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SHORT COMMUNICATION

Neural EGFL-Like 1 Is a Downstream Regulator of Runt-Related Transcription Factor 2 in Chondrogenic Differentiation and Maturation

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Recent studies indicate that neural EGFL-like 1 (Nell-1), a secretive extracellular matrix molecule, is involved in chondrogenic differentiation. Herein, we demonstrated that Nell-1 serves as a key downstream target of runt-related transcription factor 2 (Runx2), a central regulator of chondrogenesis. Unlike in osteoblast lineage cells where Nell-1 and Runx2 demonstrate mutual regulation, further studies in chondrocytes revealed that Runx2 tightly regulates the expression of Nell-1; however, Nell-1 does not alter the expression of Runx2. More important, Nell-1 administration partially restored Runx2 deficiency–induced impairment of chondrocyte differentiation and maturation *in vitro*, *ex vivo*, and *in vivo*. Mechanistically, although the expression of Nell-1 is highly reliant on Runx2, the prochondrogenic function of Nell-1 persisted in *Runx2*$^{-/-}$ scenarios. The biopotency of Nell-1 is independent of the nuclear import and DNA binding functions of Runx2 during chondrogenesis. Nell-1 is a key functional mediator of chondrogenesis, thus opening up new possibilities for the application of Nell-1 in cartilage regeneration. *(Am J Pathol 2017, 187: 963–972; http://dx.doi.org/10.1016/j.ajpath.2016.12.026)*

Chondrogenesis is an obligatory step for skeletogenesis, which results in the construction of the primary skeleton of the vertebrate embryo.[1] Chondrogenesis begins as the mesenchymal cells migrate and tightly pack at the presumptive skeletogenic sites to form cell mass condensations.[1–3] Next, the condensed cells undergoing earlier chondrogenic differentiation proliferate and produce cartilage extracellular matrix, such as collagen II and aggrecan (alias cartilage-specific proteoglycan core protein or chondroitin sulfate proteoglycan 1).[1–4] For long bone development, the proliferating chondrocytes go through prehypertrophy, hypertrophy, and terminal maturation with matrix-mineralization that serves as a template for the subsequent deposition of bone matrix.[1–4] A great number of molecules regulate chondrogenic differentiation, an essential process for bone and cartilage formation.[1–11] In particular, SRY-Box 9 and runt-related transcription factor 2 (Runx2; alias core-binding factor subunit α1 or Cbfa1) are two master transcriptional factors for chondrogenesis initiation and hypertrophic maturation, respectively.[1–4,7–9]

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Disclosures: K.T., X.Z., and C.S. are inventors of Nell-1–related patents. C.T.C. is an inventor of Nell-1–related patents filed from ORNL. K.T., X.Z., and C.S. are founders and/or board members of Bone Biologics Inc./Bone Biologics Corp. that sublicenses Nell-1 patents from the UC Regents, which also holds equity in the company. C.T.C. is a founder of NellOne Therapeutics, Inc., which licensed Nell-1–related patent applications from ORNL.

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Our previous study showed that neural EGFL-like 1 (Nell-1), an extracellular matrix molecule distributed in human uncalcified articular cartilage,[12] enhances chondrogenic marker expression and cartilage nodule formation of rabbit chondrocytes.[13] In addition, application of Nell-1 induces hyaline cartilage regeneration, as demonstrated in a rabbit knee subchondral defect model,[14] and administration of *Nell-1*−overexpressed bone marrow mesenchymal stem cells promotes articular cartilage reestablishment in critical-sized goat mandibular condyle osteochondral defects.[15] Developmentally, in comparison with wild-type (WT) littermates, newborn *Nell-1* overexpression transgenic mice exhibit premature hypertrophy and apoptosis in the chondrocranium region,[16] whereas neonatal *Nell-1*−deficient mice have shorter and deformed rib cages and vertebral bodies with compressed intervertebral spaces accompanied with reduced cartilage extracellular matrix.[17] Moreover, *Nell-1* deficiency also results in reduced expression of multiple cartilage-related genes.[17] Taken together, these findings indicate that Nell-1 may also play a role in chondrogenic differentiation and maturation. To better understand the potential regulatory roles of Nell-1 in chondrogenesis, the current study focuses on $Runx2^{-/-}$ mice to eliminate the pivotal contribution of Runx2.

Materials and Methods

Animal Maintenance and Skeletal Analysis

Mice were bred and maintained, as previously described,[7,17–19] under institutional approval by the Chancellor's Animal Research Committee at UCLA (protocol number 2012-041). *Runx2* heterozygous deficient mice ($Runx2^{+/-}$; generated by embryonic stem cells derived from the 129 mouse strain backcrossed with the C57BL/6 strain[7]) were mated with *Nell-1*−overexpressing mice (*CMV-Nell-1*; derivative of the B6C3 strain[19]) to obtain $Runx2^{-/-}$/*CMV-Nell-1* mice.[18] Because severe reduction of Nell-1 expression in homozygotes [$Nell\text{-}1^{6R/6R}$; $Nell\text{-}1^{6R}$: an *N*-ethyl-*N*-nitrosourea−induced point mutation that truncates an 810 amino acid Nell-1 protein at residue 502[20,21]] induces neonatal death,[17] $Nell\text{-}1^{6R}$ heterozygous mice ($Nell\text{-}1^{+/6R}$) were used to produce $Nell\text{-}1^{6R/6R}$ fetuses. Mouse genotypes were determined by PCR, and mRNA expression levels of Nell-1 and Runx2 were monitored using quantitative real-time PCR.

The animals in this study were euthanized with an overdose of phenobarbital (Piramal Healthcare, Maharashtra, India). After euthanization, three neonatal $Runx2^{-/-}$/*CMV-Nell-1* mice and five $Runx2^{-/-}$ littermates were skinned, dissected, and fixed in 95% ethanol for 16 hours before standard skeletal staining with Alcian Blue and Alizarin Red to provide gross distinction between cartilage and mineralized tissues. For paraffin embedding, hind limbs isolated from mouse embryos at different stages and from neonatal mice were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) at 4°C overnight. For *Runx2*-deficient mice and *N*-ethyl-*N*-nitrosourea−induced *Nell-1*−deficient mice, embryo pairs from six litters were used for each utero stage. Four neonatal $Runx2^{-/-}$/*CMV-Nell-1* mice and three $Runx2^{-/-}$ littermates were also used for histological analysis. Hematoxylin and eosin staining was performed on paraffin sections (5 μm thick) for histological analysis, whereas Safranin O staining, Alcian Blue staining, and immunohistochemical and immunofluorescence staining were performed following standard protocols.[13,14] Primary antibodies against collagen II (Developmental Studies Hybdridoma Bank, Iowa City, IA), collagen X (Developmental Studies Hybdridoma Bank), Nell-1 (Allele Biotechnology, San Diego, CA), osteocalcin (Abcam, Cambridge, UK), and Runx2 (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunohistochemical and/or immunofluorescence staining, respectively. DAPI (Sigma-Aldrich) was used for nuclear counterstaining in immunofluorescence staining.

Mouse Primary Chondrocyte Isolation and Cultivation

After removing soft tissues by 2 mg/mL protease (Roche, Nutley, NJ) and 3 mg/mL collagenase II (Roche), the rib cages of neonatal mouse embryos were digested in 1 mg/mL collagenase II for 3 hours to achieve single-cell suspension. Chondrocytes were cultured in a basal culture medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin). Medium was changed every 3 days and cells were passaged at 70% to 90% confluence. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

A total of 5×10^4 cells/well passage 2 (P2) chondrocytes were seeded in 6-well plates with basal culture medium for 6 hours. Recombinant human Nell-1 protein was synthesized by Aragen Bioscience Inc. (Morgan Hill, CA) with a purity of 92%. Before treatment, cells were synchronized by culture in starvation medium [Dulbecco's modified Eagle's medium + 1% insulin−transferrin-sodium selenite media supplement (BD Biosciences, San Jose, CA)] for 18 hours.

For adenoviral infection, P2 mouse chondrocytes at 80% confluence were infected with Ad*LacZ*, Ad*Nell-1*, or Ad*Runx2* at a multiplicity of infection of 50, 250, or 500 plaque-forming units per cell, as described previously.[22]

Mouse Primary Mesenchymal Progenitor Cell Isolation and Cultivation

Primary embryonic mesenchymal progenitor cells were isolated from limb buds of E11.5 embryonic mice, as previously described.[23] Briefly, limbs were dissected from the embryos and then digested with 1 mg/mL Dispase (Roche) for 1.5 hours at 37°C. Cells were filtered through a prewashed 40-μm cell strainer to generate single-cell suspension. After wash with Puck's Saline A solution, cells were suspended in culture medium [Dulbecco's modified

Eagle's medium:F12 = 2:3 (Invitrogen) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 mmol/L glutamine, 50 μg/mL vitamin C (Sigma-Aldrich), 10 nmol/L β-glycerophosphate (Sigma-Aldrich)] with or without 2 μg/mL recombinant human Nell-1 protein, and were plated at the density of 2×10^5 cells per tube, centrifuged at 500 × *g* for 5 minutes, then incubated at 37°C in a 5% CO_2 humidified incubator. Medium was changed every 48 hours. Pellets were cultured at 7 or 21 days for further analysis.

Mouse Limb Bud Isolation and Explant Cultivation

Mouse hind limbs were isolated from embryonic day (E)14.5 mouse embryos. Isolated limbs were cultured in BGJb medium (Invitrogen) for 24 hours on polycarbonate tissue culture inserts (pore size, 0.1 μm; EMD Millipore, Billerica, MA). For the treatment, α-modified Eagle's medium containing 5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.5 mmol/L glutamine (Invitrogen) with or without 2 μg/mL recombinant human Nell-1 protein was changed every 48 hours for 5 days to simulate the environment in neonatal mice (term, 19.5 days). Limb explants were fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, and divided into sections (5 μm thick).

Proliferation Assay

5-Bromo-2′-deoxyuridine (Invitrogen) was added 6 hours before sample collection, and pellets were fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, and divided into sections (5 μm thick). DAPI was used for nuclear counterstaining.

Quantitative Real-Time PCR

Total RNA was isolated by TRIzol Reagent (Invitrogen) followed by DNase (Invitrogen) treatment. RNA (1 μg) was injected for reverse transcription (RT) with the SuperScript II Reverse Transcriptase Kit (Invitrogen), as per the manufacturer's instructions. Real-time PCR was performed on the 7300 Real-Time PCR system with SYBR Green Mastermix (Invitrogen). The primer pair sequences were as follows: Acan, 5′-CCAGGCTCCACCAGATACTC-3′ (forward) and 5′-TGCTCATAGCCTGCCTCATA-3′ (reverse); Adamts4, 5′-ATGGCCTCAATCCATCCCAG-3′ (forward) and 5′-AAGCAGGGTTGGAATCTTTGC-3′ (reverse); Col2α1, 5′-GTCCTGAAGGTGCTCAAGGT-3′ (forward) and 5′-TTTGGCTCCAGGAATACCAT-3′ (reverse); Mmp13, 5′-TGTTTGCAGAGCACTACTTGAA-3′ (forward) and 5′-CAGTCACCTCTAAGCCAAAGAAA-3′ (reverse); Nell-1, 5′-TCCTGGGTAGATGGTGACAA-3′ (forward) and 5′-CATTGGCCAGAAATATGCAC-3′ (reverse); Runx2, 5′-AACGATCTGAGATTTGTGGGC-3′ (forward) and 5′-CCTGCGTGGGATTTCTTGGTT-3′ (reverse); and SRY-Box 9, 5′-ACGGCTCCAGCAAGAACAAG-3′ (forward) and 5′-TTGTGCAGATGCGGGTACTG-3′ (reverse). Concomitant glyceraldehyde 3-phosphate dehydrogenase was also evaluated in separate tubes for each RT reaction as a housekeeping standard [5′-ATTCAACGGCACAGTCAAGG-3′ (forward) and 5′-GATGTTAGTGGGGTCTCGCTC-3′ (reverse)]. Relative gene expression was analyzed by the $\Delta\Delta C_T$ method.[24]

Image Processing

Images were acquired at room temperature with the CellSens Standard 1.9 software (Olympus, America Inc., Center Valley, PA) on a microscope (Olympus) using 4× (dry HC Plan Apochromat; numerical aperture, 0.13), 10× (dry HC Plan Apochromat; numerical aperture, 0.30), and 20× (dry HC Plan Apochromat; numerical aperture, 0.17) objective lenses. Images were processed in Photoshop CS2 and Illustrator CS4 (Adobe Systems Computer Software Company, San Jose, CA) for merging.

Statistical Analysis

Statistical analysis was performed by OriginPro 8 (Origin Lab Corp., Northampton, MA) and included the one-way analysis of variance and two-sample *t*-test. The Mann-Whitney test was used for nonparametric data. Statistical significance was determined at the $P < 0.05$ level.

Results

Nell-1 and Runx2 Exhibit Similar Spatiotemporal Expression Patterns during Late Limb Development of Mouse Femurs

To reveal the spatiotemporal expression pattern of Nell-1 and Runx2 during long bone development, the hind limbs of fetal and neonatal WT (*Runx2*$^{+/+}$) and *Runx2*$^{-/-}$ mice were harvested for immunohistochemical examination. In WT animals, from E16.5 onward, both Nell-1 and Runx2 staining were observed in the superficial osteoblasts of the newly formed trabeculae (Figure 1A). Runx2 and Nell-1 were also coexpressed in the perichondrium and prehypertrophic chondrocytes of the WT E14.5 femurs (Figure 1A). Beginning at E16.5, Runx2 was preferentially expressed in hypertrophic chondrocytes of WT mice, where intense Nell-1 expression was also observed (Figure 1A and Supplemental Figure S1). This spatiotemporal overlap in endogenous Nell-1 and Runx2 expression at different chondrogenic differentiation zones during long bone development suggested a possible regulatory relationship between Runx2 and Nell-1 in chondrogenesis, at least during the late stages of chondrogenic differentiation.

Unlike WT mice with strong Nell-1 staining in the resting, proliferating, and prehypertrophic chondrocytes of femurs from E16.5 to newborn (Figure 1A), *Runx2*$^{-/-}$ mice barely exhibited Nell-1 staining throughout the whole

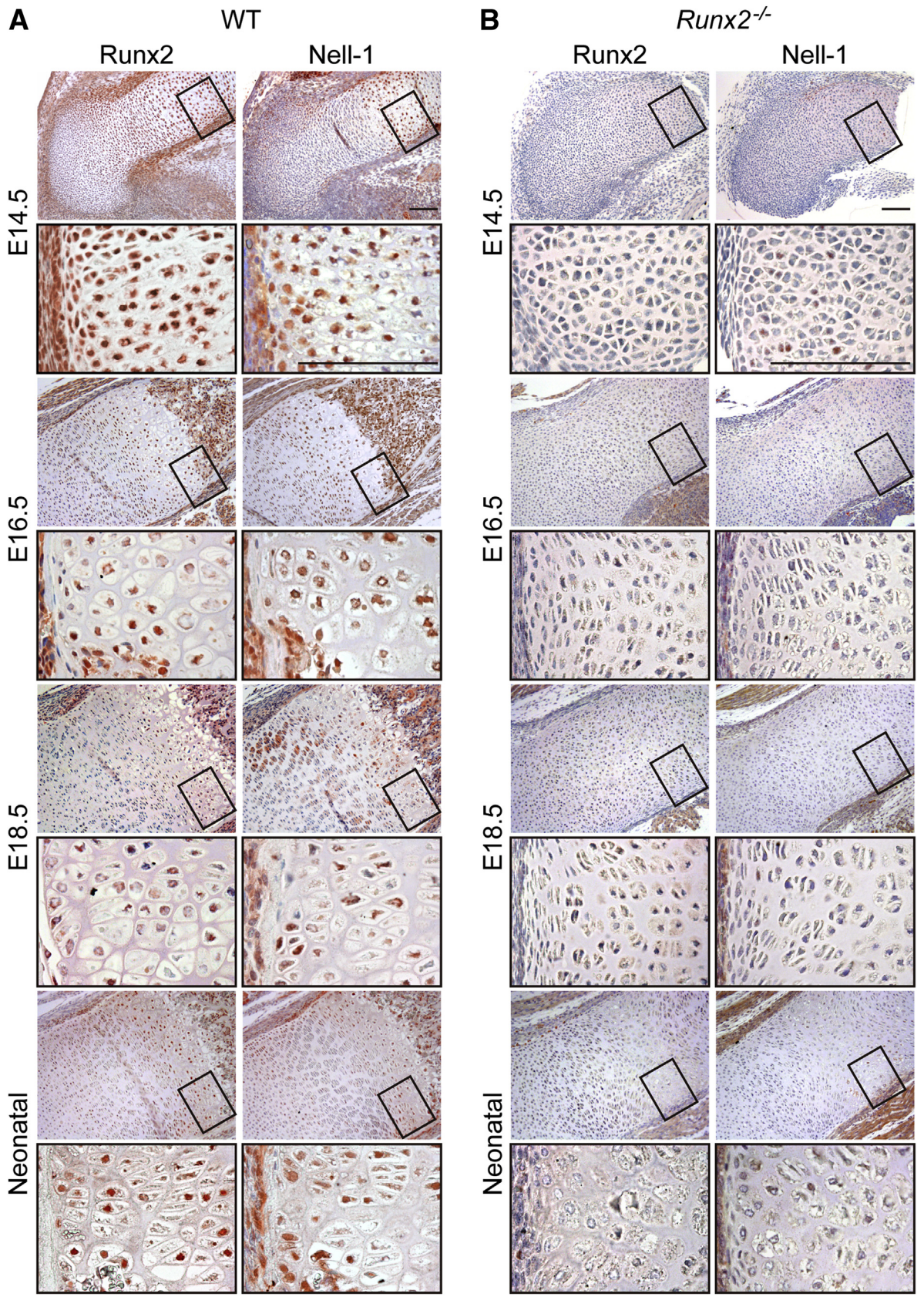

Figure 1 Spatiotemporal expression of Nell-1 and Runx2 in the femurs of mouse embryos during chondrogenesis. Nell-1 and Runx2 expression in the femoral bone of WT (**A**) and *Runx2*$^{-/-}$ (**B**) embryos at embryonic day (E)14.5, E16.5, E18.5, and neonatal stage as examined by immunohistochemical staining. **Boxed areas** are shown below at higher magnification. Scale bars = 100 μm (**A** and **B**).

femurs during the entire examining period, accompanied with impaired endochondral ossification (Figure 1B and Supplemental Figure S1). Therefore, at least during late limb development, Nell-1 expression in chondrocytes is highly reliant on Runx2.

Nell-1 Is a Downstream Target of Runx2 in Mouse Chondrocytes

To examine the regulatory relationship between Nell-1 and Runx2 in chondrocytes, we next evaluated the expression of Runx2 mRNA and Nell-1 mRNA in chondrocytes of neonatal mice with different genotypes. The expression level of Nell-1 mRNA in *Runx2*$^{-/-}$ mouse chondrocytes was only half of that in WT control (Figure 2A). In addition, forcing *Runx2* overexpression in WT mouse chondrocytes by adenovirus Ad*Runx2* significantly stimulated Nell-1 mRNA expression in a viral dose-dependent manner (Figure 2B). In contrast, expression of Runx2 mRNA was retained at similar levels in mouse chondrocytes with different *Nell-1* genotypes (Figure 2C). Moreover, neither Nell-1 protein nor Ad*Nell-1* adenovirus had a significant effect on Runx2 mRNA expression in the *Nell-1*$^{+/+}$ chondrocytes (Figure 2, D and E). These results clearly demonstrated that, in chondrocytes, Runx2 regulated Nell-1 expression but that Nell-1 did not alter Runx2 expression.

Nell-1 Plays Critical Roles in Chondrogenic Differentiation and Maturation

After 7 days of chondrogenic differentiation, *in vitro* pellet cultures of primary mesenchymal progenitor cells isolated from *Runx2*$^{-/-}$ mouse embryonic limb buds exhibited a larger undifferentiated area with less intense Alcian Blue and Safranin O staining in cartilaginous components in comparison to the pellet controls formed by cells isolated from WT littermates (Figure 3A). Interestingly, administration of Nell-1 protein not only promoted cell proliferation in *Runx2*$^{-/-}$ pellets (Supplemental Figure S2), but also increased cartilaginous nodule formation in the *Runx2*$^{-/-}$ pellets, which contained rich cartilage matrix characterized by high staining intensity of Alcian Blue and Safranin O (Figure 3A). Transcriptionally, expression of genes encoding chondrogenic markers, such as Col2α1 (encoding collagen II, α 1, an abundant and specific protein in cartilage), Acan (encoding aggrecan core protein, one of the major structural components in cartilage matrix), and SRY-Box 9,[1–4] in *Runx2*$^{-/-}$ pellets was significantly lower than that in WT pellets (Figure 3B). However, disruption of chondrogenic differentiation in *Runx2*$^{-/-}$ pellets was rescued by Nell-1 protein administration (Figure 3, A and B).

Meanwhile, after 21 days of cultivation, *Runx2*$^{-/-}$ pellets exhibited significantly decreased expression of terminal chondrogenic differentiation markers [Adamts4 (encoding aggrecanase-1)[25] and Mmp13 (encoding matrix metallopeptidase 13)[1–4]] when compared to WT pellets (Figure 3C). Mature chondrogenic hypertrophy was limited in *Runx2*$^{-/-}$ pellets with reduced proteoglycan deposition (Figure 3D). In addition, matrix mineralization observed in WT pellets after 21 days of cultivation was also absent in *Runx2*$^{-/-}$ pellets, as characterized by the lack of osteogenic marker osteocalcin[1,26] (Figure 3D). However, Nell-1 administration also restored chondrogenic maturation and ossification in *Runx2*$^{-/-}$ pellets (Figure 3, C and D).

Nell-1 Partially Restores Delayed Chondrocyte Hypertrophy in *Runx2*$^{-/-}$ Mouse Hind Limbs

To avoid systemic influences and initial differentiation stage differences,[27] we took advantage of an established *ex vivo* approach[28] to further evaluate the influence of Nell-1 on chondrocyte/cartilage maturation. Not only was collagen II observed, but also collagen X, a marker for chondrogenic maturity and hypertrophy,[1–4] in the hypertrophic zone of E14.5 WT mouse limb explants after 5 days of cultivation (Supplemental Figure S3). In contrast, absence of chondrocyte hypertrophy was observed in the age-matched *Runx2*$^{-/-}$ explants, as characterized by negligible collagen X expression but excessive collagen II expression (Supplemental Figure S3). Administration of Nell-1 protein partially reestablished collagen X expression and reduced collagen II level in *Runx2*$^{-/-}$ explants (Supplemental Figure S3), indicating the restoration of the hypertrophic chondrocyte phenotype and marker expression pattern of WT explants.

Furthermore, only Alcian Blue staining was observed throughout the whole femur of neonatal *Runx2*$^{-/-}$ mice (Figure 4A), which reflected the diminished endochondral calcification due to the *Runx2* deficiency. Histological examination further confirmed the lack of chondrogenic maturation by the complete absence of the hypertrophic zone, the disorganized chondrocyte architecture without an apparent proliferating or prehypertrophic zone, and the lack of collagen X expression in the neonatal *Runx2*$^{-/-}$ mouse femurs (Figure 4, B and C). By crossing *Nell-1*–overexpressing transgenic mice with *Runx2*$^{+/-}$ mice,[18] we obtained *Runx2*$^{-/-}$*/CMV-Nell-1* mice that overexpress *Nell-1* in a *Runx2*$^{-/-}$ background. Overexpression of *Nell-1* was unable to keep the neonatal *Runx2*$^{-/-}$ mice alive, as we previously described,[18] suggesting that Nell-1 cannot completely overcome the global influence of *Runx2* deficiency on fetal mouse development. However, the midshaft of the neonatal *Runx2*$^{-/-}$*/CMV-Nell-1* mouse femurs exhibited obvious signs of chondrocyte hypertrophy and calcification characterized by positive staining with collagen X and Alizarin Red (Figure 4).

Discussion

Nell-1 was originally identified as a craniosynostosis-associated molecule from resected human fusing and fused

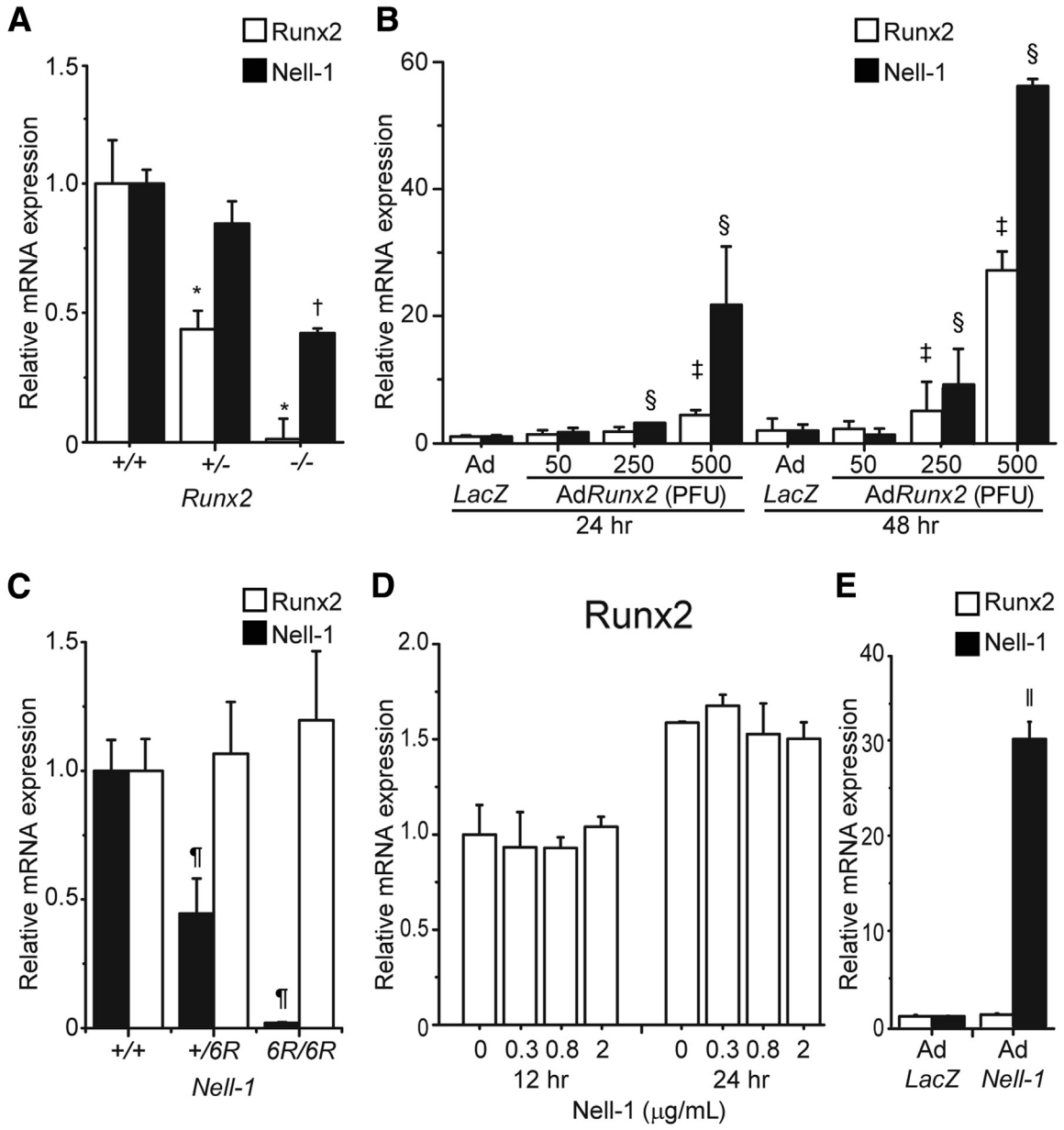

Figure 2 Nell-1 is a downstream target of Runx2 in mouse chondrocytes. **A:** mRNA levels of Nell-1 in mouse chondrocytes with different genotypes of *Runx2*. **B:** Transcriptional levels of Nell-1 and Runx2 in Ad*Runx2*-transduced *Runx2*$^{+/+}$ mouse chondrocytes. **C:** mRNA levels of Runx2 in mouse chondrocytes with different genotypes of *Nell-1*. **D:** Transcriptional levels of Runx2 in Nell-1 protein stimulated *Nell-1*$^{+/+}$ mouse chondrocytes. **E:** Transcriptional levels of Nell-1 and Runx2 in Ad*Nell-1*—transduced *Nell-1*$^{+/+}$ chondrocytes. Two sample *t*-tests were used to compare the data from two groups. Data are expressed as means + SD of three independent experiments performed in triplicate (**A**—**E**). *$P < 0.05$ versus Runx2 mRNA expression in *Runx2*$^{+/+}$ cells; †$P < 0.05$ versus Nell-1 mRNA expression in *Runx2*$^{+/+}$ cells; ‡$P < 0.05$ versus Runx2 mRNA expression in Ad*Lacz*-transduced chondrocytes for 24 hours; §$P < 0.05$ versus Nell-1 mRNA expression in Ad*Lacz*-transduced chondrocytes for 24 hours; ¶$P < 0.05$ versus Nell-1 expression in *Nell-1*$^{+/+}$ cells; ‖$P < 0.05$ versus Nell-1 expression in Ad*Lacz*-transduced *Nell-1*$^{+/+}$ chondrocytes for 24 hours. PFU, plaque-forming unit.

coronal sutures.[29] Functionally, *Nell-1* overexpression in mice leads to premature suture closure simulating nonsyndromic craniosynostosis,[19,22] whereas *N*-ethyl-*N*-nitrosourea—induced *Nell-1*—deficient mice exhibit a cleidocraniodysplasia-like defect.[30] Mechanistically, overexpression of the critical osteogenesis regulator, Runx2,[1,31,32] in osteoblast lineage cells results in up-regulated Nell-1 expression. *Runx2* deficiency, however, leads to significantly reduced Nell-1 expression,[33] which manifested the regulation of Runx2 on Nell-1 expression during osteogenesis. Further investigation demonstrated that, as a downstream regulator of Runx2 in osteogenesis, Nell-1 reciprocally induces the expression and activation of Runx2 in osteoblast lineage cells.[18,33] In fact, it is clear that Nell-1 exhibits its pro-osteogenic functions by activating the mitogen-activated protein kinase signaling pathway in osteoblast lineage cells.[18,33–35] The importance of the Runx2 → Nell-1 → mitogen-activated protein kinase axis in

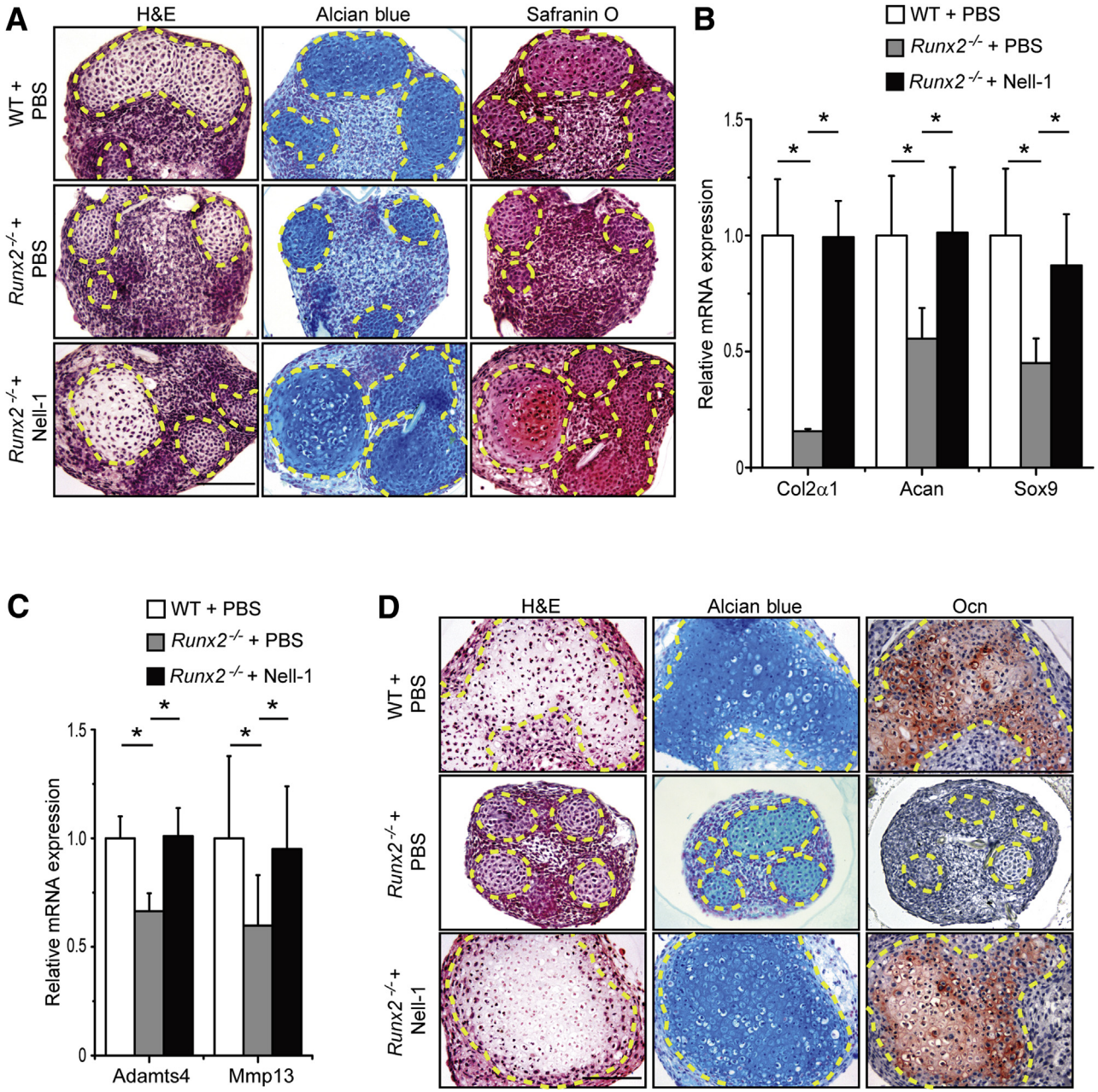

Figure 3 Exogenous Nell-1 protein promotes cartilaginous nodule formation and maturation of primary *Runx2*$^{-/-}$ mesenchymal progenitor cells. **A:** Nell-1 treatment promotes the chondrogenic differentiation of *Runx2*$^{-/-}$ cells at day 7 shown by hematoxylin and eosin (H&E), Alcian Blue, and Safranin O staining. The cartilage nodules are circled by the dotted yellow lines. **B:** Nell-1 stimulation rescues chondrogenic marker expression in *Runx2*$^{-/-}$ limb bud pellets at day 7. **C:** Nell-1 treatment promotes the terminal chondrogenic maturation markers of *Runx2*$^{-/-}$ cells at day 21 by gene expression. **D:** Nell-1 treatment promotes the chondrogenic maturation of *Runx2*$^{-/-}$ cells at day 21 shown by H&E, Alcian Blue, and osteocalcin (Ocn) staining. The cartilage nodules are circled by the **dashed yellow lines**. Two sample *t*-tests were used to compare the data from two groups. Data are expressed as means + SD (**B** and **C**). $n = 6$ (**B** and **C**). $^{*}P < 0.05$. Scale bars = 100 μm (**A** and **D**). PBS, phosphate-buffered saline; WT, wild-type.

osteogenic differentiation has been extensively studied by multiple independent research groups,[18,33,36,37] and the osteogenic activity of Nell-1 has been proven in various small and large animal models.[17,38–45]

Herein, we noticed that Nell-1 expression was devoid in neonatal mouse femurs because of the *Runx2* deficiency. Further investigation revealed that, similar to the preosteoblasts, osteoblasts, and osteosacoma cell line,[33,34] Nell-1 is a downstream target of Runx2 in chondrocytes. This similarity implies that Runx2→Nell-1 regulation may be a general characteristic shared by osteochondral lineage cells. However, unlike the mutual regulation of Nell-1 and Runx2 observed in osteoblast lineage cells,[9,13] our findings demonstrated that Nell-1 could not induce Runx2 expression in chondrocytes. This indicates a cell-specific mode of regulation between Nell-1 and Runx2.

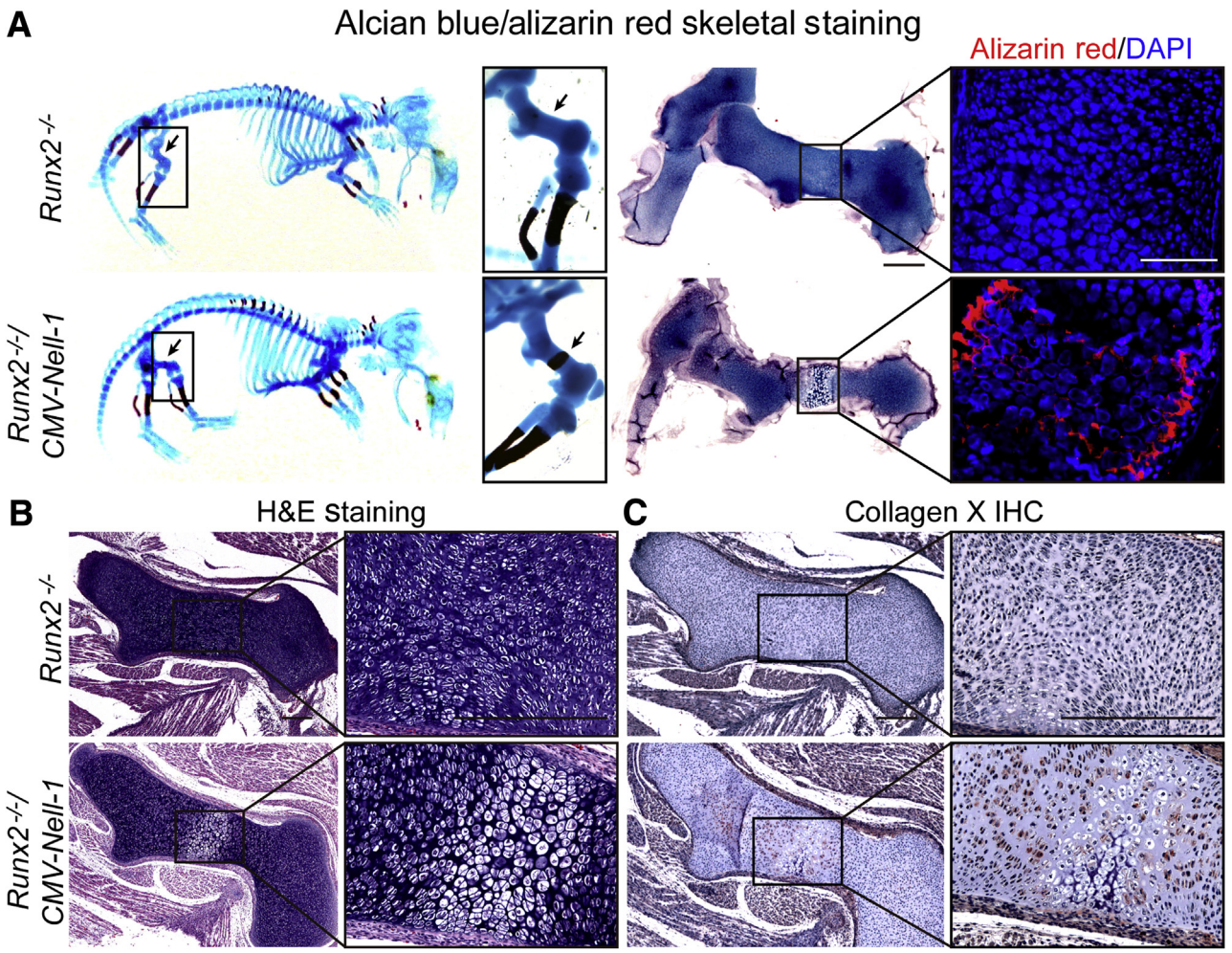

Figure 4 Partial rescue of chondrogenic differentiation in neonatal *Runx2*$^{-/-}$ by *Nell-1* overexpression. **A:** Alcian Blue/Alizarin Red skeletal staining of neonatal *Runx2*$^{-/-}$ and *Runx2*$^{-/-}$*/CMV-Nell-1* mice. The mid-shaft of the femur and lower thoracic vertebrae in neonatal *Runx2*$^{-/-}$*/CMV-Nell-1* mice clearly stain positively with Alizarin Red (**arrows**). Endochondral ossification is clearly absent in the vertebrae and proximal limbs of *Runx2*$^{-/-}$ mice. Calcified cartilage, which stains positively with Alizarin Red, is apparent in *Runx2*$^{-/-}$*/CMV-Nell-1* femurs. **B:** Hematoxylin and eosin (H&E) staining shows the cartilage morphology of *Runx2*$^{-/-}$ and *Runx2*$^{-/-}$*/CMV-Nell-1* femurs. **C:** Restoration of a distinct hypertrophic chondrocyte zone is clearly shown by immunostaining of collagen X in restricted regions of the limbs in *Runx2*$^{-/-}$*/CMV-Nell-1* mice. **Boxed areas** are shown at higher magnification to the right (**A**–**C**). Scale bars: 500 μm (**A**, **left column**); 50 μm (**A**, **right column**); 200 μm (**B** and **C**). IHC, immunohistochemistry.

Functionally, *Nell-1* deficiency results in abnormal cartilage phenotypes in the rib cage, vertebral and long bones, and chondrocranial tissues.[17,30] Nell-1 protein also exhibits prochondrogenic effects in cartilage regeneration both *in vitro*[12,13] and *in vivo*.[14,15] To better explore the possible functional role of Nell-1 in chondrogenesis from its upstream regulator, Runx2, that is a predominant transcription factor associated with chondrocyte hypertrophy differentiation,[1–4] we selected mouse subjects with *Runx2*$^{-/-}$ and *Nell-1* overexpression (*Runx2*$^{-/-}$*/CMV-Nell-1*) after crossing *Nell-1* overexpression transgenic mice and *Runx2* haploinsufficient mice. Although overexpression of *Nell-1* was unable to rescue *Runx2*$^{-/-}$ mice from perinatal lethality,[6,7] CMV-driven *Nell-1* overexpression resulted in partial rescue of the *Runx2* deficiency–induced impairments of chondrogenic differentiation and maturation. This was evidenced by mineralized cartilage in the mid-shaft of the femurs of *Runx2*$^{-/-}$*/CMV-Nell-1* mice.

Runx2 not only regulates the late stage of chondrogenic maturation, but is also involved in chondrogenic initiation.[46–48] Interestingly, although Runx2 is able to bind directly to the Indian Hedgehog (*Ihh*) promoter[9] to stimulate the Ihh-Gli pathway, the most plausible signaling pathway that induces chondrogenic differentiation,[11,48] recent studies revealed that the nuclear import and DNA binding functions of Runx2 are insufficient for chondrocyte differentiation.[31] To avoid systemic influences and initial differentiation stage differences,[27] *in vitro* and *ex vivo* approaches were applied to verify the prochondrogenic bioactivities of Nell-1 in a *Runx2*$^{-/-}$ background. In agreement with the *in vivo* results, Nell-1 protein was able to restore the chondrocyte hypertrophy of *Runx2*$^{-/-}$ limb explants and primary mesenchymal

progenitor cell pellets. In addition, we also found that Nell-1 protein enhanced primary mesenchymal progenitor cell proliferation and early chondrogenic differentiation in limb bud pellet cultivation. Taken together, in agreement with previous publications,[12,14,15,17,30] our current findings clearly demonstrated the essential roles of Nell-1 in chondrogenic commitment, differentiation, and maturation independent from Runx2. Although the expression of Nell-1 is highly dependent on Runx2, the prochondrogenic function of Nell-1 persists in *Runx2*$^{-/-}$ scenarios. This inconsistency demonstrates the complexity of the Runx2/Nell-1 regulatory network that governs chondrogenesis. Particularly, Nell-1 promotes chondrogenesis, at least partially, via an alternative pathway that is independent of the nuclear import and DNA binding functions of Runx2.

In summary, the identification of Nell-1, a secretory molecule, as a key downstream mediator of Runx2-mediated chondrogenesis opens up new possibilities for the application of Nell-1 in cartilage regeneration. This is especially important when considering the fact that cartilage regeneration continues to pose a big challenge in healing critical-sized defects.[49] Because Nell-1 has the ability to induce mesenchymal stem cell commitment to chondrogenic differentiation,[12] it could have the potential advantage to enhance stem cell–based therapies for cartilage regeneration.[14,15] In particular, we have found that the combination of Nell-1, bone morphogenetic protein-6, and transforming growth factor β-3 significantly promotes human perivascular stem cell chondrogenic differentiation while inhibiting their hypertrophic, fibrotic, osteogenic, and apoptotic differentiation.[12] Further investigation is therefore necessary not only to dissect the details on how Nell-1 orchestrates chondrogenic initiation and maturation, but also to explore the broader applications of Nell-1 as a therapeutic agent for bone and chondrogenic regeneration. The overexpression of Nell-1 was observed in multiple bone and cartilage tumors.[50,51] Moreover, our previous studies showed that the teratocarcinoma-derived chondrogenic cell line ATDC5 has distinguished responses to Nell-1 stimulation than do primary mouse chondrocytes.[52] Thus, the involvement of Nell-1 in bone and cartilage-related tumor formation also warrants further investigation.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2016.12.026*.

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