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Proliferation and Osteogenic Differentiation of Mesenchymal Stem Cells Induced by a Short Isoform of NELL-1

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Abstract

Neural epidermal growth factor-like (NEL)-like protein 1 (NELL-1) has been identified as an osteoinductive differentiation factor that promotes mesenchymal stem cell (MSC) osteogenic

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Author Contributions

S.P., J.S., and Y.L.: designed and performed experiments and wrote the manuscript; F.C., Z.Z., C.Y.H., H.Z., C.L., X.C., and H.J.: performed experiments and collected data; A.W.J., K.S.L., C.W., and X.Z.: revised manuscript; C.S. and K.T.: developed concept, designed the studies, provided overall guidance, and finalized manuscript. S.P. and J.S. contributed equally to this work. C.S. and K.T. are both co-senior authors and contributed equally to the manuscript.

Disclosure of Potential Conflicts of Interest

Drs. X. Z., K. T., and C. S. are inventors of Nell-1-related patents. Drs. X. Z., K. T., and C. S. are founders and/or board members of Bone Biologics Inc. which sublicenses NELL-1 patents from the UC Regents, which also hold equity in the company.

differentiation. In addition to full-length *NELL-1*, there are several *NELL-1*-related transcripts reported. We used rapid amplification of cDNA ends to recover potential cDNA of *NELL-1* isoforms. A NELL-1 isoform with the N-terminal 240 amino acid (aa) residues truncated was identified. While full-length NELL-1 that contains 810 aa residues (NELL-1_{810}) plays an important role in embryologic skeletal development, the N-terminal-truncated NELL-1 isoform (NELL-1_{570}) was expressed postnatally. Similar to NELL-1_{810}, NELL-1_{570} induced MSC osteogenic differentiation. In addition, NELL-1_{570} significantly stimulated MSC proliferation in multiple MSC-like populations such as murine C3H10T1/2 MSC cell line, mouse primary MSCs, and perivascular stem cells, which is a type of stem cells proposed as the perivascular origin of MSCs. In contrast, NELL-1_{810} demonstrated only limited stimulation of MSC proliferation. Similar to NELL-1_{810}, NELL-1_{570} was found to be secreted from host cells. Both NELL-1_{570} expression lentiviral vector and column-purified recombinant protein NELL-1_{570} demonstrated almost identical effects in MSC proliferation and osteogenic differentiation, suggesting that NELL-1_{570} may function as a pro-osteogenic growth factor. In vivo, NELL-1_{570} induced significant calvarial defect regeneration accompanied by increased cell proliferation. Thus, NELL-1_{570} has the potential to be used for cell-based or hormone-based therapy of bone regeneration.

Keywords

MSC proliferation; NELL-1; Short NELL-1 isoform; Osteogenesis; Secretory protein

Introduction

Mesenchymal stromal cells are populations of cells isolated from the stromal fraction of numerous tissues, including but not limited to bone marrow (BM), adipose tissue, muscle, placenta, and umbilical cord [1, 2]. Mesenchymal stromal cells contain stem cells that have clonal self-renewal and multilineage differentiation potential [3] called mesenchymal stem cells (MSCs). MSCs show potency for differentiation into osteoblasts, adipocytes, chondrocytes, and myocytes. The multipotency of MSCs indicates their important roles in overall growth and maintenance of the musculoskeletal system [1, 2]. Osteogenic differentiation of BM MSCs is postulated to be critical for bone health. MSCs in BM are a heterogeneous group of multipotent and committed progenitor cells [4]. Comitted and uncommitted MSCs together represent less than 1% of all cells present in the BM [5, 6], while osteoprogenitor stem cells represent only one in 10^4 or even less of BM nucleated cells [7, 8]. To efficiently regenerate bone tissue, in addition to inducing differentiation of MSCs into osteoblasts, first stimulating the growth of MSCs may be crucial so that a larger “stem cell reservoir” can commit to the osteoblastic lineage. Inadequate healing of bone fractures in aged patients is commonly seen, which is speculated to be due to a lack of sufficient MSCs for bone formation [9, 10].

Several growth factors have been used to stimulate MSC proliferation and osteogenic differentiation, most notably bone morphogenetic proteins (BMPs), which belong to the transforming growth factor-β superfamily [11–13]. However, BMPs may also induce other lineage differentiation including MSC adipogenesis [14, 15]. Our studies have demonstrated

that neural epidermal growth factor-like (NEL)-like protein 1 (NELL-1) is highly specific to the osteochondral lineage and induces MSC osteogenic differentiation both in vitro and in vivo [16]. NELL-1 regulates Runt-related transcription factor 2 (RUNX2) by enhancing RUNX2 phosphorylation [17]. Activation of RUNX2 in turn triggers many downstream genes involved in bone regeneration including the gene that transcribes NELL-1, which can stimulate MSCs to differentiate into osteoblast progenitor cells [18, 19]. Sequence analysis demonstrated that NELL-1 contains several highly conserved structural motifs, including a secretory signal peptide, an NH_2-terminal thrombospondin-1-like module (also known as laminin globular domain [LamG]), four chordin-like cysteine-rich domains (or WF domains), and six epidermal growth factor (EGF)-like domains [16].

Although full-length NELL-1 induces differentiation of MSCs into osteoblasts, the protein alone does not significantly stimulate MSC proliferation [20]. According to the Genbank database, more than 10 *NELL-1*-related transcripts with truncation of exon 1 have been identified (*NELL-1* and NELL-1 indicate human gene and protein, respectively; *Nell-1* and Nell-1 indicate animal gene and protein, respectively). The expression patterns suggest the possibility that NELL-1 has different splicing to regulate cell fate, such as differentiation and/or proliferation. We first investigated whether any of these transcripts could generate open reading frames (ORFs). Subsequently, if some of these transcripts could generate ORFs, functional studies of the proteins derived from these *NELL-1* transcripts were performed such as determining whether deletion of exon 1, which is related to the LamG domain of NELL-1, could affect the function of NELL-1 in either MSC differentiation or proliferation. We first used rapid amplification of cDNA ends (RACE) to obtain *NELL-1*-related transcripts. Subsequently, we used in silico analysis to identify the potentially functional transcripts. We found one transcript that can produce a peptide of 570 aa residues. In both cell culture and animal models, we found that this NELL-1 isoform of 570 aa residues not only induces MSC osteogenic differentiation but also stimulates MSC proliferation.

Materials and Methods

Animals

Sprague-Dawley rats, purchased from Charles River Laboratories (Wilmington, MA, http://www.criver.com/), were housed in light- and temperature-controlled facilities as previous described [21]. Three-millimeter-diameter trephine defects were created unilaterally in the calvaria of 3-month-old male animals under constant irrigation, with care to avoid injury to the underlying dura. Each defect was flushed with saline solution to remove bone debris. Subsequently, scaffolds (see below) containing lentiviral vectors carrying NELL-1 gene isoforms or the green florescent protein (GFP) control gene were implanted. All animals and surgical procedures were approved and handled in accordance with the guidelines of the Chancellor's Animal Research Committee of the Office for Protection of Research Subjects at the University of California, Los Angeles (UCLA).

Cells

Murine C3H10T1/2 (cat. CCL-226) and human Saos-2 (cat. HTB-85) cell lines were obtained from (American Type Culture Collection, Manassas, VA, http://www.atcc.org/) and maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Invitrogen, Carslbad, CA, http://www.invitrogen.com). Newborn mouse calvarial cells (NMCCs) were freshly isolated from mice, as described previously [22]. 293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS. Human BM MSCs were purchased from ScienCell (Carlsbad, CA, http://www.sciencellonline.com/) and maintained in RPMI 1640 medium with 10% FBS for less than five passages. Human perivascular stem cells (PSCs) were obtained from the UCLA Medical Center without identification information under federal and state regulations with the approval of UCLA's Institutional Review Board.

Lentiviral Vectors

The coding sequences of NELL-1_{810} and NELL-1_{570} were obtained from our previously isolated NELL-1_{810} clone, as described [23], using polymerase chain reaction (PCR). The ends with appropriate restriction sites were subsequently generated using PCR. The resulting gene fragments were inserted into the pFG12 plasmid [24] to substitute for the *GFP* gene. These inserts were controlled by the ubiquitin C promoter. The *BMP2* gene was obtained from an adenoviral BMP2 vector [25] by PCR cloning, which is similar to previously described *NELL-1* gene cloning [20]. The *GFP* fused genes of *NELL-1* isoforms and *BMP2* were constructed using a similar approach of inserting the *GFP* gene at the 3′ terminus of these genes. Appropriate PCR primers were designed to remove the termination codons of *NELL-*1_{810}, *NELL-*1_{570}, and *BMP2*, so that the translation of the respective genes could be extended to the *GFP* coding region. These generated transduction plasmids were used to cotransfect 293T cells with plasmids containing the lentiviral packaging proteins and envelope proteins, as described previously [24]. Viruses from 293T cell cultures were collected and concentrated by ultracentrifugation at 17,000 rpm for 60 minutes at 4°C, using the SW32 rotor of a Beckman centrifuge Beckman Coulter (Brea, CA, http://en.wikipedia.org/wiki/Beckman_Coulter). The lentiviral GFP vector, FG12, was titrated by transduction of 293T cells with limited dilutions to determine the tissue culture infective dose of 50% chance ($TCID_{50}$) and by measuring the viral p24 protein contents in preparations. Titers of other viral vectors with the p24 content in viral preparations were used to determine titers, with FG12 as the reference. Generally, 1 pg of p24 content in a viral preparation was equivalent to 5–20 $TCID_{50}$ units.

Generation of NELL-1_{570} Protein

The NELL-1_{570} cDNA coding region was inserted into the plasmid vector pSecTag2 from Invitrogen (Invitrogen, Carlsbad, CA, www.invitrogen.com). The obtained plasmid, pSecTag2-$NELL_{570}$, was then used to transfect CHO-K1 cells. The NELL-1_{570} expression cells were selected out using 400 μg/ml Zeocin in F-12 medium with 10% FBS. The cell culture medium that contained NELL-1_{570} was collected and purified by affinity columns and verified by Western blot. The selected NELL-1_{570}-expressing CHO-K1 cells were colony-purified to optimize the yield of NELL-1_{570}.

Transfections and Transductions

Transfection of 293T cells to generate lentiviral vectors was performed by calcium precipitation using a kit purchased from Promega (Madison, WI, http://www.promega.com/). Transfection of other cells, including NMCC and Saos-2, was accomplished using Lipofectamine from Invitrogen (Invitrogen, Carslbad, CA, http://www.invitrogen.com) according to the manufacturer's instructions. Lentiviral transduction was performed by addition of lentiviral vectors to cell cultures at a multiplicity of infection (MOI) of 2:1 for human cells and 10:1 for murine cells. Three hours prior to transduction, the medium was changed to either serum-free or specific medium (depending on the requirements of the particular experiments). Immediately before transduction, the culture medium was replaced with serum-free medium. Three hours post-transduction, the cell cultures were washed to remove virus, and fresh medium was added.

Reverse Transcription-PCR, Western blot, and Bone Mineralization Staining

RNA was isolated using the Invitrogen TRIzol reagent, and the isolated RNA samples were reverse-transcribed using oligo-dT primer and reverse transcriptase from Qiagen (Valencia, CA, http://www.qiagen.com/) according to the protocols provided. The primers for NELL-1_{570} were 5′-GCTTCATGACTCCTGGCTGC and 3′-GCTAACTGACAGTGCAACC, and the resulting PCR fragment was 344 bp long. The primers for NELL-1_{810} were 5′-TGACCTTCAGATGGATATCG and 3′-GCTAACTGACAGTGCAACC (same as the 3′ primer for NELL-1_{570}), and the PCR product was 394 bp long. Quantitative reverse transcription-PCR (RT-PCR) primers for osteocalcin (OCN), osteopontin, alkaline phosphatase, Runx2, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Applied Biosystems (Foster City, CA, http://www.appliedbiosystems.com/) and analyzed by real-time PCR, as previously described [22]. RT-PCR primers for the hairy/enhancer-of-split related to the YRPW motif protein 1 (HEY1) mRNA were 5′-GACGAGAATGGAAACTTGAG and 3′-TCAATGATGCTCAGATAACG; for peroxisome proliferator-activated receptor gamma (PPARγ) mRNA were 5′-TCCTTCCCGCTGACCAAAGC and 3′-ATCTTCTCCCATCATTAAGG; and for β-actin mRNA were TCGACAACGGCTCCGGCATG and GTCTCAAACATGATCTGGGT. The antibody to detect both NELL-1 isoforms was purchased from ABCAM (cat. AB55548; Cambridge, MA, http://www.abcam.com/). Mineralization staining by Alizarin Red S was performed as described [26].

Oil Red O Staining

For oil red O staining of cultured cells, monolayers were rinsed with phosphate buffered saline, fixed with 10% formalin for 1 hour at room temperature, and stained with 0.3% oil red O solution in 60% isopropyl alcohol/40% H_2O for 15 minutes. Adipocyte number was quantified by counting oil red O-positive cells in four wells per experimental condition. The results were reported from 16 random fields at ×200 magnification.

Rapid Amplification of cDNA Ends

The *SMART RACE* kit was purchased from Clontech Laboratories, Inc. (Mountain View, CA; cat. 634914, http://www.clontech.com/) and used to amplify the ends of the cDNA of the NELL-1-related transcripts according to the manufacturer's instructions.

Scaffold Fabrication

Disc-shaped 85/15 scaffolds (thickness, 500 μm; diameter, 3 mm) were fabricated from poly (lactic-*co*-glycolic acid) (PLGA) (inherent viscosity of 0.64 dl/g in chloroform; Durect, Co., AL) by solvent casting and a particulate leaching process, as previously described [22]. Briefly, the porogen (sucrose 200–300 μm diameter) and PLGA/chloroform solution were packed into a Teflon mold to achieve 92% porosity (volume fraction). After porogen leaching and ethanol sterilization, all scaffolds were dried under a laminar flow hood. Prior to implantation into mice, lentiviral vectors of GFP (control), NELL-1_{810}, and NELL-1_{570} were diluted in 0.025% type I collagen solution (Sigma, St. Louis, MO, http://www.sigmaaldrich.com/united-states.html) and incorporated into the PLGA scaffold for 60 minutes.

Radiographic Imaging and MicroCT Scanning

Radiographic imaging and microCT scanning were performed as described previously [27].

Statistical Analysis

The results were graphically depicted as the mean ± SEM. Statistical significance was computed using ANOVA (13.0 for Windows, SPSS, Chicago, IL). Independent-sample *t* test was used to compare results of two groups. *p* value <.05 was considered statistically significant.

Results

Identification of the Short Isoform of NELL-1

Using the BLASTN program to search Genbank, more than 50 *NELL-1*-related sequences were obtained. Among them, some had a deletion of exon 1 (i.e., BC096100, AK127805, BC069674, D83017, AL535053, BI822086, AU121129, CD622061, DA172639, and DA134334). To obtain potential NELL-1 isoforms with different N-terminals, we used the SMART RACE method to clone the 5′ end of the *NELL-1*-related transcripts and recover them from the human Saos-2 cell line. We chose this cell line because it is known to express NELL-1 [28]. The first round of PCR revealed four bands (Fig. 1A), which we cloned and sequenced. Based on the sequences of these PCR clones, new PCR primers were designed and used to obtain the full-length cDNA of NELL-1 isoforms. In addition to the previously identified full-length *NELL-1* [16, 23], a new *NELL-1* isoform with 2,746 nucleotides was identified (submitted to GenBank ACC# JX565026), which was derived from the 0.9-kb SMART RACE PCR band (Fig. 1A). Further studies with sequence analysis confirmed the results. The 1.15-kb PCR band was consistent with full-length *NELL-1* (Fig. 1A), and the other two bands of 0.4 and 1.35 kb were derived from transcripts not related to *NELL-1*. The cDNA of a 2,746-bp *NELL-1* isoform encodes a peptide of 570 aa residues (Fig. 1B). We

compared the two *NELL-1* isoforms and found that the new isoform contains a different exon 1 and a long 5′ untranslated sequence. Based on the human short NELL-1 isoform mRNA sequence, we found that the exon 1 of the short NELL-1 isoform is located within the intron between exons 2 and 3 of the long NELL-1 isoform and we expected that the promoter for NELL-1_{570} is located upstream of this location [29] (Fig. 1B). The new *NELL-1* isoform cDNA was 264 nucleotides (nt) shorter on the 5′ end than the full-length *NELL-1* cDNA, and the peptide sequence of the new isoform was 240 aa shorter than that of the reported full-length NELL-1. Importantly, truncation of the N-terminal 240 aa completely deleted the LamG domain [16]. To distinguish the N-terminal-truncated NELL-1 from the full-length form, we termed the new isoform NELL-1_{570} and the full-length isoform NELL-1_{810}.

Differential Nell-1 Isoform Expression in Newborn Mice

We used a mouse model to investigate whether the short isoform of Nell-1 is expressed in vivo. We isolated proteins from the heads of C57BL/6 mice and performed Western blotting. Full-length Nell-1 (130 kDa), known to be highly expressed in brain tissues, was consistently expressed during and after gestation, as shown by Western blotting (Fig. 1C). Although expression of the short isoform (105 kDa) was not detected in the samples from embryonic mice, expression levels gradually increased postnatally (Fig. 1C). The molecular weight of this short Nell-1 isoform was approximately 25 kDa less than that of full-length Nell-1 as expected, which suggests that this isoform is expressed naturally. In later studies (Fig. 5A), we found that human NELL-1_{570} is 105 kDa, suggesting that the 105 kDa Nell-1 is the mouse counterpart of human NELL-1_{570}. Further studies of the expressions of these two isoforms in calvarial bone plates of mice were also performed using immunohistochemical staining (IHC) of E18.5, newborn (P1), and 6-day postnatal (P6) mice (Supporting Information Fig. S1). NELL-1_{810} appears to localize along the calvarial bone plates and NELL-1_{570} distributes on osteogenic fronts as well as in the suture mesenchyme.

NELL-1_{570} Stimulation of MSC Proliferation

To efficiently express NELL-1_{570}, we cloned the coding region of the short isoform using a lentiviral vector. The ubiquitin C promoter was inserted at the 5′ terminus of the *NELL-1_{570}* gene. To evaluate the effect of NELL-1_{570} on MSC proliferation, a murine MSC line, C3H10T1/2, was transduced by the NELL-1_{570} expression lentiviral vector in serum-free medium. Elimination of serum from growth medium minimized the potential effects of growth factors to stimulate MSC proliferation. To compare levels of cell proliferation, we used a BMP2-expressing lentiviral vector and a NELL-1_{810}-expressing lentiviral vector as controls, due to the positive effects of BMP2 on MSC proliferation [30] and the inductive effect of NELL-1_{810} on MSC and osteoblast differentiation [17, 20, 22, 31]. Expression of the *NELL-1_{570}* transgene showed an effect similar to that of the *BMP2* transgene for stimulating C3H10T1/2 proliferation, but *NELL-1_{810}* expression did not (Fig. 2A). When we repeated the experiments using human primary bone mesenchymal stem cells (BMSCs), significant stimulation of cell proliferation by both NELL-1_{570} and BMP2 was observed (Fig. 2B). NELL-1_{810} had no significant effect on cell proliferation. Thus far, our studies have demonstrated that NELL-1_{570} stimulates MSC proliferation, but NELL-1_{810} does not.

Molecular Characterization of NELL-1_{570}

To determine how truncation of the N-terminal 240 aa significantly alters the effect of NELL-1, we investigated the role of NELL-1 isoforms in regulating Notch signaling and expression of PPARγ. The relationship between NELL-1 and Notch was explored, as the Notch pathway is involved in cell proliferation and differentiation in the early stages of development [32]. We used RT-PCR to study the interaction between NELL-1_{570} and the key gene in the Notch pathway, *HEY1*, in C3H10T1/2 cells. We found that both NELL-1_{570} and BMP2 significantly increased HEY1 expression, but NELL-1_{810} only weakly increased it (Fig. 3A). These results correspond to the mitogenic effects of signal activation by NELL-1_{570} for Notch. We then studied the effect of the NELL-1_{570} isoform on the expression of PPARγ which is an adipogenic regulator [33]. Both NELL-1 isoforms significantly inhibited gene expression of PPARγ (Fig. 3B), suggesting that both NELL-1 isoforms are able to inhibit adipogenesis.

Since both OSX and RUNX2 are required for osteoblastogenesis [34, 35], we investigated the responsiveness of Nell-1 short isoform expression to OSX and RUNX2. We transfected plasmids carrying either *OSX* or *RUNX2* gene into NMCCs, which contained osteoblast progenitor cells, and analyzed the expression profiles of NELL-1_{570}, as well as the control, NELL-1_{810}. Similar to NELL-1_{810}, NELL-1_{570} expression could be upregulated by RUNX2 (Fig. 3C) with even higher responsiveness. However, unlike NELL-1_{810}, NELL-1_{570} expression showed positive responsiveness to OSX (Fig. 3C), suggesting that the NELL-1_{570} promoter contains different elements than the NELL-1_{810} promoter, and that the role of NELL-1_{570} may be different from that of NELL-1_{810}. The results demonstrated that NELL-1_{570}, at least in some stages of osteoblastogenesis, responds positively to both RUNX2 and OSX. Sequence analysis demonstrates that putatively, there are two osteoblast-specific binding elements 2 (ACCACA) 2.5 kb upstream from the *NELL-1_{570}* transcriptional initiation site (Fig. 3D). AP2, SP1, E2F, AGP/EBP, and MAPF1 motifs were also found in this region (Fig. 3D).

Promotion of Osteogenic Differentiation and Inhibition of Adipogenesis by NELL-1_{570}

The responsiveness of NELL-1_{570} expression to RUNX2 and OSX suggests that NELL-1_{570} can promote MSC osteoblastogenesis. We transduced the murine MSC cell line, C3H10T1/2, with *NELL-1_{570}* and control genes. Similar to NELL-1_{810}, NELL-1_{570} showed a significant effect of promoting osteogenesis and mineralization as well as inducing expression of key genes including *Opn* and *Alp*, as also indicated by the Alizarin red staining assay for bone nodule formation (Fig. 4A). NELL-1_{570} inhibition of PPARγ expression significantly reduced adipogenesis in NELL-1_{570}-transduced C3H10T1/2 cells (Fig. 4B). We also found that osteogenesis promoted by NELL-1_{570} may involve the increase of Runx2 expression (Fig. 4C).

NELL-1_{570} as a Secretory Protein

NELL-1_{810} is known to be a secretory protein [31]. To study whether NELL-1_{570} is also a secretory protein, we transfected 293T cells with the plasmid vector containing NELL-1_{570} cDNA. Two days post-transfection, both the culture medium and the transfected cells were collected. A NELL-1_{570} protein of 105 kDa was detected in both the culture medium and the

cell lysates, suggesting that NELL-1_{570} was secreted from the host cells (Fig. 5A). Since no obvious signal sequence was identified in the cDNA of NELL-1_{570}, NELL-1_{570} was presumed to be secreted via unconventional secretory machinery (USM) as either interleukin 1β [36] or fibroblast growth factor 2 (FGF2) [37] and to remain in the plasma membrane before secretion [36]. To investigate whether NELL-1_{570} accumulates in plasma membrane before secretion, we attached GFP to the C-termini of both NELL-1 isoforms. We used BMP2 with GFP at the C-terminus as a control. After transfection into 293T cells, the GFP-fused NELL-1_{570} isoform was visualized on the membrane of the transfected cells (Fig. 5B), suggesting that NELL-1_{570} may use a mechanism that causes it to transiently remain in the plasma membrane before secretion, similar to FGF2 [38]. Since NELL-1_{810} also showed accumulation on the cell membrane, such USM secretory mechanism may also be used by NELL-1_{810}. To identify the sequence that is involved in the transportation of NELL-1_{570} to cell membrane, we performed deletion studies. When the first von Willebrand type C (VWC) domain was removed from the N terminus, NELL-1_{570} was unable to accumulate in the cell membrane (Fig. 5B) suggesting that this sequence is required for NELL-1_{570} secretion. The deletion of coiled-coil (CC), VWC, or EGF regions significantly abrogated the ability of NELL-1_{570} to stimulate MSC proliferation (Fig. 5C, Supporting Information Fig. S2). BMP2 also showed membrane localization in some but not a high proportion of transfected cells, while the control cells transfected with the plasmid containing only GFP showed almost no GFP specifically localized in the membrane (Fig. 5B).

NELL-1_{570} Protein Demonstrating the Same Functions as the NELL-1_{570} Gene Expression Vector

There are two potential mechanisms for NELL-1_{570} to stimulate MSC proliferation and differentiation: (a) the expression of NELL-1_{570} in cytoplasm activates genes that can stimulate MSC proliferation and/or differentiation; and (b) NELL-1_{570} protein released to the medium binds to cell surface receptors and trigger signal transductions for MSC proliferation and differentiation. To identify the mechanism by which NELL-1_{570} stimulates MSC proliferation and differentiation, we cloned the *NELL-1_{570}* gene into CHO-K1 cells. In the CHO-K1 culture, NELL-1_{570} protein released to the medium was collected and purified using affinity column. The recovered NELL-1_{570} protein was verified and quantified by Western blot.

The results demonstrated that NELL-1_{570} protein significantly stimulated the proliferation and differentiation of human and mouse BMSCs (Fig. 6A, 6B) and mouse MSC line C3H10T1/2 (Fig. 6C), indicating that NELL-1_{570} protein can interact with MSC surface receptors to initiate MSC stimulation cascade. NELL-1_{570} stimulated the expression of OCN, a protein involved in osteoblastogenesis in human BMSCs (Fig. 6D), suggesting that NELL-1_{570} protein is similar to its lentiviral vector in stimulating cell differentiation. These results propose that NELL-1_{570} can directly be used to stimulate MSC proliferation and differentiation in vivo and ex vivo.

We also used NELL-1_{570} to treat PSCs isolated from human fat tissue. PSCs have been characterized as a perivascular origin for MSCs [37]. NELL-1_{570} demonstrated similar effects in PSC proliferation (Fig. 6E) and osteogenic differentiation (Fig. 6F, 6G),

confirming that this protein functions as a growth factor in cell proliferation and differentiation of MSCs or related stem cells such as PSCs.

NELL-1_{570} Promotion of Calvarial Bone Healing

We next examined the in vivo effects of NELL-1_{810} and NELL-1_{570} on calvarial bone defect healing using a previously described critical-sized rat calvarial defect model [21]. NELL-1_{810} has shown effects on calvarial bone growth [22, 39], and a similar approach was used to assess NELL-1_{570}. Radiographic imaging data showed that both NELL-1_{810} and NELL-1_{570} induced significant defect reossification, whereas the GFP control did not (Fig. 7A). Quantitative analysis of the high-resolution microCT scans and three-dimensional reconstructions of new bone volume and surface area demonstrated that in vivo growth stimulation by NELL-1_{570} was significant, compared with the GFP control and NELL-1_{810}-treated animals (Fig. 7A). We also performed IHC of the proliferating cell nuclear antigen (PCNA) to quantify NELL-1_{570}-induced cell proliferation in vivo. Similar to in vitro studies, the number of PCNA-expressing cells was significantly higher among those treated with the NELL-1_{570} lentiviral vector (Fig. 7B), supporting the results from our cell culture studies in which NELL-1_{570} stimulated MSC proliferation. To study whether the stimulation of MSC proliferation by NELL-1_{570} induces tumor formation, we transplanted NELL-1_{570}-transduced MSCs into SCID mice by subcutaneous injection [40]. No tumor formation was observed at 12 weeks post-transplantation (Supporting Information Fig. S3). We also found that NELL-1_{570}-transduced MSCs did not cause any colony formation in soft agar assay (Supporting Information Fig. S3), suggesting that NELL-1_{570} does not induce tumorigenesis.

Discussion

NELL-1 is a protein involved in osteogenic differentiation [16, 17, 20, 31, 39], neural development and function [41, 42], B-cell development [43], as well as inflammatory bowel disease [44]. During bone growth, high expression levels of NELL-1 in relevant cells have been reported, suggesting that expression of the *NELL-1* gene is increased during osteogenesis [23].

Nell-1_{810} plays a major role in rodent embryologic skeletal development [45]. Expression of Nell-1_{570} in mice after birth suggests that this isoform is programmed to regulate organ development and homeostasis postnatally. Since NELL-1_{570} retains most of the aa sequence of NELL-1_{810}, it is not surprising that both isoforms share many features such as the capacity to stimulate differentiation of MSCs into osteoblast progenitor cells and further into osteoblasts or osteocytes. However, NELL-1_{570} also displays different biological properties compared with NELL-1_{810}. As shown in this study, NELL-1_{570} demonstrated a mitogenic effect on MSCs, but NELL-1_{810} did not.

Mechanistic studies indicated that NELL-1_{570} upregulates the expression of genes involved in the Notch pathway, whereas the full-length isoform NELL-1_{810} does not. Since the Notch pathway is involved in cell proliferation and differentiation, upregulation of Notch proteins or other proteins in that pathway should alter the proliferation profile of cells. Notch proteins are cell surface transmembrane-spanning receptors that mediate critically important cellular

functions through direct cell-to-cell contact [46, 47]. Interactions between Notch proteins and their ligands initiate a signaling cascade that governs cell fate, such as differentiation, proliferation, and apoptosis, in numerous tissue types [47–49]. In some reports, the Notch pathway was found to maintain cells in a proliferative state [50]. On the molecular level, the Notch pathway increases expression of *c-MYC* and *cyclin D1* [51, 52], two critical genes required for stem cell proliferation. Our results demonstrated that both BMP2 and NELL-1_{570} upregulate the Notch pathway in MSCs, and the higher expression levels of Notch corresponded to the increased proliferation of the cells treated with these two proteins. The low-level stimulation of Notch by NELL-1_{810} paralleled the low proliferation of cells treated with NELL-1_{810}. Although both BMP2 and NELL-1_{570} demonstrated stimulation of the Notch pathway, we expect that these two growth factors use different mechanisms because NELL-1 isoforms contain EGF-like domains that are similar to those in Notch proteins while BMP2 does not. NELL-1 isoforms may thus interact with Notch ligands more directly. NELL-1_{570} may use a mechanism similar to that of the interaction between thrombospondin 2 and Notch3 [53], since both proteins contain EGF-like domains and VWC sequences (Fig. 5C and website http://www.ebi.ac.uk/inter-pro/ISearch?query=P35442).

Truncation of the LamG domain in NELL-1_{570} (Fig. 5C) suggests that this domain is important for diversifying *NELL-1* gene functions. The LamG domain has a wide variety of roles in cell adhesion, signaling, migration, assembly, and differentiation [54–56]. For example, the LamG domain in NELL-1 is required for formation of hetero-multimers between NELL-1 and NELL-2, which regulate neural development and function [41]. Truncation of the LamG domain in NELL-1_{570} may abrogate the binding that involves the domain, allowing NELL-1_{570} to interact with molecules that do not interact with NELL-1_{810}. NELL-1_{570} induction of osteoblastogenesis suggests that the N-terminal LamG domain of NELL-1_{810} may not be essential for osteoblastogenesis. However, the shared C-terminal region of both NELL-1 isoforms that binds to integrin $\alpha 3\beta 1$ [57] may be related to osteoblastogenesis.

It is also important to note the different expression levels of these two isoforms during various stages of mouse development. In embryonic mice, expression of the short Nell-1 isoform is undetectable, while that of the long isoform predominates, suggesting that NELL-1_{810} may be required for embryonic cells to differentiate. NELL-1_{570} may function as a regulator for postnatal organ development. Overall, the expression levels of NELL-1_{570} were lower compared with those of NELL-1_{810} (Fig. 1C). Based on the immunohistochemical staining of mouse calvaria, NELL-1_{810} appears to localize along the calvarial bone plates, while NELL-1_{570} distributes on osteogenic fronts as well as in the suture mesenchyme (Supporting Information Fig. S1). Additional studies demonstrated that the turnover rate of NELL-1_{570} is quicker than that of NELL-1_{810} (Supporting Information Fig. S4). We also performed the studies to detect NELL-1_{570} in adult blood serum, but NELL-1_{570} was not found (data not shown).

In addition to the mechanistic significance of NELL-1_{570} in understanding the roles of NELL-1 in development and bone metabolism, the potential application of this isoform in clinical settings is significant. NELL-1_{570} can be easily synthesized and used for treatment;

for example, it can be injected into bone fractures or BM to stimulate proliferation of MSCs. For many aged individuals, both the quantity and quality of bone MSCs may not be sufficient for bone fracture healing or for bone regeneration to suppress osteoporosis [9, 58]. Currently, BMP2 and other BMPs are used to stimulate MSC proliferation. As mentioned previously, BMP2 is a strong growth factor for stimulating MSC proliferation and osteoblastogenesis [11], but it induces side effects such as promoting adipogenesis [14, 15]. Conversely, inhibition of PPARγ by NELL-1_{570} suggests that this isoform can block MSCs from differentiating into adipocytes [33]. Therefore, it is likely that BMP2 alone may not be an adequate treatment for some patients, and using NELL-1_{570} to stimulate MSC proliferation and induce osteoblastogenesis may be an alternative or adjunct treatment option for bone regeneration.

Summary

Our results demonstrated for the first time that the short isoform of NELL-1 (NELL-1_{570}) can stimulate MSC proliferation and osteogenic differentiation. Similar to the full-length isoform of NELL-1 (NELL-1_{810}), the short isoform is a secretary protein. We expect that this protein can be used as a growth factor to stimulate bone regeneration in treatment of osteoporosis and fracture. Since the short isoform inhibits the expression of PPARγ and thus it should also block adipogenesis of MSCs, the use of NELL-1_{570} alone or combined with BMP2 for treatment may increase the quality of the regenerated bone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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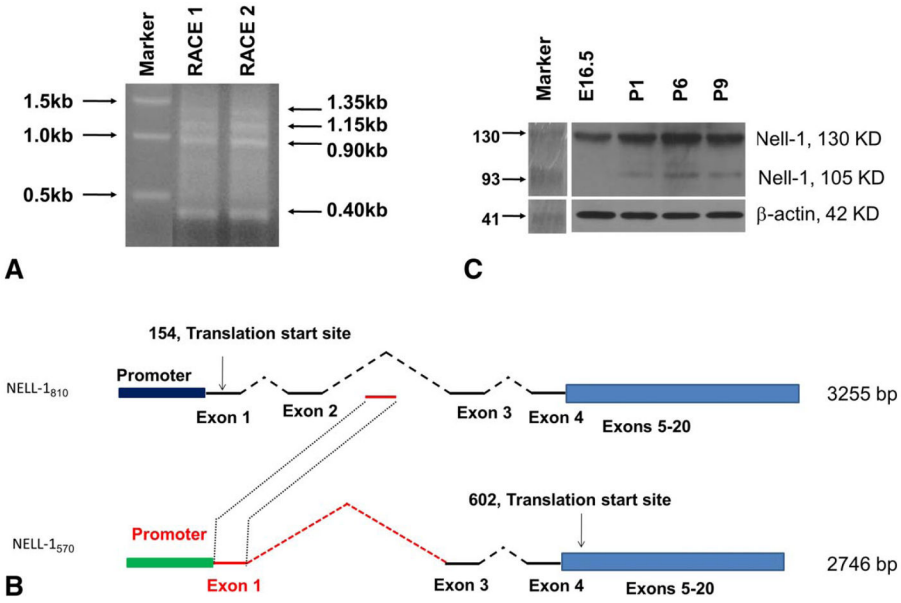

Figure 1.

Identification and isolation of NELL-1 isoforms. **(A):** 5′ RACE was used to obtain cDNA of NELL-1 isoforms. Two separate RACE experiments demonstrated similar results. **(B):** Combining the sequence data from 5′ RACE and our published NELL-1 sequence data (NELL-1_{810}), we obtained a short NELL-1 isoform cDNA (NELL-1_{570}) using PCR. Sequence data demonstrated that there is a long 5′ untranslated region. Red text indicates variations. The exon 1 of NELL-1_{570} is located in intron 2 of NELL-1_{810}. **(C):** Protein was isolated from whole mouse heads at embryonic (E16.5) and postnatal time points (P1, P6, and P9). The isolated protein samples were investigated using Western blot. Both NELL-1 isoforms were visualized using a monoclonal antibody. Abbreviations: NELL-1, NEL-like protein 1; RACE, rapid amplification of cDNA ends.

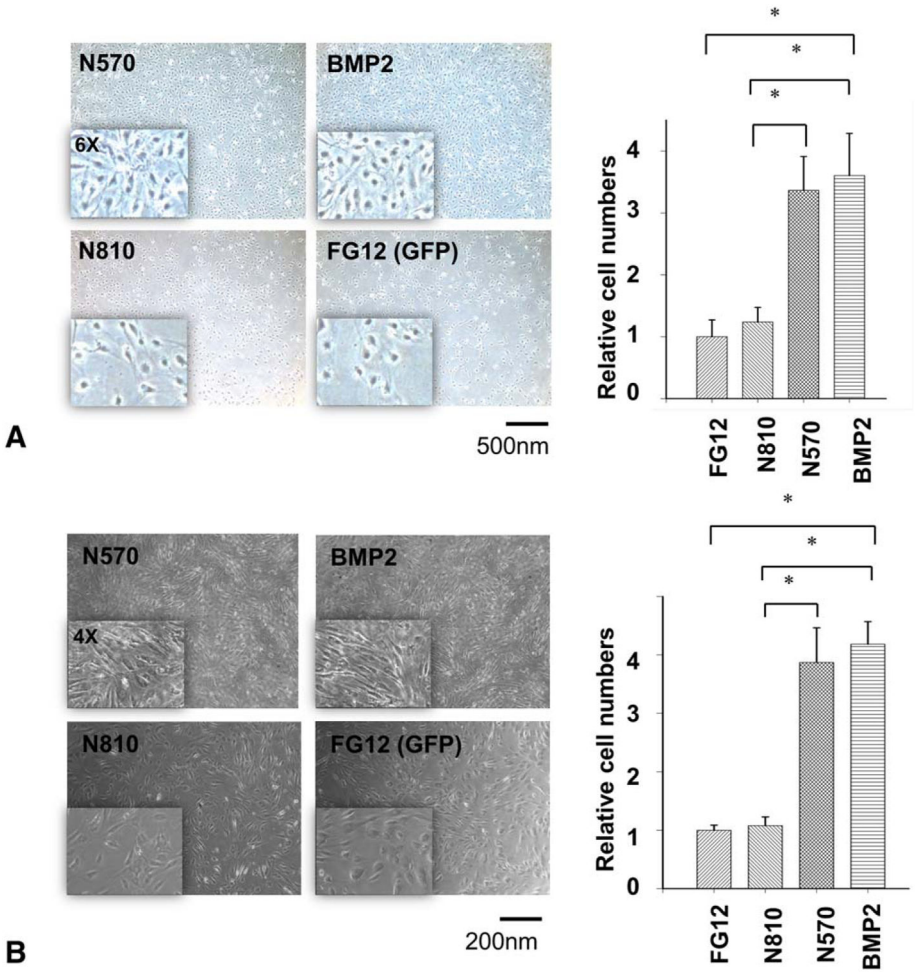

Figure 2.

Differential regulation of cell proliferation by NEL-like protein 1 (NELL-1) isoforms. Long and short NELL-1 isoforms (designated N810 and N570) as well as BMP2 cDNA were inserted into a lentiviral vector driven by the ubiquitin C promoter. A control lentiviral vector that contains the *GFP* gene under the ubiquitin C promoter (FG12) was also used. **(A):** Proliferation of C3H10T1/2 cells. Cells were plated 19 hours prior to transduction in RPMI medium with 10% FBS (multiplicity of infection [MOI] =10). Three hours prior to transduction, the culture medium was removed and serum-free RPMI medium was added to the cell cultures. The culture medium was changed prior to transduction. Photos were taken at 10 days post-transduction, with quantification of relative cell numbers presented based on determination by fluorescent-activated cell sorting. **(B):** Proliferation of human mesenchymal stem cells by NELL-1_{570}. Methods were the same as those described in (A), except that lower doses of vectors were added (MOI =2). FG12, control GFP vector; N810, long isoform of NELL-1; N570, short isoform NELL-1; and BMP2, bone morphogenetic protein 2. *, p <.05, n =4. Abbreviation: GFP, green florescent protein.

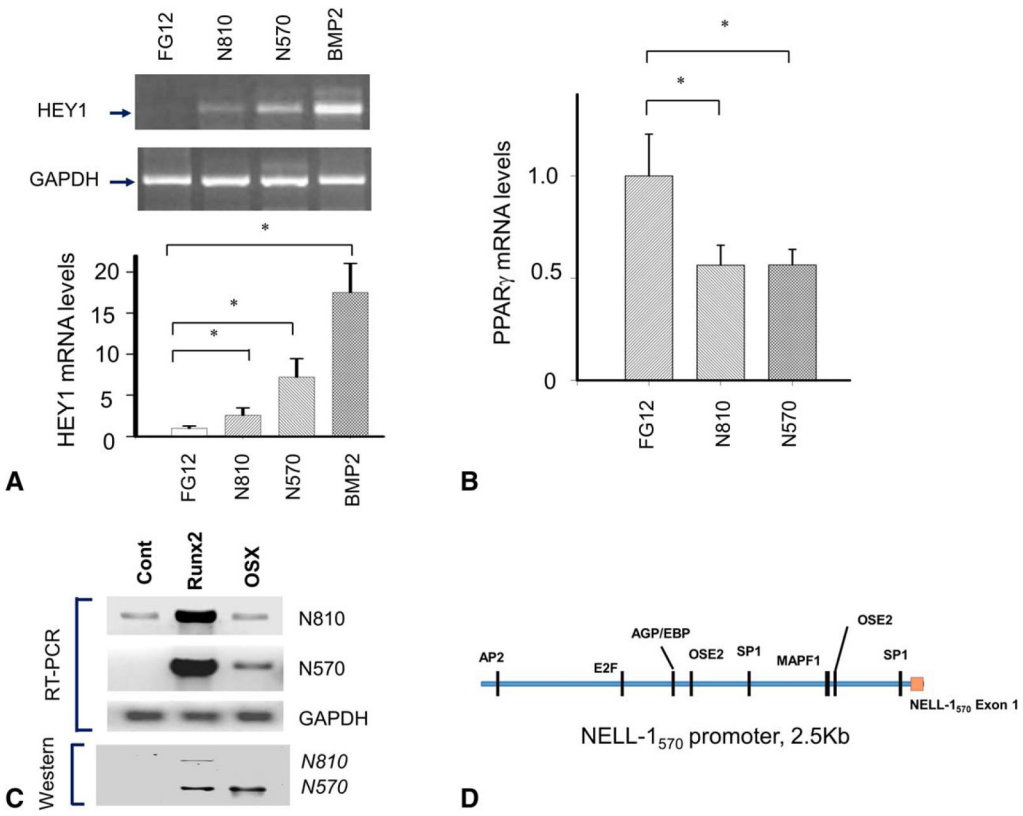

Figure 3.

Expression of genes involved in the Notch pathway and adipogenesis, and responsiveness of NELL-1 isoforms to RUNX2 and OSX. **(A):** RT-PCR to detect the expression of *HEY1*. C3H10T1/2 cells were transduced by FG12, NELL-1_{810}, NELL-1_{570}, or BMP2 lentiviral vectors. Two days post-transduction, mRNA was isolated for RT-PCR. The histogram was from two separate experiments. **(B):** Quantitative RT-PCR of PPARγ expression in NELL-1 isoform-transduced cells. **(C):** Responsiveness of NELL-1 isoforms to RUNX2 and OSX. Mouse primary calvarial cells were transfected with control (Cont), OSX, or RUNX2 plasmids. Forty-eight hours post-transfection, RNA samples were extracted for RT-PCR, and medium was collected for immunoprecipitation (IP) assays using the NELL-1 C-terminal Ab cross-linked with protein G beads. (Top) RT-PCR results. (Bottom) Proteins recovered from IP were visualized by Western hybridization using the antibody recognizing both NELL-1 isoforms. **(D):** Sequence motifs involved in transcriptional regulation and responsiveness to RUNX2. *, $p < .05$. $n = 4$. Abbreviations: NELL-1, NEL-like protein 1; RT-PCR, reverse transcription-polymerase chain reaction.

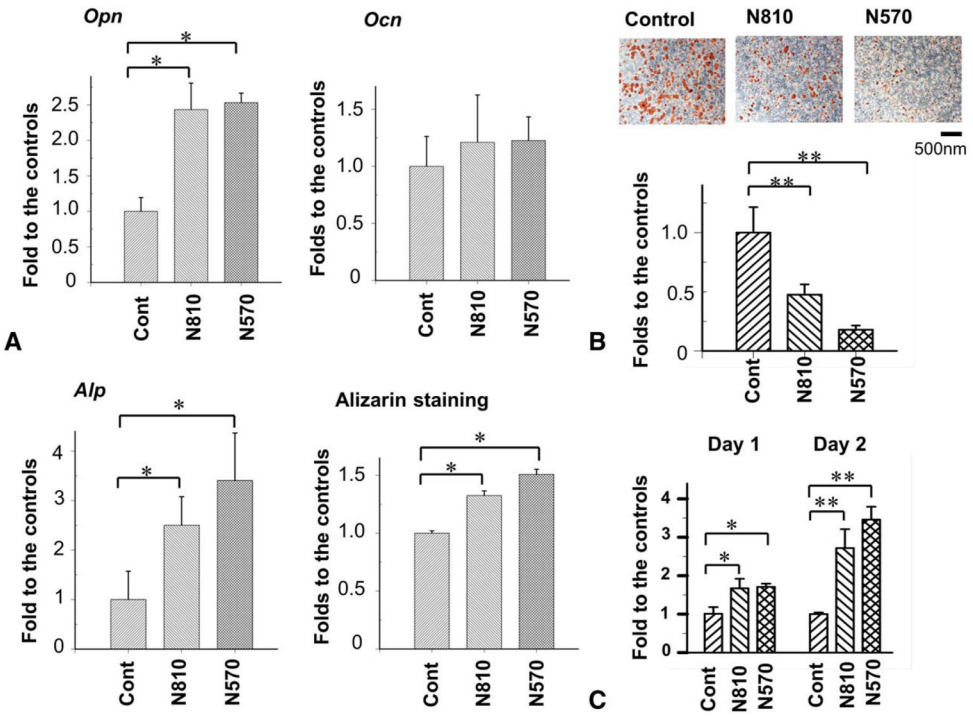

Figure 4.

Differential regulation of osteogenic differentiation by NEL-like protein 1 (NELL-1) isoforms in C3H10T1/2. **(A):** C3H10T1/2 cells were transduced with NELL-1 isoform vectors or the green florescent protein (GFP) lentiviral control (FG12). Both NELL-1 isoforms demonstrated effects of upregulating genes related to osteogenic differentiation, including *Opn* (osteopontin), *Ocn* (osteocalcin), and *Alp* (alkaline phosphatase). Data presented are derived from normalized quantitative reverse transcription-polymerase chain reaction. RNA samples were isolated from lentiviral-transduced cells 2 days post-transduction. Alizarin staining (21 days post-transduction) also demonstrated the effects of NELL-1 isoforms on osteogenic differentiation. n =4. **(B):** Oil red O staining of C3H10T1/2 cells transduced with lentiviral vectors expressing NELL-1_{570}, NELL-1_{810}, and GFP control protein (FG12). The bar graph represents the results of two experiments. **(C):** The expression of Runx2 mRNA in transduced C3H10T1/2 cells. Results were also from two experiments. *, p <.05; **, p <.01 compared to the control.

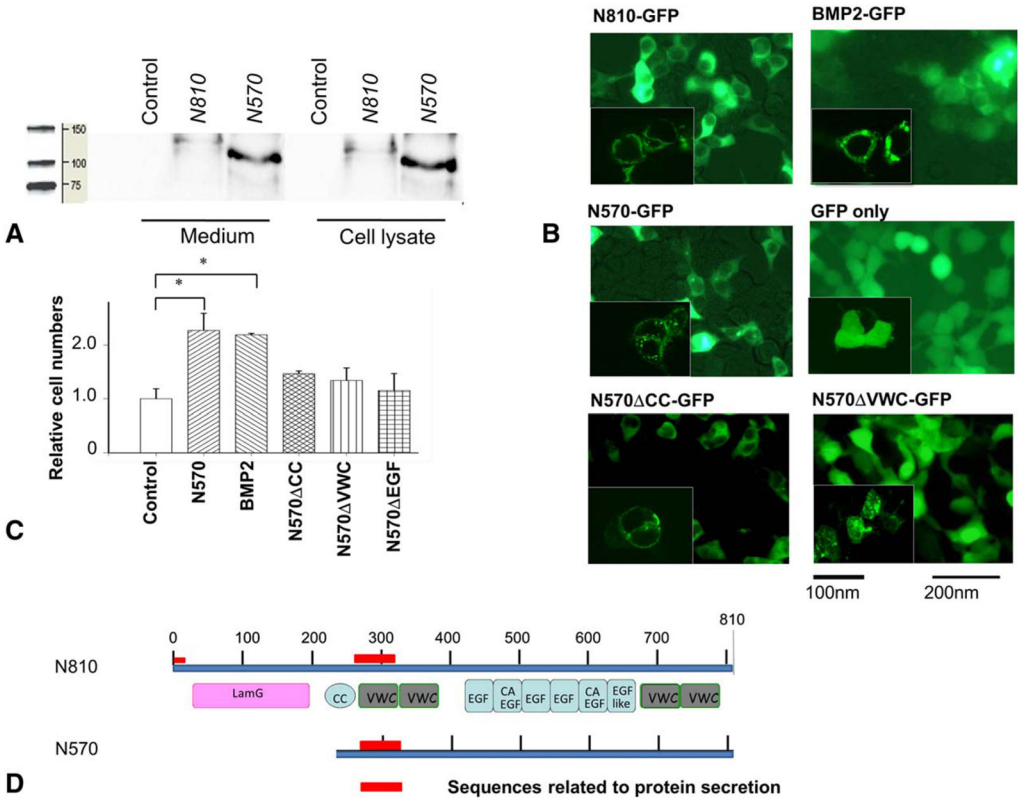

Figure 5.

Secretory properties of NELL-1_{570}. **(A):** Western hybridization of NEL-like protein 1 (NELL-1) isoforms. Similar to NELL-1_{810} (N810), NELL-1_{570} (N570) was detected in both cell lysates and culture medium fractions. **(B):** Expression of NELL-1 isoforms fused with the GFP protein in 293T cell membrane. Photos were taken 2 days post-transfection. Large images were from regular light fluorescent microscopy while the smaller photos in the bottom left of individual larger images were taken using confocal laser microscopy. **(C):** Stimulation of C3H10T1/2 cells by lentiviral vectors carrying FG12, BMP2, N570, or different N570 deletion constructs. The method was described in Figure 2 legend. **(D):** Sequence comparison of NELL-1_{570} and NELL-1_{810}. Distinct domains within the protein structure included LamG, VWC, CC, and CA EGF. The red bar that overlaps with the first VWC domain is the sequence related to NELL-1_{570} transportation to cell membrane. *, $p <$. 05, compared to the control. Abbreviations: CA EGF, calcium-binding type EGF-like domains; CC, coiled-coil regions; GFP, green florescent protein; LamG, laminin G domain; VWC, von Willebrand type C domain.

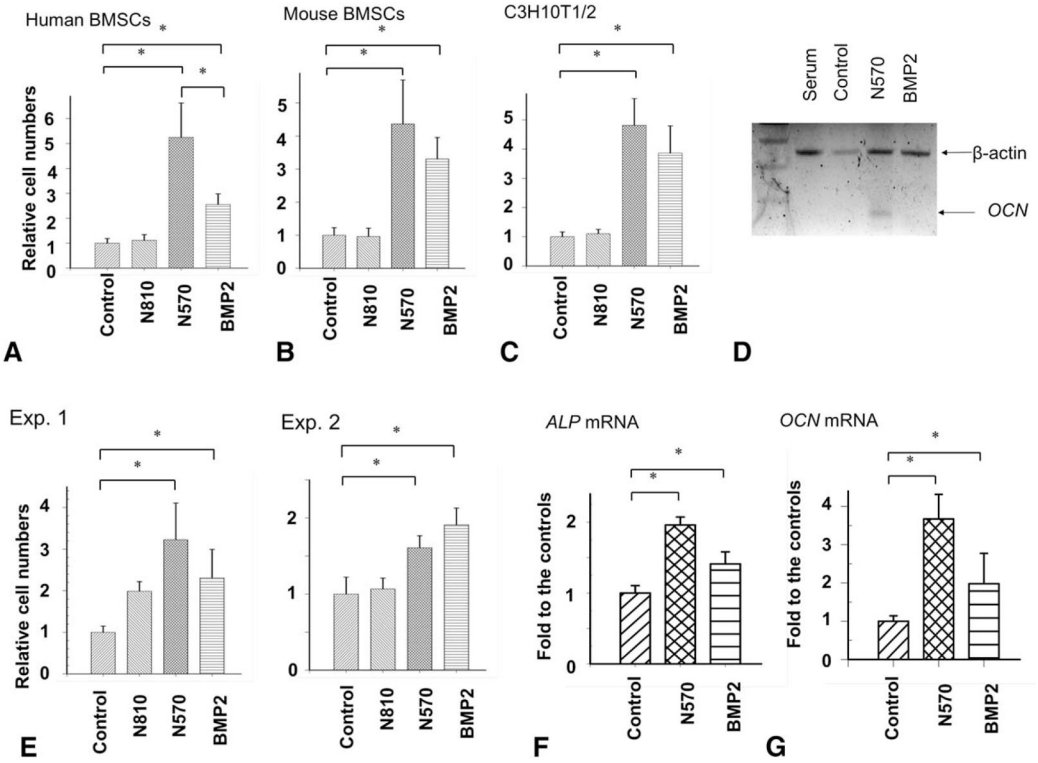

Figure 6.

NELL-1_{570} protein stimulation of mesenchymal stem cell and perivascular stem cell proliferation and differentiation. **(A):** Human BMSCs growth was stimulated by NELL-1_{570} protein. Cells were plated as described in the Figure 2 legend. After the culture medium was changed to serum-free medium, 800 ng/ml NELL-1_{810}, 800 ng/ml NELL-1_{570}, or 100 ng/ml BMP2 were added into the medium. The medium was changed every 3 days with appropriate NELL-1 or BMP2 proteins. At 10 days post-treatment, cells were collected and counted using a cell counter. **(B):** Mouse BMSCs growth was stimulated by NELL-1_{570} protein. **(C):** C3H10T1/2 cell proliferation was stimulated by NELL-1_{570} protein. **(D):** Reverse transcription polymerase chain reaction (RT-PCR) to detect NELL-1_{570} stimulation of OCN mRNA expression in treated human BMSCs. In this experiment, β-actin mRNA was used as a control. Cellular mRNA was isolated 3 days post-treatment. **(E):** PSCs from two subjects were treated with NELL-1_{570} or other growth factors. **(F):** RT-PCR of alkaline phosphatase mRNA expression levels in treated cells. **(G):** OCN mRNA expression levels in treated cells. *, p <.05. n =4. Abbreviations: ALP, alkaline phosphatase; OCN, osteocalcin.

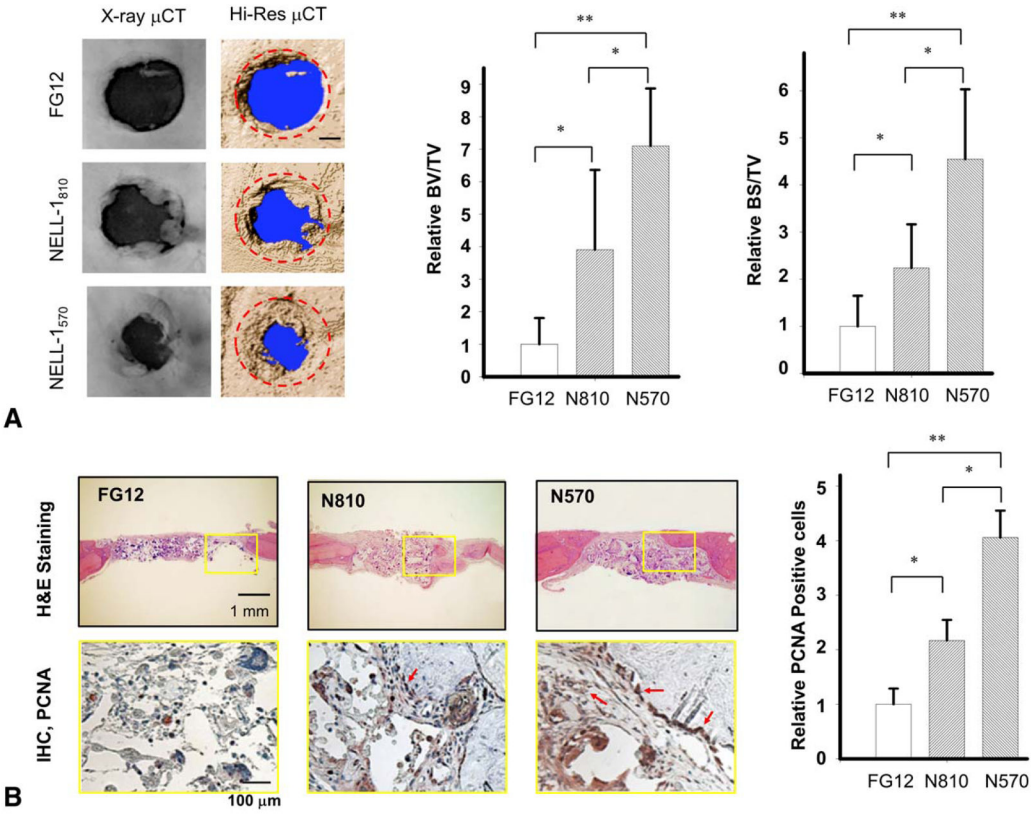

Figure 7.

Differential calvarial defect healing by NELL-1 isoforms. Lentiviral vectors carrying NELL-1_{810}, NELL-1_{570}, or green florescent protein were delivered into a 3-mm circular calvarial defect in the parietal bones of rats. **(A):** Bone healing was assessed by microcomputed tomography (μCT) imaging and analysis. A top-down view of the defect is shown by x-ray imaging and high-resolution μCT reconstruction. Red dashed circles represent the original 3-mm defect, used as a region of interest for CT analyses. Scale bar =1 mm. μCT analyses included relative BV/TV and BS/TV. **(B):** Histology and immunohistochemical detection of PCNA of the treated calvarial defects. Red arrows indicate examples of PCNA-positive cells (left panels). The diagram on the right demonstrates the quantification of relative numbers of PCNA-positive cells shown on high-power fields derived from four animals. *, p <.05 (n =4 calvarial defects per treatment type); **, p <.01. Abbreviations: BS/TV, bone surface/total volume; BV/TV, bone volume/total volume; NELL-1, NEL-like protein 1; PCNA, proliferating cell nuclear antigen.