Nell-1 is a critical downstream Runx2 functional mediator insofar as Runx2-regulated Nell-1 promotes osteoblastic differentiation through, in part, activation of MAPK and enhanced phosphorylation of Runx2 and that Runx2 activity is significantly reduced when Nell-1 is blocked or absent.

In our previous report, NELL-1 localized to more mature osteoblasts at the osteogenic front in fusing and newly fused sutures from UCS patients. This study confirmed a close correlation between levels of endogenous Runx2 and Nell-1 expression during development at the tissue level (eg, lower Nell-1 in long bone versus calvaria) and genotype levels (eg, higher Nell-1 in Runx2+/−/− versus Runx2+/+), which suggests that the multifunctional OSE2 sites on the Nell-1 promoter described previously are highly relevant to controlling Nell-1 transcription in vivo. The CCD-like calvarial defect phenotype of Nell-1-deficient mice further indicates that Nell-1 has a critical role in the Runx2 osteoblastic differentiation pathway.

The importance of Nell-1 as a downstream mediator of Runx2 activity was clearly demonstrated when siRNA inhibition of Nell-1 significantly reduced Runx2-induced mineralization of Runx2+/+/− NMCCs as well as Runx2-induced upregulation of Opn and Ocn but not necessarily Alp. In addition, a significant reduction in Runx2-induced mineralization also was observed in Nell-1-deficient NMCCs. These data are consistent with Nell-1 being an important downstream Runx2 mediator that preferentially

**Fig. 6.** Nell-1 affects Runx2+/− NMCC differentiation. (A) Alp histochemistry on Runx2+/− NMCCs transduced with AdNell-1 or AdLacZ. Note increased Alp staining on day 9 after AdNell-1 transduction (whole-plate image at left and higher-magnification images at right of each panel). Scale bar: 50 μm. (B) Relative quantitation of Alp activity in Runx2+/− NMCCs transduced with AdNell-1 (red) and AdLacZ (green) or no-treatment (blue) control on days 3, 6, 9, and 12. *p < .05. (C) Graph displaying real-time RT-PCR results for Nell-1, Alp, Opn, and Ocn expression in Runx2+/− NMCCs transduced with AdLacZ or with AdNell-1 on days 3, 6, and 9. **p < .01. (D) Mineralization (ARS) of Runx2+/− and wild-type NMCCs transduced with AdNell-1 or AdLacZ on day 27 (whole-plate image at top and higher-magnification image at bottom of each panel).
promotes late rather than early osteoblastic differentiation in the presence of Runx2. The inability of Nell-1 to induce bone formation in Runx2<sup>−/−</sup> NMCCs is of interest. In particular, BMP-2 treatment of Runx2 null cells also failed to induce complete osteogenic differentiation. Komori and colleagues were able to induce increased Alp activity and low-level Ocn expression in Runx2<sup>−/−</sup> calvarial cells at pharmacologic dosages of rhBMP-2 above 300 ng/ml; however, they noted that those cells did not form bone and were not considered mature osteoblasts. Similarly, long-term culture of E17.5 Runx2<sup>−/−</sup> mouse calvarial cells in osteogenic medium or with BMP-2 also failed to induce terminal osteoblastic differentiation. Meanwhile, we demonstrated that Runx2<sup>−/−</sup> NMCCs infected with adenoaviral Nell-1 displayed increased Alp transcripts and activity and, to a lesser degree, Ocn transcripts during osteoblastic differentiation in vitro. Increased Alp or Ocn expression demonstrates that Runx2<sup>−/−</sup> NMCCs are responsive to Nell-1 stimulation and that Nell-1 can induce a certain degree of osteoblastic differentiation in those cells through Runx2-independent pathways; Runx2, however, is still absolutely required for complete osteoblastic differentiation.

Mechanistically, our previous data indicated that Nell-1 induces osteoblastic differentiation in rat and human osteoblasts by activating MAPK pathways as well as promoting Runx2 phosphorylation. In this study, we evaluated the responsiveness of MAPK pathways in NMCCs with different Runx2 genotypes (+/+ , +/− , and −/−) and the changes in Runx2 phosphorylation/transactivating activity on rhNell-1 stimulation. Surprisingly, Runx2<sup>−/−</sup> NMCCs respond to Nell-1 stimulation as rapidly as Runx2<sup>+/+</sup> and Runx2<sup>+/−</sup> NMCCs do with respect to ERK and JNK pathway activation. However, the intensity and sustainability of ERK1/2 and JNK1 activation in Runx2<sup>−/−</sup> NMCCs were severely reduced. In addition, Runx2 phosphorylation and transactivating activity were enhanced on Nell-1 stimulation in Runx2<sup>+/+</sup> and Runx2<sup>+/−</sup> NMCCs. In contrast, Runx2<sup>−/−</sup> NMCCs exhibited increased Runx2 phosphorylation and transactivating activity by Nell-1 stimulation only after introduction of exogenous Runx2 by transfection. Meanwhile, although rhNell-1 stimulation activated ERK1/2 and JNK1 but not p38, the inhibition of ERK, JNK, or p38 separately reduced Runx2 phosphorylation and mineralization in Nell-1-treated Runx2<sup>+/−</sup> NMCCs. These data suggest that Nell-1 promotes osteoblastic differentiation partially through MAPK pathways that cross-talk with Runx2 (eg, Nell-1 increases Runx2 phosphorylation and transactivating activity) and pathways that do not necessarily cross-talk with Runx2 (eg, Nell-1 increases osteoblastic markers Alp and Ocn expression in Runx2<sup>−/−</sup> NMCCs).

Runx2 phosphorylation is thought to be crucial for its activity. We postulate a mutually dependent mechanism between Runx2 and Nell-1 based on the fact that Runx2 directly regulates Nell-1 transcription and Nell-1 significantly modulates Runx2 activity during osteoblastic differentiation. Therefore, Nell-1 can induce osteoblastic differentiation by at least one of two mechanisms: (1) enhancing overall Runx2 phosphorylation and activity (Runx2-dependent) and (2) other effects not related to modulating Runx2 activity (Runx2-independent). Conspicuously, ENU-induced Nell-1-deficient mice, while exhibiting similar CCD-like calvarial phenotypes as Runx2<sup>−/−</sup> mice, also display rib cage vertebral abnormalities not described in Runx2<sup>+/−</sup> mice. This, coupled with our current data demonstrating Nell-1 induction of Alp and Ocn expression in Runx2<sup>−/−</sup> NMCCs, indicates that a subset of Nell-1 effects is not necessarily related to Runx2 modulation.

Although Runx2 may exert some of its effects mechanistically through Nell-1 and vice versa, the exact mechanism by which Nell-1 can promote osteoblast differentiation through effects not related to modulating Runx2 activity is unclear. Structurally, NELL-1’s N-terminal domains include a laminin G-like domain (previously known as an N-terminal thrombospondin 1 (TSP-1)-like module) that likely interacts with heparan sulfate proteoglycans and integrin-related molecules. The EGF-like domains of rat Nell-1 are phosphorylated by protein kinase C (PKC) β1, but it is unknown at this point whether Nell-1 phosphorylation increases or decreases its activity and whether binding to PKC changes Nell-1’s intracellular distribution or function or its secretion into the extracellular matrix. In addition, MAPK may not be the only pathway by which Nell-1 promotes Runx2 phosphorylation. Given the fact that Nell-1 contains TSP-N and EGF-like repeat domains, other pathways for promoting Runx2 phosphorylation may involve Nell-1–integrin interactions involving focal adhesion kinase (FAK) and PKC or calcium-binding–mediated kinase activation.

NELL-1 was originally found as a local factor with upregulation at fusing and fused sutures from UCS patients. Subsequently, we discovered that NELL-1 transcription is tightly regulated by Runx2, a key mechanistic convergence point for CS development. Interestingly, while syndromic and nonsyndromic CS differ in extracraniofacial presentation and in the pattern and degree of suture involvement, the histomorphometric phenotype at the level of the pathologic closed/closing suture is virtually indistinguishable. This implies that even widely disparate regulatory factors such as fibroblast growth factor receptors (FGFRs) 1, 2, and 4 and Twist causing distinctly different CS syndromes nonetheless may converge mechanistically at the level of the calvaria to affect suture fusion. In fact, altered FGFRs and Twist activity largely associate with downstream modulation of Runx2 expression and/or activity—making Runx2 a potentially key molecule of mechanistic convergence for CS. Overall, Runx2’s activities are etiopathologically involved in many congenital craniofacial anomalies, including CCD, in humans caused by Runx2 mutations.

The identification of Nell-1, a secretory molecule, as a key component of the Runx2-mediated bone-formation network opens up exciting possibilities for future NELL-1-blocking therapies to treat CS or other conditions involving undesirable bone formation (eg, heterotopic ossification). On the other hand, use of NELL-1 as an osteoinductive molecule may have even wider applications than anti-NELL-1 therapeutics. Thus far we have shown comparable Nell-1–versus BMP-induced bone regeneration in multiple animal models from rat palatal distraction, calvarial defect, and spinal fusion to sheep spinal fusion. More notably, Nell-1, by virtue of being transcriptionally “downstream” of Runx2, is a more highly selective osteoinductive molecule in vivo than BMP-2 and is capable of inducing high-quality bone regeneration from BMSCs. The development of more osteoinductive growth
factors with divergent but complementary bone-formation pathways can serve to maximize biologic efficiency—which, in turn, may improve clinical efficacy, lower dose requirements (and costs), and minimize potential adverse effects of current osteoinductive therapeutics.

Disclosures

CS, KT, and XZ are the inventors of Nell-1-related patents filed from UCLA. CTC is an inventor of Nell-1-related patents filed from ORNL. All the other authors state that they have no conflicts of interest.

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