Title

NELL-1 regulates BMSC osteogenic and adipogenic differentiation by interacting with Integrin and regulating cell focal adhesion

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in Oral Biology

by

Jong Uk Chung

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ABSTRACT OF THESIS

NELL-1 regulates BMSC osteogenic and adipogenic differentiation by interacting with Integrin and regulating cell focal adhesion

By

Jong Uk Chung

Master of Science in Oral Biology

University of California, Los Angeles 2012

Professor Shen Hu, Co-chair

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Previous studies demonstrated that NELL-1 promoted osteogenic differentiation of bone marrowl stromal cells (BMSCs). BMSC differentiation into osteoblasts and the signaling pathways involved are poorly understood. We hypothesized that NELL-1 binds to Integrin and promotes osteogenic differentiation through FAK signaling pathway.

To test this hypothesis, we examined the physical interaction of NELL-1 with Integrin beta-1 and inhibition of NELL-1's cell binding and adhesion using knockdown of Integrin beta-1 siRNA. We also assayed for phosphorylation of FAK as well as expression of osteogenic differentiation markers (*OCN*, *OPN*, ALP staining) and adiopogenic differentiation markers (ORO staining) in BMSC.

We found that NELL-1 physically binds to Integrin beta-1 and Integrin beta-1 siRNA knockdown reduced NELL-1 cell binding significantly. NELL-1 stimulated expression of Integrinβ1 and activated FAK phosphorylation. Finally, NELL-1 enhanced expression of osteogenic genes, whereas it inhibited expression of adiopogenic genes. Taken together, our results suggest that NELL-1 may initiates transduction signals through Integrin, and FAK pathway may play an important role in regulating NELL-1 induced osteogenic and adipogenic differentiation of BMSC.

The thesis of Jonguk Chung is approved.

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University of California, Los Angeles

2012

DEDICATION

This work is dedicated to my family and friends.

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ACKNOWLEDGEMENT

First and foremost, I would like to thank my parents, Dr. Kun Sung Chung and Mrs. Mi Hee Chung, for their sacrifice, love and support over the years.

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INTRODUCTION

A. NELL-1 Protein

Nel-like molecule type 1, also known as NELL-1, has similar structure to Nel that has epidermal growth factor-like repeated sequence when expressed in neural tissues [1]. NELL-1, which is a homolog of Nel, was found in a human fetal brain cDNA that was part of the Human Genome Project; they were first discovered in a nine-day-old chick using phage display screen. Interestingly, an 1800-bp fragment of Nell-1, which was not initially found in the Human Genome Project but detected in human cranial bone tissues with unilateral coronal synostosis, was expressed in higher amounts in patients with coronal synostosis [2]. In addition, human Nell-1 gene has been located at chromosome 11p15.1-p15.2 and was consisted of 906 kb with 20 coding exons using the Human Genome project [3].

The homologous region of NELL-1 and Nel are similar and yet very distinct to suggest difference in structure and function [3]. Through homology studies, 57% nucleotide and 50% amino acid of NELL-1 were discovered to be homolog to those of Nel. This triggered in depth study of the domains in NELL-1 and Nel [4].

Translation of *Nel* gene may generate two different forms of Nel protein. The *Nel* gene is known to have different domains consisting of the following: a histine-rich region, a cysteine-rich that is similar to the C terminal domain of von Willebrand factor, five EGF-like repeats, and two cysteine-rich regions [1]. A hydrophobic trans-membrane domain close to the C terminal of its last EGF-like repeats had suggested the possibility of Nel protein being a membrane-integrated protein [5]. Another possible form of Nel is with a frame shift of the gene. An extra EGF-like repeat can be added into the gene and result in forming a signal sequence [4].

Before N-glycosylation and oligomerization, NELL-1 protein has an 810-amino acid open reading frame with a molecular weight of 90 kDa. The protein contains a conserved structural motif that includes a secretory signal peptide, an NH2-terminal thrombospondin-1 (TSP-1)-like module that overlaps with a laminin G domain, 4 chordin-like cysteine-rich (CR) domain or vWF domains, and 6 EGF-like domains [6].

The Nell-1 protein secreted from rat and NELL-1 secreted from human have similar but yet different appearances. The rat Nell-1 form has a phosphorylated homotrimeric oligomer with molecular weight over 400 kDa. Although both forms were 92.6% homologous, they differed in length when exposed to reducing and non-reducing conditions. When recombinant NELL-1 was expressed from CHO cells, it was expressed as 140 kDa under reducing conditions and 700 kDa in non-reducing conditions. The possibility of NELL-1 existing as a pentamer in native condition explains a smaller sized NELL-1 when exposed in reducing conditions [3].

In addition, Nell-1 has a herapin-binding capacity due to the TSP-1 herapin-binding domain at the N terminus. However, it lacked type I and type III TSP repeats and RGD-binding domains, and C-terminal domain. The EGF repeats in Nell-1 are known to have interactions with PKC subunit [6].

B. Expression of Nell-1 and its Role in Development

Expression of *NELL-1* was detected in developing human B cells and in human fetal cDNA, suggesting the possibility of *NELL-1* gene expressed in different parts of the body [7]. In a two-day-old chick embryo, *Nel* was detected in various tissue types, showing the highest expression in the brain. Its expression level was maintained at a steady rate and detected highest in the brain until the chick is hatched [1]. Northern blot analysis indicated a single 4.5 kb piece

of mRNA was detected in all tissues in various time points of chick development. However, human *NELL*-1 RNA transcript, a 3.5 kb sized transcript, was found exclusively in fetal and adult brain [4]. From this data, one can infer that human *NELL-1* expression may be detected in specific type or population of cells at different stages of development [3].

In depth northern blot analysis on rat embryos at different developmental timepoints detected a 4.0 kb transcript at Day 11 of embryo development. There was a steady increase of this transcript from Day 14 to Day 17 of mouse embryogenesis [6]. In the head area from middle to later stages of development showed high *Nell-1* expression. Despite RNA expression in other areas, no sufficient amount of RNA expression was detected using northern-blot analysis [8]. However, the analysis suggests Nell-1's role in neurological development in mouse development [3].

Mice with overexpression of *Nell-1* showed a lot of apoptosis of neural cells at Day 15.5, which suggests the possible cause of acrania, a syndrome that seems to involve sever defects in both brain and neural-crest derived tissues [8]. In addition, overexpression of *Nell-1* in transgenic mice showed more abnormalities in the skeletal system, specifically with craniofacial bones [9]. Overexpression of *Nell-1* in transgenic mice showed that Nell-1 plays an important role in craniofacial bone and cartilage development [3].

For further investigation of Nell-1's role in the biological system, a knockdown model was conducted using ENU, a highly potent mutagen that can induce new mutation in every 700 loci. ENU created Nell-1 deficient mouse by shortening of Nell-1 from an 810 amino acid sequence into a 502 primary sequence via nonsense point mutation. Subjects with homozygous mutated allele of *Nell-1* were found to be lethal with abnormalities such as enlarged cranial vaults, short body length and anomalous curvature in the cervical spine, without detectable

abnormalities in other organs compared with their wild-type and heterozygous littermates. In addition, less mineralized calvarial bones, compressed cervical intervertebral spaces and sever deformity of ribcage were other symptoms observed when the skeletal system of homozygous litters was stained [10]. Histological analysis also confirmed compressed intervertebral spaces of homozygous litters when compared to that of normal wild-type litters [10]. Finally, microCT analysis of these deformed litters confirmed and revealed differences found in calvarial, vertebral, and long bones between *Nell-1* deficient newborn and wild-type pups [11]. In addition, *Nell-1* deficient newborns also showed parietal bones were much thinner and that sagittal sutures were much wider in *Nell-1* deficient mice compared to wild-type [12]. This was significant since *Nell-1* not only expressed in calvarial bones, but also in the head region of mice [6,10]. This loss-of-function study model alludes Nell-1 role in inducing osteogenesis and its potential usage in bone regenerative therapy.

In observing the differences between *Nell-1* deficient mice and wild-type, thoracic and lumbar vertebral bodies were smaller, less mineralized, with compressed intervertebral spaces in *Nell-1* deficient mice. *Nell-1* deficient mice showed less-mineralized trabeculae in the center region of the vertebral bodies in MicroCT analysis. Histological findings showed delayed ossification at the central vertebral bodies and formed fewer than thinner trabeculae compared to wild type. Moreover, long bones exhibited deformities in the cortical bone. Results suggest Nell-1 to be part of mouse bone formation via intramembraneous and endochondral ossification [13]. As indicated, Nell-1 can be a critical factor with relative specificity to promote differentiation of neural-crest-cells derived osteoblasts. Compared to other growth factors such as BMP2, Nell-1 may even be more highly specific to a specific type of bone development [3].

In animal models of bone repair, rats with calvarial defects treated with exogenous NELL-1 coated scaffold demonstrated its ability to induce bone healing, repair of rat femoral segmental defects, as well spinal fusion in animal models [14,15,16]. After several animal studies done with same experimental protocol, results demonstrated a reproducible preclinical osteoinductivity of NELL-1 in multiple small and large animal models.

C. Translational Studies of NELL-1 in Enhancing Osteogenesis

i) The Effect of NELL-1, BMP2, and their synergistic effect on Bone Regeneration

BMP2, an osteogenic growth factor being widely used in orthopedic surgeries, plays an important role in migration and proliferation of mesenchymal cells and have a unique ability to alter differentiation of osseous and nonosseous mesenchymal cells toward the osteogenic lineage [17]. Thus, human BMP-2 recombinant protein has been used to regenerate bone defects with results comparable to autogenous particulate bone and marrow [18,19,20]. However, the current usage of BMP2 has raised some safety issues revolving its appropriate dosage when applied in humans. NELL-1, as mentioned previously, is a novel osteoinductive protein being investigated for its ability to increase osteoblast differentiation and mineralization [21,22]. Two studies have been conducted to investigate NELL-1's effectiveness in inducing bone formation as well as its synergistic effect when combined with BMP2.

Among various skeletal defect models, calvarial defect was selected among all as NELL-1 is overexpressed during premature bone formation in calvarial sutures of craniosynostosis patients. A 3-mm thickness craniotomy is conducted to create the defect in each parietal bone. After treating these defects with either NELL-1 or BMP2 coated PLGA scaffold, it revealed the osteognic potential of Nell-1 to induce bone regeneration compared to control samples for a 12-

week time period. Furthermore, calvarial defects treated with Nell-1 showed equivalent bone healing as that of BMP2 [21].

In addition to rat calvarial model, a similar study has been done on a rat femoral segmental defect model. From a previous study, NELL-1 has successfully induced bone formation in the rat calvaria without side effects and ectopic bone formation [21]. As the calvarial defect model showed Nell-1's effectiveness in calvaria, other defect models need to be investigated as well to test the *in vivo* osteoinductive capacity of NELL-1.

Previous studies have shown regulation of NELL-1 increased on transforming growth factor β1 and fibroblast growth factor 2 but not BMP2. Studies on downstream regulation of NELL-1 showed reduced expression of early markers of osteoblastic differentiation and increased expression of intermediate and late markers [22]. This was significantly different from BMP-2 induced effects on BMSC, which suggests that NELL-1 and BMP2 may induce osteoblastic differentiation through a different signaling pathway [22,23]. Thus, another study was conducted to observe the effect of NELL-1 and BMP2's synergistic effects on calvarial bone defect model.

When Nell-1 and BMP2 are synergistically used for bone regeneration, results show better bone fusion. Using the same experimental protocol from previous study, BMP2 and combination of BMP2 and Nell-1 coated PLGA scaffolds were administered into the 5-mm calvarial defect. Histology and high resolution microCT analysis indicated the combination of two osteogenic factors showed more mature and complete bone fusion compared to that of BMP2 treatment alone [22].

In addition to the benefit of having higher quality bone regeneration, combined treatment with BMP2 and Nell-1 may alleviate some side effects known to occur with BMP2 treatments.

When high dosage of BMP2 is used in orthopedic surgeries, some side effects have been reported such as ectopic bone formation with spinal cord impingement [24], osteoclast activation with transiently elevated bone resorption, cyst-like bone void formation [25], and soft-tissue swelling [26]. Conducting *in vivo* study with BMP2 reproduced some side effects such as cyst-like bone void formation and soft tissue inflammation [27]. Developing osteoinductive molecules with different but yet complementary pathways of bone formation increases efficiency of these molecules and prevent potential side effects. Further investigation of BMP2 and Nell-1 combined therapy is needed [22].

In our *in vitro* studies, Nell-1 showed specificity towards cells of osteochondrogenic lineage. Due to the three Runx2 binding sites on Nell-1, Runx2 has direct promoter regulation of Nell-1, and this regulation results in the osteospecificity of Nell-1 [28,29]. Upregulating Nell-1 accelerated differentiation and bone formation; however, downregulation of Nell-1 resulted in inhibited osteoblast differentiation [2,9,6]. To further investigate Nell-1's osteogenic properties, Bone Marrow Stromal cells, which includes osteochondrogenic cells found in bone marrow, ovary, uterine mucosa and prostate, will be used.

ii) Different type of Scaffold and their effectiveness in delivering Nell-1

Orthopedic surgical treatment rely on bone graft consisted of various material useful for bone regeneration. For an effective bone graft, three elements are necessary to generate and maintain bone: scaffolding for osteoconduction, growth factors for osteoinduction, and progenitor cells for osteogenesis [30].

Autograft is the gold standard for the three requirements mentioned above. However, there are some limitations to using autograft. Acquiring autogenous bone is a difficult time itself

and thus can cause delay in surgery [31]. In addition, there are limited amount autograft in donor site for surgery, and the viability of cells taken from the harvest site tend to decline rapidly after being separated from blood supply [32,33]. Allograft can substitute for autograft, but the cost, varability in results, risks of bacterial contamination, viral transmission and immunogenicity makes the usage of allograft a risk.

Synthetic bone grafts have been developed to overcome the disadvantages from using autografts and allografts. Ceramics, collagen, noncollagenous proteins, bioactive glasses, and biodegradable polymers are materials used to make different kinds of synthetic bone grafts [34]. Although theses substitutes may have the potential for poor resorbability and inclusion of processed animal components, its ability to combine with osteoconductive matrices, osteoinductive growth factors, and osteogenic cells may also be the advantages of using synthetic bone grafts [35]. Our lab mostly relied on demineralized bone matrix (DBM) and poly(lactic-co-glycolic acid), also known as PLGA.

DBM is decalcified cadaveric cortical bone treated to reduce the risk of transferable infection and immunological response. DBM is effective because the trabecular structure of the bone tissue is intact and can be used as a scaffold [35]. Having gone through demineralization, DBM contains some bone growth factors and can possess more osteoinductive potential than that of allografts [32,36]. For the sheep spinal fusion model, our lab used sheep DBM to deliver Nell-1 and to see its effect on bone regeneration. To prevent leftover osteoinductive factors from contributing to bone formation, sheep DBM was heat inactivated before use. This study was able to show how both DBM and heat-inactivated DBM were effective in inducing bone formation when coated with Nell-1. Both groups showed 50% increase in bone formation and bone mineral

density. However, heat-inactivated DBM group did seem to have delayed the osteogenic effect of Nell-1 by one month [16]. Nonetheless, DBM can be suitable as a Nell-1 delivery vehicle.

Synthetic bone grafts are made of composite scaffolds such as poly(lactic-co-glycolic acid). These type of scaffolds are useful as it can be used to control the concentration of osteoinductive components and to have those osteoinductive components delivered to the graft site by way of an osteoconductive carrier [32]. PLGA is a copolymer that has high biodegradability and biocompatibility. The presence of water causes hydrolysis of PLGA into two monomers, lactic acid and glycolic acid, and they are also by-products of various metabolic pathways. Since the body knows how to dispose the two acids, there is minimal systemic toxicity associated with the use of PLGA [37]. Our lab also used PLGA in rat calvarial defect model to deliver Nell-1. PLGA is neither osteinductive nor osteoconductive, and it allows one to observe Nell-1's effect on calvarial defect without any other osteoinductive growth factor that may reside in biologic processed scaffolds. Although other studies evaluated PLGA's sustained release of growth factor such as BMP2 to increase the bioactivity of growth factors, studies on Nell-1 and its sustained release in PLGA needs to be verified [22].

D. Nell-1 Associated Signaling Pathways

Runx2

As of today, there are a couple studies that have focused on the signaling pathways induced by Nell-1's osteogenic activity. Bone specific cDNA microarray analysis of Nell-1's effect on bone marrow stem cells such as MC3T3, a pre-osteoblast cell line obtained from calvarial bone, showed increase of late-stage osteogenic markers such as OPN, OCN, and BMP-7. However, no difference was observed in mRNA expression level of Runx2, TGFβ-1,2,3,

FGFβ and its corresponding receptors [9]. However, analyzing NELL-1 promoter binding proteins showed Runx2 directly bind onto the osteoblast specific-binding elements 2 (OSE2) [38]. Since Runx2 is an important transcriptional factor in controlling osteoblastogenesis and bone formation, identifying NELL-1 as Runx2 downstream gene proved NELL-1's contribution to osteogenesis and its significance in further research on its signaling pathways.

These proteins play a key role in Runx2 activity in the cell. Mutation of FGF receptor has shown decrease in Runx2 expression and upregulating FGF in a FGF mutated cell showed dramatic increase in Runx2 expression [39]. In addition, Nell-1 induced Runx2 phosphorylation can enhance Runx2 transactivation capacity [3]. Although Runx2 is upstream gene of *NELL-1*, there is some speculation that Runx2 phosphorylation via NELL-1 maybe through a feedback loop. This maybe the reason why NELL-1 shows a different kind of bone formation compared to BMP2, as NELL-1 is more precise in regulation [28].

Wnt Signaling

Wnt signaling also plays a role in osteogenic differentiation in undifferentiated cells. When LRP5, one of Wnt signaling receptors on cell membrane, is mutated, it has been associated with osteoporosis-pseudoglioma syndrome, which results in low bone mineral density and skeletal fragility [40]. When overexpressed, it showed high osteogenic activity and reduced amount of osteoblast apopotsis [41]. Recent studies have found β-catenin dependent Wnt signaling to play integral roles in stemness and mesenchymal cell differentiation, as well as bone development and homeostasis [42,43,44]. Overall, activation of the β-catenin dependent Wnt pathway showed enchanced osteogenesis via activation of Runx2 [35,43,44]. Nell-1's contribution to Wnt signaling has yet to be verified, but experimental data from our lab suggests Nell-1's

activation may activate beta-catenin dependent Wnt signaling to promote bone formation and reduce adipogenesis.

MAPK Pathway

Mitogen-Activated Protein Kinase Pathway **(**MAPK) has been known to play various roles in the cells by modulation from growth factors, cytokines, and physical stresses [45,46]. Since Nell-1 is a growth factor, studies focusing on Nell-1's signaling pathways have placed emphasis on its relationship with MAPK pathway.

When significant amount of Nell-1 is administered into rat fetal calvarial cells, ERK ½ and JNK 1/2/3 kinases were significantly activated after 10 minutes of Nell-1 protein addition. After a transient activation of those two kinases, OPN, a late marker for osteogenesis, was upregulated. The activation of ERK and JNK were verified to cause upregulation of OPN after Nell-1 protein addition. Knockdown of Ras prior to Nell-1 administration resulted in lower expression of OPN. This suggests that ERK activation depends on Ras, which is a key molecule for the MAPK pathway [47,48].

Unlike BMP2, which is a similar yet different osteoinductive growth factor, Nell-1 doesn't play a role in activating Smad1/5/8 pathway to initiate osteogenesis. BMP signaling pathway involves Smad playing a crucial role in transcription of the downstream genes. In addition, Smad has been implicated in osteoblast differentiation by activating MAPK pathway [49,50,51]. As Nell-1 did not show any up or down regulation of Smad, Nell-1 must participate in a different pathway to induce expression of osteogenic genes [3].

E. Integrin: a New Signaling Pathway to NELL-1?

i) Integrin may be a possible receptor for NELL-1

Integrins are cell surface receptors that mediate cellular adhesion to extracellular proteins and to other cells. Integrin receptors are heterodimeric receptor complexes consisting of non-covalently associated α and β chains, and each subunit is categorized as a single-pass type I transmembrane protein [52]. In mammals, there are 17 alpha subunits and 8 beta subunits identified [53]. It has been reported that Integrin beta-1 interacts with the LDVP (Leu-Asp-Val-Pro) sequence in the N-terminal region of thrombospondin-1 [54]. The LDVP sequence is conserved at the corresponding position in most Laminin G domain modules, including NELL-1 [55]. Thus, NELL-1 with the TSP-1 domain may interact with extracellular domain of Integrin beta-1 and initiate signaling pathways (**Fig. 1**).

ii) Critical roles of Integrin—cytoskeleton reconstruction

Cell adhesion mediated by Integrin is vital for cell proliferation, survival and function [56]. As Integrin receptors bind with protein ligands, Integrin receptors begin to aggregate near each other and to recruits focal adhesion structures, which is very important roles when modulating cell adhesion and morphological changes of the cells [57]. After activation of Integrin receptors, they can transmit signals into the cell and provide information on its location, local environment, adhesive state, and surrounding matrix to the cell [58,59]. The information carried in the signal determines the response of the cell by undergoing migration, survival, differentiation and motility. In addition, they can also activate and inactivate other receptors such as growth factor or G-protein coupled receptors [52].

Upon activation of Integrins, it initiates talin-mediated connection between cytoskeleton and extracellular matrix. This further contributes to establish the link between extracellular and cytoskeleton, and also to recruit additional cytoskeletal proteins such as actin [60,61].

Cell-matrix interactions via Integrins are essential for osteoblastic differentiation. Various ECM proteins (e.g. laminin 5, fibronectin, vitronectin, and TSP1/2 have been shown to interact with the Integrin family [62]. The ligand-bound Integrin transduces the signals through activation of the focal adhesion kinase (FAK), followed by indirect activation of the Ras-MAPK cascade, and then leads to the osteogenic differentiation through Runx2 [63].

iii) Primary intracellular signaling pathways regulated by Focal Adhesion Kinase

One of the first Integrin signaling molecules to be identified was focal adhesion kinase (FAK), which acts as a phosphorylation-regulated signaling scaffold and is important for adhesion turnover, Rho-family GTPase activation, cell migration and cross-talk between growth-factor signaling and Integrins [64]. For osteoblastic differentiation stimulation, FAK activity is essential for BMP-Smad signaling [65]. Unlike BMP, the NELL-1 protein does not activate the Smad signaling cascade. Instead, a study done on NELL-1 and its signaling pathway suggested a binding of NELL-1 to its receptor may activate the osteogenic signaling via Tyr-kinase associated Ras-MAPK cascade [48]. Since FAK is an immediate downstream signal of Integrin beta-1, it is imperative to look at Integrin beta-1 related molecules, receptors and tyrosine kinase involved in NELL-1's signaling transduction pathway [65].

Studies have shown the disruption of osteoblastic differentiation occurs when FAK has been inactivated [63]. FAK is a non-receptor protein tyrosine kinase (NRPTK) that is associated with focal adhesion complexes. As Integrin beta-1 and FAK work closely with each other, FAK

initiates the activation of Integrin beta-1 when FAK binds onto the cytoplasmic domain of Integrin beta-1 receptor. Upon activation of Integrin beta-1, it autophosphorylates itself and binds Src kinase. Activated FAK makes the C-terminal domain of Integrin into a docking site for focal adhesion complexes to initiate signaling of cell growth, survival and morphogenesis [66]. In addition, FAK activates PI3K.PKD/Akt/PKB and Grb2/SOS/Ras/Raf-1/MEK/ERK pathways [67]. Notably, it has been shown that a ligand bound Integrin protein can send signals via activating FAK, followed by subsequently activating MAPK cascade, and then leads to osteogenic differentiation through Runx2 [48]. By determining if there is direct binding /interaction between NELL-1 and Integrin beta-1 and its functional effects as the first critical step in current study, we may be able to further explain NELL-1's pathways leading to osteogenic differentiation.

Hypothesis and Specific Aims

Working hypothesis:

Integrin is a cell surface receptor that is known to mediate attachment between cells, tissues or growth factors surrounding it. Integrin also serves additional roles in regulating cellular shape, mobility and cell survival. Recently, Integrin has been shown to interact with extracellular matrix proteins. Once an ECM protein is bound with Integrin, it activates focal adhesion kinase and indirectly activates the Ras-MAPK cascade. This activation can lead to osteogenic differentiation via upregulation of transcription factor Runx2. A previous study has reported interaction between LDVP (Leu-Asp-Val-Pro) sequence positioned in the N-terminal region of thrombospondin-1 and Integrin $\alpha 4\beta 1$. Since NELL-1 has an NH2-terminal thrombospondin-1 (TSP-1) like module that overlaps with a Laminin G domain (**Fig. 1**), we plan to investigate whether Integrin beta-1 may be a possible Nell-1-interacting receptor and signal pathways to induce osteoblastic differentiation of bone marrow stromal cells.

Specific Aim: To identify the interaction of NELL-1 with Integrin beta-1 and role of NELL-1 in osteogenesis and adipogenesis via FAK pathway

1. Demonstrate the interaction of Nell-1 with Integrin beta-1.
2. Demonstrate the role of Nell-1 in cell adhesion and FAK phosphorylation.
3. Demonstrate Nell-1's role in cell proliferation.
4. Demonstrate Nell-1's regulatory role in osteogenic and adipogenic differentiations.

Materials and methods

Cell Culture

Cultures were visualized using an inverted microscope to assess the degree of confluence and to confirm the absence of bacterial and fungal contaminants. The cell monolayers were washed twice with 5 ml PBS without Ca_{2+}/Mg_{2+} (Sigma Aldrich: Prod. No. D8537). 2 ml Trypsin/EDTA (Sigma Aldrich: Prod. No. T4049) was pipetted onto the washed cell monolayer and the cell culture dish was gently rocked to cover the monolayer with trypsin. The cell culture was then returned to the incubator and for 2-5 minutes. The cells were examined using an inverted microscope to ensure that the majority of the cells were in the process of detachment. Next, the cells were resuspended in a small volume of fresh serum-containing medium to inactivate the trypsin. A 10 μl representative aliquot was removed and a cell count was obtained using a hemocytometer. The cells were centrifuged, resuspended in fresh media, and the required number of cells was transferred to new cell culture plates containing pre-warmed medium. The cells were returned to the 37˚ C CO_2 incubator with 5% CO_2 in 95% air filtered through a 0.22 μm filter.

Cell Lines

ST2 Cells

The ST2 cell line, a clone derived from mouse bone marrow was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in growth medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin). The cells were incubated in 95% air

and 5% carbon dioxide at 37˚C. A sub-cultivation ratio of 1:4 was used and medium renewal was performed 2-3 times per week.

M2-10B4 Cells

The M2-10B4 cell line is a clone derived from bone marrow stromal cells from a (C57BL/6J X C3H/HeJ) F1 mouse. Cells were maintained in growth medium (RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 100 U/ml penicillin/streptomycin). The cells were incubated in 95% air and 5% carbon dioxide at 37˚C. A sub-cultivation ratio of 1:4 was used and medium renewal was performed 2-3 times per week.

ATDC5 Cells

ATDC5 is a chondrogenic cell line that has been derived from teratocarcinoma AT805. These cells showed sequential transition of phenotype in vitro and encompass stages from mesenchymal condensation to calicification. Cells were maintained in growth medium (DMEM: Ham's F12 (1:1), 5% Fetal Bovine Serum (FBS) and 100 U/ml penicillin/streptomycin). The cells were incubated in 95% air and 5% carbon dioxide at 37˚C. A sub-cultivation ratio of 1:4 was used and medium renewal was performed 2-3 times per week.

C3H10T(1/2) Cells

Mouse C3H 10T(1/2) cells, a mesenchymal fibroblast-like cell line of embryonic origin, was grown in DMEM with 10% fetal bovine serum at 37°C, were treated with or without ascorbic acid and beta-glutaldehyde to induce differentiation. 100 U/ml penicillin/streptomycin

was added. The cells were incubated in 95% air and 5% carbon dioxide at 37˚C. A sub-cultivation ratio of 1:4 was used and medium renewal was performed 2-3 times per week.

HEK 293T Cells

HEK293T (human embryonic kidney 293T) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated FBS (fetal bovine serum), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 ℃ under humidified air containing 5% CO2. The cells were incubated in 95% air and 5% carbon dioxide at 37˚C. A sub-cultivation ratio of 1:4 was used and medium renewal was performed 2-3 times per week.

Immunoprecipitaion

Preparation of Cell Lysates: Membrane Protein Extraction

HEK 293T were grown on 10 cm^2 plates until they were 80-90% confluent. After trypsinizing and isolating cells from the plate, 5×10^6 cells per sample were isolated by centrifuging harvested cell suspensions at 850 × g for 2 minutes. Pellet cells are washed in PBS in 1.5 mL microcentrifuge tubes. After carefully removing and discarding the supernatant, 50 μL of Reagent A was added to the cell pellet and pipette up and down to obtain a homogeneous cell suspension. With occasional vortexing, samples were incubated 10 minutes at room temperature. Having the samples on ice, a mix of Reagent C and Reagent B (ratio of 2:1) was added. After adding, they were incubated on ice for 30 minutes, vortexing every 5 minutes. Afterwards, tubes were centrifuged at 10,000 × g for 3 minutes at 4 ℃. Supernatant is transferred to new tubes and incubate 10 minutes in 37 ℃ water bath to separate the membrane protein fraction. To enhance phase separation, incubation time was extended to 20 minutes. Tubes with supernatant were

centrifuged at room temperature for 2 minutes at 10,000 × g to isolate the hydrophobic fraction from the hydrophilic fraction. The bottom phase consisted of the hydrophobic protein and saved in a new tube.

Covalently binding NELL-1 and interacting proteins

Sulfo-SBED Biotin Label Transfer Reagent (Thermo Scientific) was used to crosslink NELL-1 and cell surface Integrin beta-1. Briefly, 500 μl NELL-1 (0.4 mg/ml) was mixed with 11 μl Sulfo-SBED solution (44.8 mg/ml) and incubated at room temperature for 30 minutes to let NELL-1 react with Sulfo-SBED crosslinker. Slide-A-lyzer MINI dialysis devices (Thermo Scientific) were used to remove the non-reacted Sulfo-SBED. The photoactive conjugation of Sulfo-SBED-linked NELL-1 with cell surface Integrin beta-1 was performed by using a long-wave UV lamp (365 nm). Then the interacting complex was pulled down by incubating Streptavidin conjugated magnetic beads (Solublink) overnight.

The incubated Streptavidin conjugated magnetic beads was separated from the membrane protein supernatant by placing them on the magnet for 2 minutes. Then, 500 uL of immunoprecipitation washing buffer (was used to resuspend the beads and left on rotating shaker for 5 minutes. This step was repeated three times. The immunocomplex was subjected to SDS-PAGE and Western blotting using and Integrin beta-1 was detected by western blot (Abcam) from elute of above magnetic beads upon reduction of the disulfide bonds in SBED.

Western Blot Analysis

30 μg of total protein combined with 5X loading buffer was boiled for 10 minutes, separated by SDS-PAGE (4% stacking and 12% resolving gel), and electrotransferred to a

nitrocellulose membrane (GE Healthcare, Piscataway, NJ, USA) at 100 V for 1 hour at 4℃. The membrane was blocked for 1 hour with 5% milk in Tris-buffered saline plus 0.05% Tween 20 (TBST) incubated with anti-phosphorylated FAK (Cell signaling), anti-FAK (Cell signaling), and GAPDH (Santa Cruz) primary antibody at 1:1000 dilution in blocking buffer overnight at 4℃, washed with TBST, and incubated with peroxidase–conjugated secondary antibody at 1:10,000 dilution in blocking buffer for 1 hour. Following incubation, the membrane was washed with TBST, and proteins were visualized using the Immun-Star WesternC Chemiluminescent Kit (Bio-Rad).

Cell Adhesion Assay

Cell adhesion assay was performed as previously described [68]. NELL-1 protein at the indicated concentrations were added to 96 well plates (100 μl/well) and incubated at 4℃ overnight. After washing, the plates were blocked with 10 mg/ml heat-denatured BSA (200 μg/well) in divalent cation-free PBS for 30 minutes (10 mg/mL heat-denatured BSA in divalent cation-free Dulbecco's PBS. After dissolving the BSA, filter through a 0.22-μm filter to remove undissolved protein, and incubate in a water bath at 85℃ for 10–12 min).

Then, ST2 cells in RPMI medium, C3H10T1/2 cells in DMEM medium, M2-10B4 in RPMI medium and ATDC5 cells in DMEM/F-12 (1:1) medium at the concentration of 5×10^5/ml (100 μg/well) were resuspended and incubated at 37°C in a 15 mL polypropylene tube for 10 minutes. Then, cells were added to each well for 15 minutes. Nonadherent and loosely attached cells were removed by either tapping the plate or gently washing the wells with one or more 100 uL of PBS. Attached cells were fixed in 5% glutaraldehyde (w/v) and incubated for 20 minutes at room temperature. Crystal violet stain was used to stain adherent cells and incubated for 60

minutes. After washing the wells with 100 uL of water three times, quantification was performed by solubilizing in acetic acid; absorbance was measured at 570 nm.

Real Time PCR

Total RNA from ST2 and C3H10T1/2 cells for real-time PCR evaluation was extracted using TRIzol Reagent. Following ethanol precipitation, total RNA was applied to an RNeasy column (Qiagen) for further purification and treated with DNase per the manufacturer's protocol. cDNA was synthesized from 1 g of total RNA using the SuperScript III Reverse-Transcriptase Kit (Invitrogen) in a final volume of 20 μL. Real-time PCR reactions were performed routinely in triplicate using the 7300 Real-Time PCR System instrument (Applied Biosystems). For gene expression studies of osteogenesis, SYBR Gene Expression Master Mix primer sequences of *Osteocalcin* (*Ocn*) were sense: GCAATAAGGTAGTGAACAGACTCC and anti-sense: AGCAGGGTTAAGCTCACACTG, and those of *Osteopontin (Opn)* were sense: CAGCCTGCAAGATCCTA and anti-sense: GCGCAAGGAGATTCTGCTTCT. The primer sequences of *GAPDH* control are sense TGCACCACCAACTGCTTAGC and anti-sense CCACCACCCTGTTGCTGTAG

Small Interfering RNA Experiments

RNA knockdown experiments were performed using chemically synthesized and annealed small interfering RNA (siRNA) specific to Integrin beta-1 (Santa Cruz). When ST2 cells reached 30% confluence, cells were transfected with 50 nM Integrin beta-1 siRNA or nontarget negative control siRNA (Santa Cruz) using Lipofectamine RNAiMax (Invitrogen). Efficiency of knockdown was validated using Western blot.

NELL-1 Cell Surface Binding Assay

After culturing a plate of ST2 cells at 90% confluence, 5×10^5 ST2 cells were counted and prepared for three groups: unstained, Integrin beta-1 siRNA knockdown, and scrambled siRNA ST2 cell groups. Scrambled and Integrin beta-1 siRNA ST2 cells were incubated in 100 μl of his-tagged NELL-1 (1 μg/ml) for 30 minutes on ice. Afterwards, cells from three groups were washed twice with 1 ml of PBS, re-suspended in 100 μl of the FITC-conjugated mouse monoclonal antibody (1 μg/μl) against 6 X His tag (Thermo), and incubated for 30 minutes on ice in the dark. Following 1 ml of PBS washes, the samples from three groups were analyzed by flow cytometry (LSRII, BD Biosciences).

Immunocytochemistry

Millicell EX slides (Millipore) were coated with NELL-1 protein (10 μg/ml) or PBS overnight and then blocked with 10% BSA. ST2 cells were added at 2×10^4 cells/well in RPMI 1640 for 2 hours. Attached cells were fixed with ice-cold acetone for 10 minutes and blocked with 1% BSA. Anti-active Integrin beta-1 antibody (Millipore) was applied at a dilution of 1:200. ABC complex (Vector Laboratories) was applied to the sections following incubation with biotinylated secondary antibody (Dako). AEC substrate (Dako) was used as chromogen. The intensity of staining was analyzed using commercial software Image-Pro Plus 6.0. Relative active Integrin beta-1 staining was quantified by the mean optical density of staining signal X per percent area positively stained X 100 [69]. For immunofluorescent staining of the cytoskeleton, Vinculin was stained with primary anti-Vinculin antibody (Abcam) followed by Alexa 594 labeled anti-mouse IgG while F-actin was stained with Alexa 488-phalloidin. Nuclei were

showed by DAPI. Photomicrographs were acquired using Olympus IX71 and BX51 microscopes and quantified using Image-Pro Plus 6.0.

Cell Proliferation Assay

NELL-1 protein at the indicated concentrations (0, 0.5, 1, 5, 10, 50 μg/mL) were added to 96 well plates (100 μl/well) and incubated at 4 ℃ overnight. After washing, the plates were blocked with 10 mg/ml heat-denatured BSA (200 mg/well) in divalent cation-free PBS for 30 minutes (10 mg/mL heat-denatured BSA in divalent cation-free Dulbecco's PBS. After dissolving the BSA, filter through a 0.22-μm filter to remove undissolved protein, and incubate in a water bath at 85 ℃ for 10–12 min).

After culturing a plate of ST2 cells at 90% confluence, 5×10^5 ST2 cells were counted and seeded. For different time points (Day 1, Day2, Day 3), cell proliferation of ST2 cells were quantified using MTS assay (Promega). Upon adding 20 μl of the CellTiter 96® AQueous One Solution Reagent, the cell culture plates were incubated at 37 ℃ for 1–4 hours in a humidified, 5% CO_2 atmosphere. Afterwards, the absorbance was measured at 490 nm using a plate reader.

Osteogenic Differentiation and Alkaline Phosphatase (ALP) Staining

For osteogenic differentiation, ST2 cells were seeded in 24-well plates at a density of 5×10^4/well. ALP assay were performed in triplicate wells. After one day of attachment, cells were treated with osteogenic differentiation medium consisting of RPMI1640, 10% FBS, 50 μg/ml ascorbic acid and 3 mM-glycerophosphate for 9 days. ALP staining was performed as previously described [70].

Adipogenic Differentiation and Oil Red O (ORO) Staining

For adipogenic differentiation, C3H10T1/2 cells were seeded in 24-well plates at a density of 5×10^4/well. Oil red O assay was performed in triplicates. After one day of attachment, cells were treated with MesenCult MSC Basal Medium and MesenCult Adipogenic Stimulatory Supplements (STEMCELL Technologies Inc.). Oil red O stain was performed for 9 day and 21 day time point as previously described. For Oil Red O staining of cultured cell, each well was rinsed with PBS and fixed with 10% formalin for 1 hour at room temperature. After washing with 60% isopropanol, cells were incubated with Oil red O working solution (as above) for 10 minutes at room temperature and washed with running water for 10 mins. The well was air dried and photographed afterwards.

Statistical Analysis

Means and standard deviations were calculated from numerical data. To allow multiple comparisons between groups, statistical analyses were performed using one-way ANOVA for single-variable comparisons followed by post-hoc Tukey's range test to directly compare two groups. T-test was also conducted to compare two groups.

RESULTS

NELL-1 directly interacts with Integrin beta-1

In order to determine if NELL-1 directly or indirectly binds to Integrin beta-1, we used the Sulfo-SBED biotin label transfer reagent to crosslink NELL-1 with its cell surface binding membrane proteins. Initially, HEK 293T cells were treated with NELL-1 to have NELL-1 interact with the cell membrane proteins. Streptavidin-conjugated sepharose beads were used to pull down protein complexes that consisted of an unknown protein bound with NELL-1. Western blot analysis using anti- Integrin beta-1 antibody (Millipore) showed a band that resembled a reduced form of Integrin beta-1, which showed to be around 90 kDa. Integrin beta-1 was identified as one of the biotinylated proteins by western blot, indicating that NELL-1 directly binds to the extracellular domain of Integrin beta-1 (**Fig. 2**).

NELL-1 protein stimulates Integrin beta-1 activation and FAK phosphorylation.

Extracellular domain of Integrin mediates cell adhesion when it binds to various protein ligands that initiate conformational changes on the extracellular domain of the Integrin heterodimer. These rapid and reversible changes in the extracellular domain activates Integrin, followed by activation of focal adhesion kinase (FAK) [71] and regulation of cytoskeleton rearrangement [72]. Since NELL-1 promotes cell adhesion and contains Laminin G domain, we inquired if NELL-1 can result in Integrin beta-1 activation. Results showed that ST2 cells, when seeded on NELL-1 pre-coated cell culture plates, exhibited not only faster adhesion, but also significantly stronger staining for active Integrin beta-1 than PBS control at 2 hrs (**Fig. 3a**). Quantification of the percentage of positively stained cells for active Integrin beta-1 per high-

powered field confirmed a significant increase in Integrin beta-1 with NELL-1 (**Fig. 3b,** $^{**}p<0.01$).

As FAK is phosphorylated upon Integrin activation at the sites of focal adhesions [73], we investigated the effect of NELL-1 (10 μg/ml NELL-1) pre-coated on the bottom of each well on FAK phosphorylation after ST2 cells were seeded. Using western blot analysis, results observed at all timepoints from 1 to 8 hours after cell seeding showed that NELL-1 increased FAK phosphorylation (**Fig. 4a**). In contrast, there was no difference detected in total FAK between NELL-1 and control groups (**Fig. 4a**). Quantification of western blot confirmed a statistically significant induction of FAK phosphorylation by NELL-1, observed from 1 to 8 hours after cell seeding (**Fig. 4b**, $^{**}p<0.01$). Thus, in summary, NELL-1 pre-coating resulted in Integrin beta-1 activation and FAK phosphorylation in ST2 cells *in vitro*.

We next characterized the cytoskeletal organization of ST2 cells either on control or NELL-1-coated plates. ST2 cells seeded on NELL-1-coated plates showed evidence of greater F-actin and Vinculin staining in comparison to control at 2 hours after seeding (**Fig. 5**). Therefore, in summary, NELL-1 pre-coating resulted in Integrin beta-1 activation, FAK phosphorylation, and increased cytoskeleton assembly and adhesion size in ST2 cells *in vitro*.

NELL-1 protein promotes ST2, M2-10B4, C3H 10T(1/2) cells adhesion.

The Integrins are a family of transmembrane glycoproteins. The best known function of Integrin is to mediate adhesion of cells to tissue/extracellular molecules surrounding it [56]. We first studied that pre-coating of NELL-1 on cell culture plates, (a novel way to apply NELL-1), could increase cell adhesion. ST2 cells were used for all studies, a clone of bone marrow stromal cells from BC8 mice which have been used in previously reported cell adhesion assays [74]. In

addition, other cell lines such as M2-10B4 and C3H 10T(1/2) cells were used to verify ST2's response to NELL-1's effect on cell adhesion. NELL-1 increased cell attachment in ST2 cell line dose-dependently with a significant increase in adhesion at concentrations exceeding 1 μg/ml (**Fig. 6a,b**). The cell adhesive activity of NELL-1 was also observed with other cell lines such C3H10T1/2 (multipotential mesenchymal cells), M2-10B4 (bone marrow stromal cells), and ATDC5 (chondroprogenitor cells) (**Fig. 6b**). In summary, NELL-1 promoted cell adhesion in culture across a number of different cell lines as well as increased cell adhesion in dose-dependent manner.

NELL-1's cell binding and adhesion is inhibited by knockdown of Integrin beta-1

Having demonstrated that NELL-1 binds Integrin beta-1 and subsequently stimulates expression of Integrin beta-1 and phosphorylation of FAK, we next inquired that Integrin beta-1 expression is required for NELL-1 induced cell adhesion. When Integrin beta-1 was blocked, cell adhesion to ECM was hindered and thus cell adhesion decreased. This data suggests that Integrin beta-1 be crucial in cell adhesion. We knocked down the expression of Integrin beta-1 using siRNA in ST2 cells. With using western blot, we confirmed decreased amount of Integrin beta-1 in Integrin beta-1 siRNA group compared to scrambled siRNA group; moreover, quantification of the western blot also showed a significant decrease in Integrin beta-1 expression in siRNA treated ST2 cells .(**Fig. 7a,b,** $*p<0.01$).

Next, flow cytometry was performed on the three groups of ST2 cells: unstained, Integrin beta-1 siRNA, and scrambled siRNA. After treating the first two groups with his-tagged NELL-1, all three groups were incubated with FITC-conjugated mouse monoclonal antibody (1 μg/μl) against 6 X His tag (Thermo). Using flow cytometry, we analyzed the percentage of NELL-1

binding cells in each group (**Fig. 8a**). Our results showed a reduction in the number of NELL-1 bound cells from 61% to 32% in Integrin beta-1 siRNA group (**Fig. 8b**). This indicates that Integrin beta-1 siRNA significantly attenuated but did not eliminate NELL-1 cell binding completely. We next inquired that Integrin beta-1 expression is necessary for NELL-1 mediated cell adhesion by using Integrin beta-1 siRNA knockdown. siRNA knockdown inhibited ST2 cell adhesion under control conditions (**Fig. 8c**). More importantly, Integrin beta-1 siRNA also inhibited NELL-1 induced cell adhesion at all examined concentrations (**Fig. 8c**). Therefore, data suggest that Integrin beta-1 may be a receptor for NELL-1 and Integrin beta-1 is required for NELL-1 induced cell attachment.

NELL-1 protein promotes proliferation of ST2 cell

After observing that NELL-1 significantly increased cell attachment, we next examined the effects of NELL-1 pre-coating on cell proliferation of ST2, M2-10B4, C3H 10T(1/2). Proliferation was evaluated with a standard MTS assay. Increasing concentrations of NELL-1 significantly increased the proliferation of ST2 cells from 1 day to 3 days (**Fig. 9**). An interesting observation was that even the lowest dose of NELL-1 pre-coating tested (0.5 μg/ml) significantly promoted ST2 cell proliferation. Cell proliferation assays done with other cell lines also showed an increasing trend. However, the quantifications of proliferation for M2-10B4, C3H10T(1/2) were not statistically significant (Data not shown). Thus, NELL-1 pre-coating significantly increases proliferation of ST2 cell, in similar to previous observations in other cell types, including rabbit chondrocytes [75] and purified human perivascular cells [76].

NELL-1 pre-coating promotes osteogenic differentiation of ST2 cells.

NELL-1 protein has been known to positively regulate osteogenic differentiation of bone marrow stromal cells [77]. In all cases NELL-1 protein was added to the supernatant in cell culture. Here we instead cultured ST2 cells on plates pre-coated with NELL-1. ST2 cells were induced toward osteogenic differentiation, as it has been previously reported, by culturing cells in RPMI medium supplemented with ascorbic acid and β-glycerophosphate [78]. Interestingly, NELL-1 pre-coating was observed to enhance ST2 cell osteogenic differentiation (**Fig. 10**). This was observed by alkaline phosphatase (ALP) staining at 9 days osteogenic differentiation (**Fig. 10a**), as well as photographic ALP quantification (**Fig. 10b,** $^{**}p<0.01$). The pro-osteogenic effect of NELL-1 pre-coating was confirmed by quantitative RT-PCR for *Osteocalcin (Ocn)*, a marker of terminal osteogenic differentiation, and *Osteopontin (Opn*), an early marker for osteogenic differentiation (**Fig. 10c,d**). Thus, NELL-1 pre-coating was observed to be an effective method for increasing the osteogenic differentiation of ST2 cells.

NELL-1 pre-coating inhibits adipogenic differentiation of C3H 10T(1/2) cells

Recently, NELL-1 protein has been shown to be anti-adipogenetic towards adipose-derived stromal cells [79]. However, instead of adding NELL-1 protein in the medium, the NELL-1 protein was pre-coated on 24-well plates. We cultured C3H10T(1/2) cells on plates pre-coated with NELL-1. C3H10T(1/2) cells were induced toward adipogenic differentiation by culturing cells in MesenCult MSC Basal Medium supplemented with MesenCult Adipogenic Stimulatory Supplements. C3H 10T(1/2) cells seeded on NELL-1 pre-coated plates showed inhibition of adipogenesis. This was observed by Oil red O (ORO) staining at 21 days adipogenic

differentiation (**Fig. 11a**). Quantification of Oil Red O staining of the cells confirmed suppression of adipogenesis (**Fig. 11b**, $*p<0.01$). Thus, NELL-1 pre-coating was observed to be an effective method for decreasing the adipogenic differentiation of C3H 10T(1/2) cells.

DISCUSSION

NELL-1 is an osteoinductive protein that is regulated by Runx2, which controls the osteogenic differentiation of mesenchymal stromal cells. It is a critical molecule for osteoblast differentiation and function. A NELL-1 overexpression study showed significant increase of bone formation in calvarial sutures of transgenic animals [9]. Homozygous mouse with ENU-induced recessive mutations of NELL1 resulted in gross cranial defects as well as other skeletal abnormalities [10]. Notably, the interactions between extracellular matrix and osteoblast play a key role in stimulating signaling pathways that together promote osteoblast-specific gene expression and differentiation [80,81]. NELL-1, a secreted protein, has been proposed to be an ECM from its structural property by in silicon analysis. Experimentally, NELL-1 has also been verified to promote osteogenic effect when it's used as an adhesive molecule [82]. However, the underlying mechanism of this effect remained unknown. In this study, we took multiple approaches to investigate a possible binding of NELL-1 to Integrin beta-1 and its functional impact in inducing osteogenic differentiation in bone marrow stromal cells.

Integrin as a potential NELL-1 receptor

Integrin has been known to be a biological mediator in cell adhesion, differentiation and other signal transduction pathways. We think that Integrin receptor seems to be one of promising candidates for NELL-1 binding receptor because NELL-1 has an NH2-terminal thrombospondin-1 (TSP-1) like module that overlaps with a Laminin G domain. TSP-1 was found to interact with Integrin $\alpha 4\beta 1$ via recognition of LDVP motif [54]. As NELL-1 protein has a TSP-1 like domain with LDVP motif (**Fig. 1**), we believed NELL-1 might interact with Integrin.

Our immunoprecipitation assay showed that NELL-1 directly binds to the extracellular domain of Integrin beta-1 (**Fig. 2**). Furthermore, flow cytometry data showed that knockdown of Integrin beta-1 expression using siRNA decreased the binding of NELL-1 in ST2 cells (**Fig. 8a,b**). This data suggests that Integrin beta-1 may be a receptor for NELL-1. However, the binding specificity of NELL-1 on α subunit of Integrin has yet to be determined. We also demonstrated that Integrin beta-1 siRNA treatment blocked ST2 cell adhesion induced by NELL-1 at various concentrations (**Fig. 8c).** This result indicated that when Integrin beta-1 was knocked down, NELL-1 mediated cell adhesion capacity decreased drastically. Thus, this data suggest that NELL-1 promotes ST2 cell adhesion by directly interacting with Integrin beta-1. Currently, the investigation of specific binding domains of NELL-1 to Integrin beta-1 is ongoing in our lab. A recent study showed that NELL-1 could bind to apoptosis related protein 3 (APR3) in the osteoblast, and affect osteoblastic proliferation and differentiation [83]. However, the intracellular distribution of APR3 and the location of interaction between NELL-1 and APR3 didn't reveal the APR3 is a cells surface receptor of NELL-1. The direct binding of NELL-1 to Integrin beta-1 on cell surface likely indicated that the Integrin beta-1 may function as a NELL-1 receptor or one component of the receptor complex.

A new signal pathway mediating NELL-1's osteoinductivity:

Another important finding in this study was that NELL-1 might initiate signaling pathway through enhancing phosphorylation of FAK upon NELL-1's binding to Integrin beta-1. FAK is known to have association with β1 subunit of Integrin receptors [52]. Our data showed that NELL-1 increased the amount of phosphorylated FAK in ST2 cells (**Fig. 4a**). FAK mediates a variety of biological processes including proliferation, adhesion, and differentiation through

activating its downstream targets such as MAPK, ERK and JNK [84] and PI3K/PKD1/Akt/PKB and Grb2/SOS/Ras/Raf-1/MEK/ERK pathways [85].

Several signal pathways may be involved in the positive effect of NELL-1 on osteoblastogenesis in multiple cell lines [48,76]. Previous studies showed that NELL-1 protein activated ERK1/2 and JNK1/2/3 kinases in primary rat fetal calvarial cells after 10 minutes of exposure [48]. NELL-1 was shown to promote osteoblastgenesis through MAPK pathway and Runx2 phosphorylation in new born calvarial cells [76], RFC and mouse mesenchymal cells [48], but not BMP2 mediated Smad or p38 pathways in C2C12 cells [23,48].

A recent study suggested that Integrin and FAK activation synergized with Wnt signaling may go through a Grb2-rac-jnk-c-jun pathway [86]. Canonical Wnt signaling has been reported to promote osteoblastogenesis and inhibit adipogenesis in BMSCs [43,44,87]. Our previous *in vivo* studies found a significant nuclear accumulation of β-catenin after treatment of NELL-1 and NELL-1+BMP2, but no sign of β-catenin in BMP2 treatment group (Data not shown). This finding suggests Nell-1 may promote bone formation by regulating Runx2 via canonical Wnt signaling. However, further investigations are required to verify NELL-1's role in activation of Wnt signaling when osteogenic signals are transduced through Integrin /FAK pathway.

The activation of FAK or its downstream mediators induces cell adhesion, migration, proliferation and differentiation [88,89,90]. In this study, NELL-1 promoted cell adhesion in culture across a number of different cell lines such as C3H10T1/2 (multipotential mesenchymal cells), ST2 and M2-10B4 (bone marrow stromal cells), and ATDC5 (chondroprogenitor cells) **(Fig. 6a,b**). In our cell proliferation assay, NELL-1 significantly increased cell proliferation in only ST2 cell line (**Fig. 9**) but not in the M2-10B4 or C3H10T(1/2). We believe this difference in NELL-1 induced cell proliferation among the three cell lines may be caused by the difference in

cell types, as different cell types may respond differently. In addition to cell proliferation, we saw NELL-1 promoting cell migration by recruiting cytoskeleton as a result of Integriβ1 activation and FAK phosphorylation (**Fig. 5**).

Role of NELL-1 in osteogenesis and adipogenesis

The osteogenic differentiation of BMSCs is characterized by osteoblast markers such as alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN) and type I collagen (Col1A1) [91,92]. NELL-1 has exhibited its pro-osteogenic effects with several types of cells when it's applied as a soluble factor in our previous studies [16,93]. In this study, we used pre-coated NELL-1 cell plates instead of adding NELL-1 into the cell medium. As a novel way of administering NELL-1, the pre-coated Nell-1 significantly promoted osteogenic differentiation in ST2 cell lines which was confirmed by increase in OCN expression and ALP staining in addition to promoting its adhesion and proliferation. Similar to soluble NELL-1 in cell medium, pre-coated NELL-1 also inhibited adipogenic differentiation in C3H10T(1/2) cell lines which was confirmed by Oil red O (ORO) staining (**Fig. 11**). These findings warrant further investigations on the efficiency of NELL-1's role in promoting osteogesis and inhibiting adipogenesis when using pre-coated NELL-1 scaffold *in vivo*. In addition, the identification of NELL-1 directly binding to Integrin beta-1 and activating FAK signaling may just open a new avenue for further exploration of the underlying molecular mechanisms of NELL-1's effects. Consistent with *in vitro* finding which NELL-1enhances osteogenic differentiation in BMSC, *in vivo* study showed that NELL-1 formed a mature bone in the muscle when NELL-1 gene transduced BMSCs were injected into thigh muscle of nude mice [77]. In addition, our present data showed that NELL-1 alone significantly inhibited PPARγ expression in BMSC. It suggests that NELL-1 mayt have an

inherent suppressive effect on adipogenesis (Unpublished data).

Clinical significance of NELL-1 in tissue engineering

Mesenchymal stromal cells (MSCs) have capabilities to differentiate into osteogenic, chondrogenic, or adipogenic lineages. Their multipotent ability is thought to be the reason why many believe they can be the potential source for tissue regeneration. These cells require other materials such as scaffolds and growth factors to induce differentiation [94]. NELL-1, as a soluble osteoinductive protein, has been successfully applied to diverse animal models to promote bone formation. Application of NELL-1 in bioengineered scaffolds has been predominately used in orthopedic surgeries [14,15,16], and has been successful in promoting osteogenesis in many of our *in vivo* studies. In order to apply NELL-1 and to develop NELL-1 into potential therapy for bone regenerative orthopedic surgeries, the identification of molecular mechanisms which NELL-1 may direct osteogenic differentiation and inhibit adipogenic differentiation is crucial. The current findings of NELL-1's role in promoting cell adhesion and proliferation to solid surface provided a new angle to reveal its mode of action in bone tissue engineering.

Our lab is currently investigating the potential mechanisms through which NELL-1 may enhance osteogenic differentiation and suppress adipogenic differentiation in BMSC. Recently, we conducted studies using combination of BMP2 and NELL-1 pre-coated on PLGA scaffold in *in vivo* segmental bone defect model to investigate its synergistic effects. Our data suggested that synergistic appliance of NELL-1 and BMP2 had better quality of bone formation and prevented adipogenesis induced by high dose BMP2. In this context, suppression of adipogenic factors PPARγ by NELL-1 can favor ostegenic differentiation of mesenchymal stem cells (MSCs) rather

than the adipocyte lineage. This finding may extend the combinatory application of NELL-1 and BMP2 to age related bone diseases such as osteoporosis in which balance between bone formation and resorption is altered. With the new finding of NELL-1 in promoting cell adhesion and proliferation to tissue culture plates, the further optimization of scaffold fabrication and NELL-1 delivery will be our next step to make better use of NELL-1 in bone tissue engineering field.

Conclusion

Collectively, we found that NELL-1 directly binds to Integrin beta-1. Pre-coated NELL-1 on culture plate significantly promotes adhesion and proliferation of BMSCs cells. The activation of Integrin and FAK signaling pathway through the direct interaction between Integrin beta-1 and NELL-1 may play critical role in NELL-1-induced osteoblast differentiation although the possible connection of FAK and MAPK activation by NELL-1 remained unknown. Our current *in vitro* findings may expand the clinical applications of NELL-1 into tissue engineering fields, especially bone formation and regeneration. Further investigations need to be conducted not only to verify the data obtained from this *in vitro* study, but also to develop a better therapeutic protocols for promoting *in vivo* bone regeneration through tissue engineering by utilizing these new findings.

FIGURES

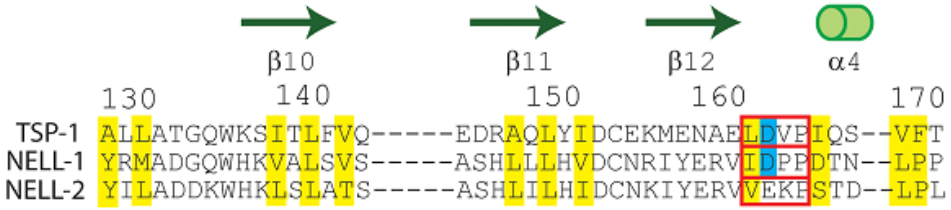


Figure 1. LDVP Sequence in NELL-1 and TSP-1. This diagram aligns the sequence of NELL-1, NELL-2 and TSP-1 to highlight in red the presence of LDVP sequence.

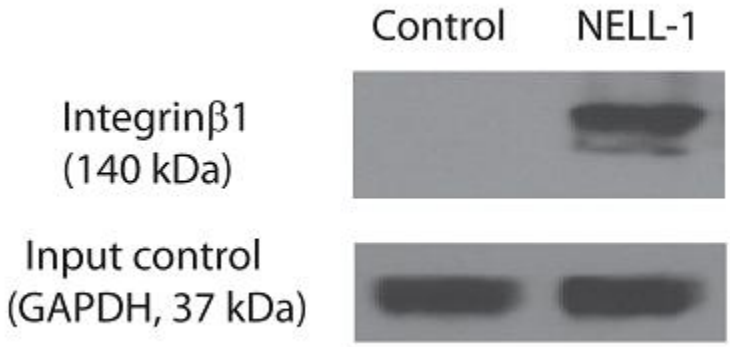
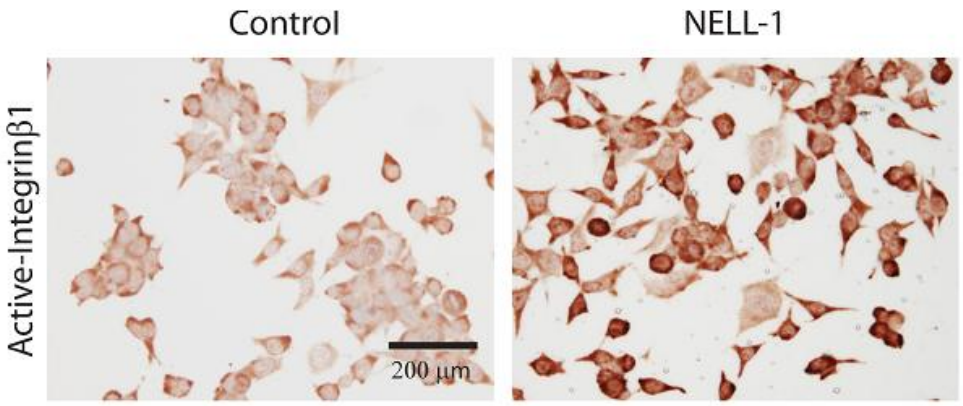


Figure 2. NELL-1 Directly Interacts with Integrin beta-1. Immunoprecipitation followed by western blot analysis indicates the physical binding of NELL-1 protein onto extracellular domain of Integrin beta-1. Sulfo-SBED biotin label transfer reagent cross linked NELL-1 and Integrin beta-1. The bait protein, Integrin beta-1, becomes associated with biotinylated interacting complex. This complex was pulled down by Streptavidin-conjugated sepharose beads and detected for the existence of Integrin beta-1 by the western blot. PBS without NELL-1 was used as negative control. No Integrin beta-1 band was seen in control. The level of GAPDH before pulldown was used as input control.

a)



b)

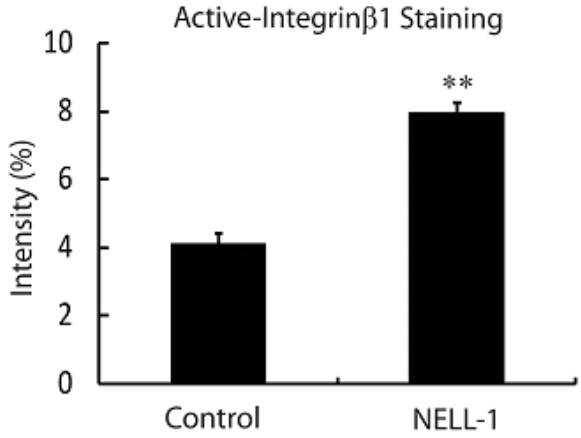
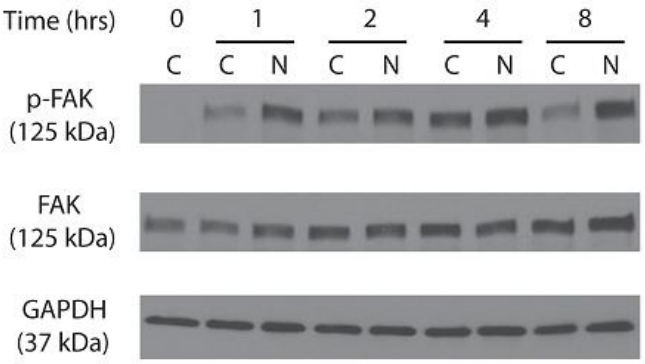


Figure 3. NELL-1 Activates Integrin beta-1 in ST2 cells. a) Immunocytochemistry analysis of ST2 cells incubated in NELL-1 protein (10 μg/ml) coated Millicell EX 8 well chamber slides exhibited significantly stronger staining for active Integrin beta-1 than PBS control. These images were taken two hours after cells were seeded. **b)** Quantification of active Integrin beta-1 was done using Image-Pro Plus software on 8 separate random fields.

a)



b)

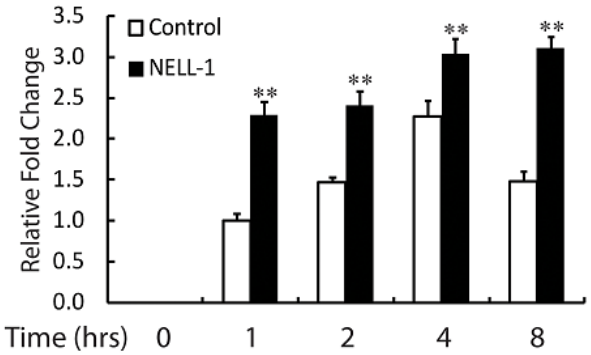


Figure 4. NELL-1 Protein Promotes Phosphorylation of FAK in ST2 cells. a) ST2 cells were seeded in 24-well plates coated with NELL-1 protein (N, 10 μg/ml). For different time points (0, 1, 2, 4, 8 hours), each group had three repeats. Western blot analysis of NELL-1 treated and non treated detected an increase in phosphorylated FAK in NELL-1 from 1-8 hours after seeding the cells when compared to control. The control group substituted NELL-1 protein with PBS. **b)** Quantification of the level phosphorylation of FAK in **a)** on three replicates using Image J software confirmed western blot analysis. ** $p < 0.01$ compared to control at corresponding time points.

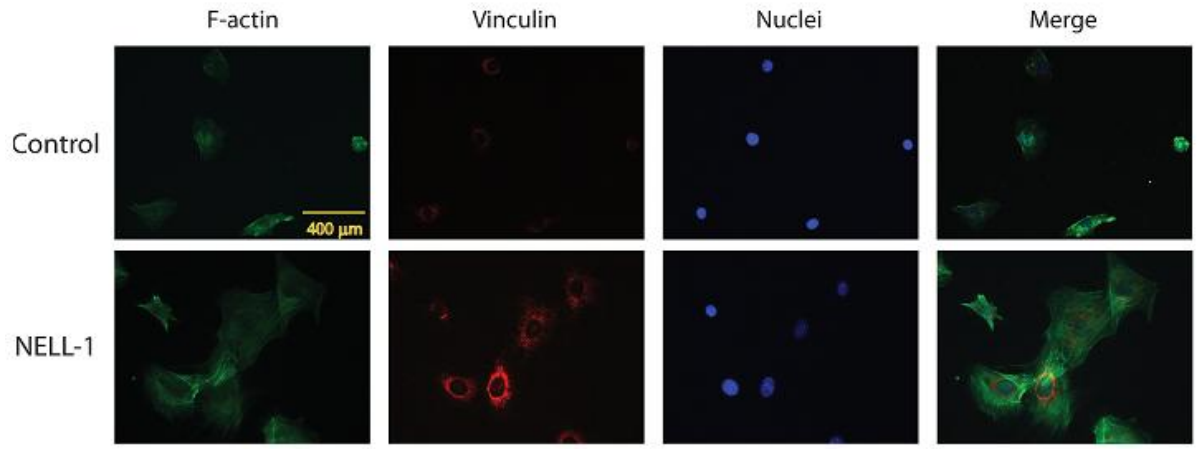


Figure 5. NELL-1 Induces Accumulation of Cytoskeleton as a Result of Integrin beta-1 Activation and FAK Phosphorylation. ST2 cells were seeded in Millicell EX chamber slides that were coated with PBS (control) or NELL-1 protein (10 µg/ml). After two hours, F-actin and Vinculin, both proteins that bind onto activated FAK, showed greater amount of staining in wells coated with NELL-1 compared to that of control.

a) **b)**

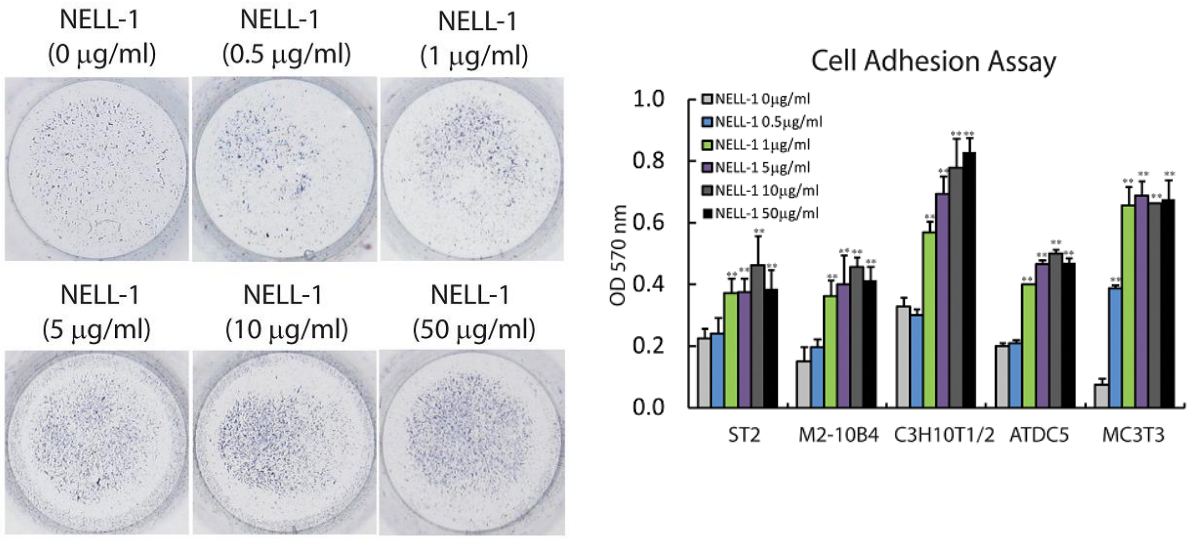
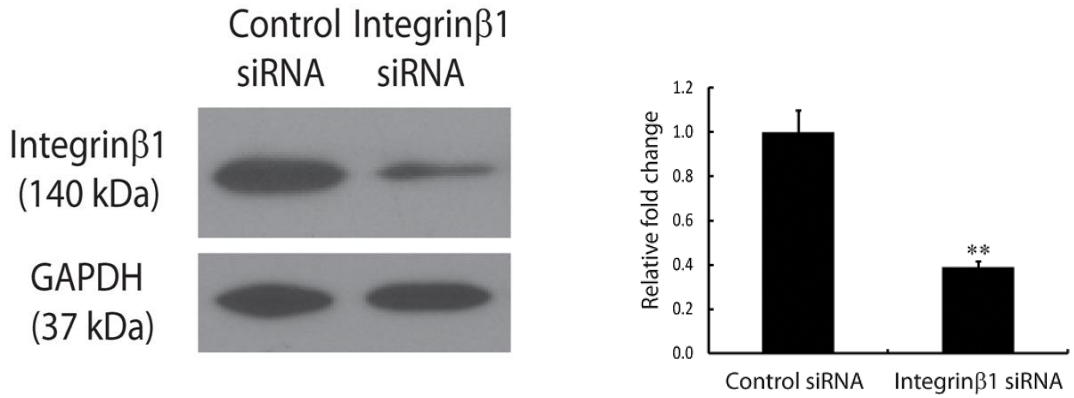


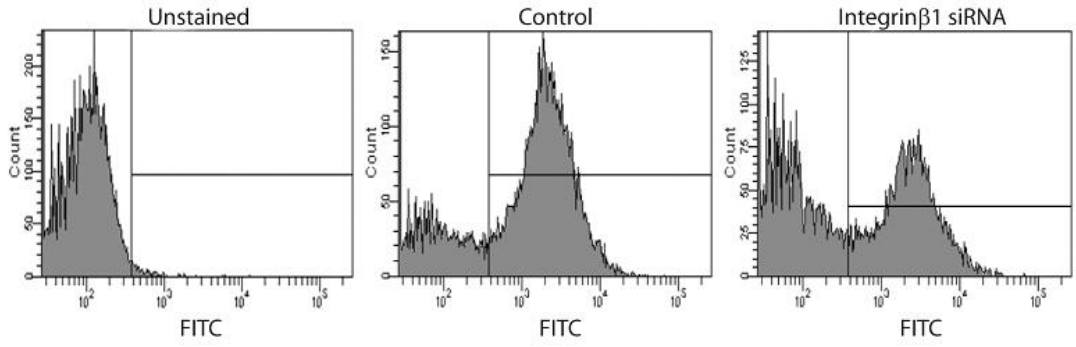
Figure 6. NELL-1 Induces Cell Adhesion. a) On the left, ST2 cells were seeded on a 96 well plate coated with increasing NELL-1 dosage (0, 0.5, 1, 5, 10, 50 μg/mL) to observe NELL-1 promoting cell adhesion. Afterwards, crystal violet stain was used to stain adhered cells and photographed. **b)** After staining, each well was treated with 10% acetic acid to quantify cell adhesion at 570 nm.



a) **b)**

Figure 7. siRNA Knockdown of Integrin beta-1. a) To ensure siRNA knockdown of Integrin beta-1 in ST2 cells, western blot analysis was done. This figure verified decrease in Integrin beta-1 expression in ST2 cells. GADPH was used as a negative control. **b)** Quantification of the western blot using Image J Pro Plus indicates significant drop in Integrin beta-1 expression after treating with Integrin beta-1 siRNA compared to scrambled siRNA as a negative control.

a)



b) **c)**

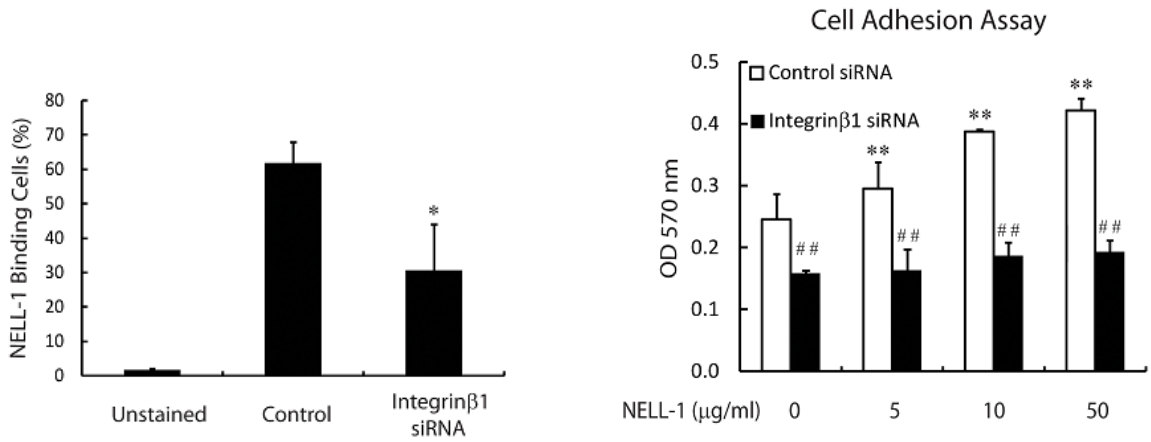


Figure 8. Knockdown of Integrin beta-1 Affects Cell Surface Binding of NELL-1. a) Flow cytometry analysis of NELL-1 binding on ST2 cells with or without knocked down expression of Integrin beta-1. After treatment with unstained control, scrambled siRNA, and Integrin beta-1 siRNA, ST2 cells were incubated with His-tagged NELL-1 protein stained with FITC. Negative control ST2 cells were not incubated with NELL-1. Data showed that Integrin beta-1 knockdown cells had lower number of cells binding with His-tagged NELL-1 than cells with normal Integrin beta-1 expression. **b)** Quantification of flow cytometry data showed a significant decrease from 61% to 32% in binding of NELL-1 when Integrin beta-1 expression is lowered in ST2 cells. n=4. *p<0.05 compared to control. **c)** On the right, knockdown of Integrin beta-1 affected NELL-1 mediated ST2 cell adhesion. As siRNA Integrin beta-1 blocked the binding of NELL-1, NELL-1 cannot promote cell adhesion without presence of Integrin beta-1. ** $p< 0.01$ compared groups of different doses of NELL-1 treatment to NELL-1 (0 μg/ml) group. ## $p< 0.01$ compared control siRNA and Integrin beta-1 siRNA groups at the same dose of NELL-1 treatment.

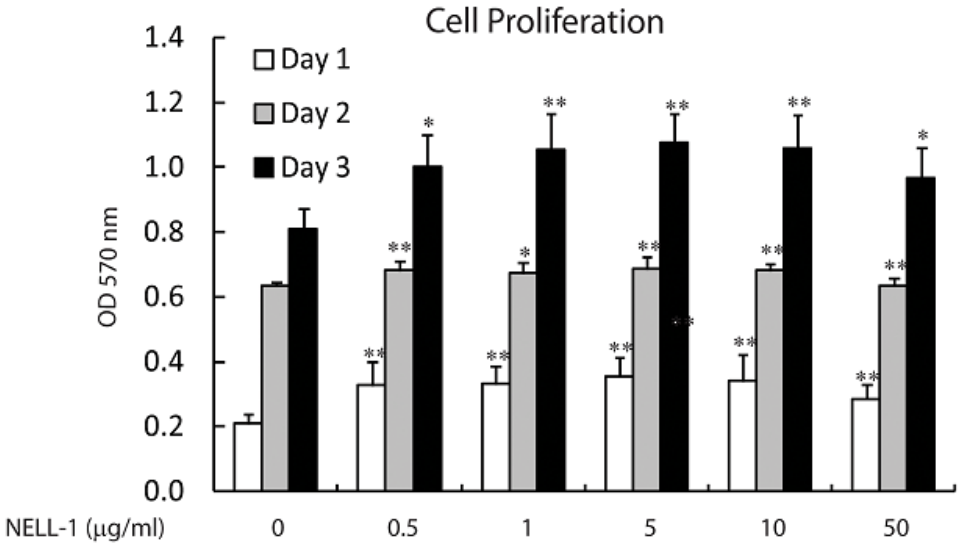


Figure 9. NELL-1 Promotes Cell Proliferation. ST2 cells were seeded in a 96 well plate with pre-coated NELL-1. Each group had different dosage of NELL-1 to observe its ability to promote cell proliferation. Using MTS assay, cell proliferation was measured from day 1 to day 3. The readings (at 570 nm) indicate increase in proliferation between NELL-1 and control during the three days. This data confirms cell proliferation promoted by NELL-1. * $p< 0.05$, ** $p< 0.01$ compared to 0 μg/ml NELL-1 group at corresponding time point.

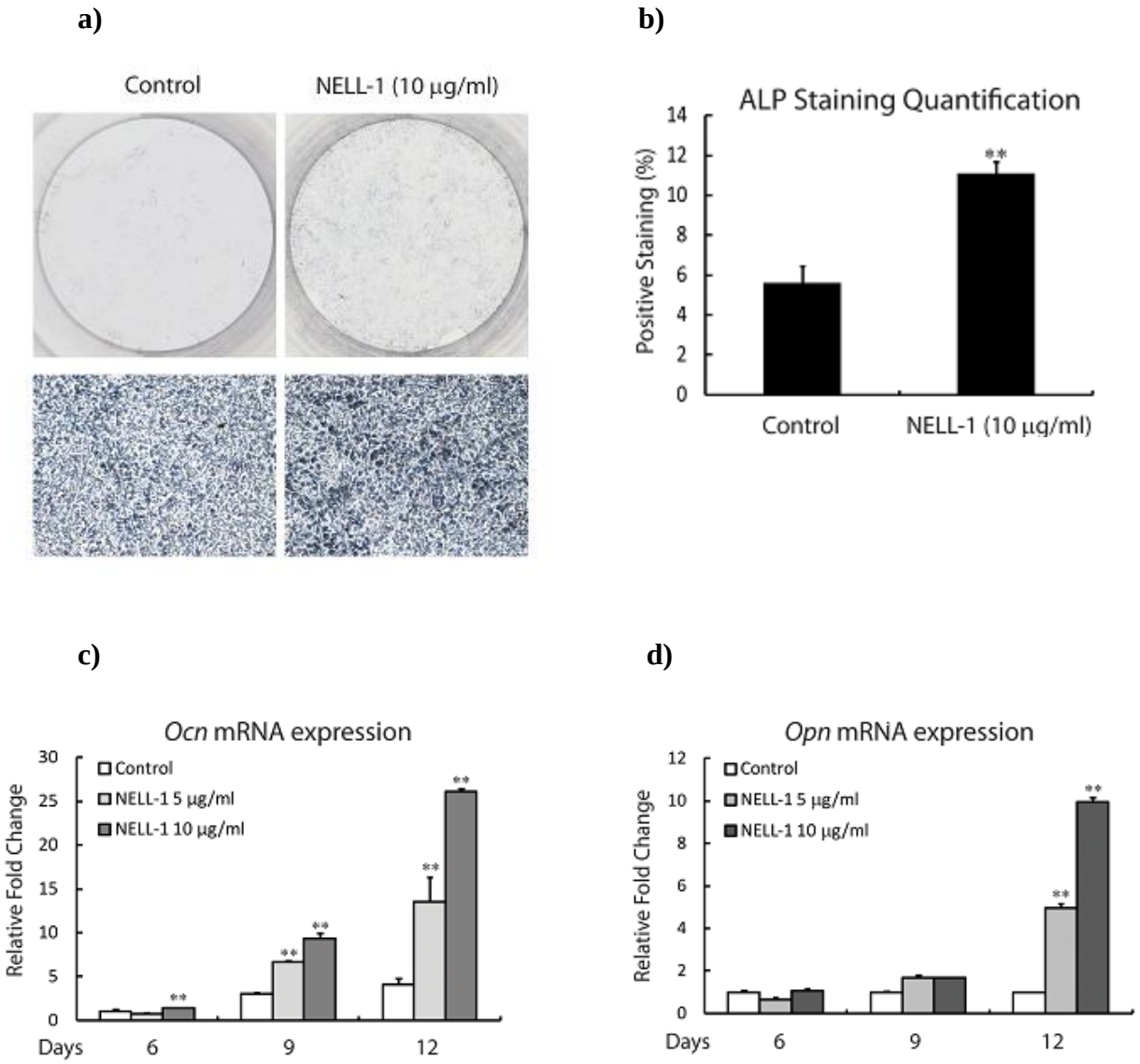
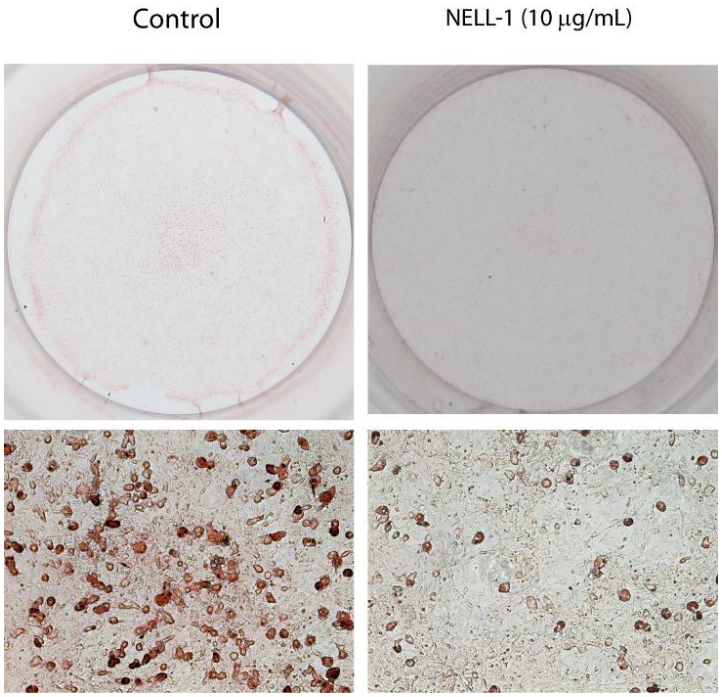


Figure 10. NELL-1 Promotes Osteogensis in ST2 cells. a) After ST2 cells were seeded on 24-well plates coated with PBS or NELL-1 (10 μg/ml) and cultured in osteogenic differentiation medium for 9 days, ALP was performed on the cells. Each group had n=3. **b)** ALP staining was quantified using Image-Pro Plus 6.0 software on 8 separate random fields. **c)**, **d)** ST2 cells were seeded on 24-well plates coated with PBS or indicated NELL-1 dosage. Three different time points (6, 9, and 12 days) were chosen to look at the expression level of osteogenic differentiation markers (*Ocn* and *Opn*). Real time PCR analysis showed significant increase in both early and late osteogenic markers. ** $p < 0.01$ compared to control group at corresponding time point.

a)



b)

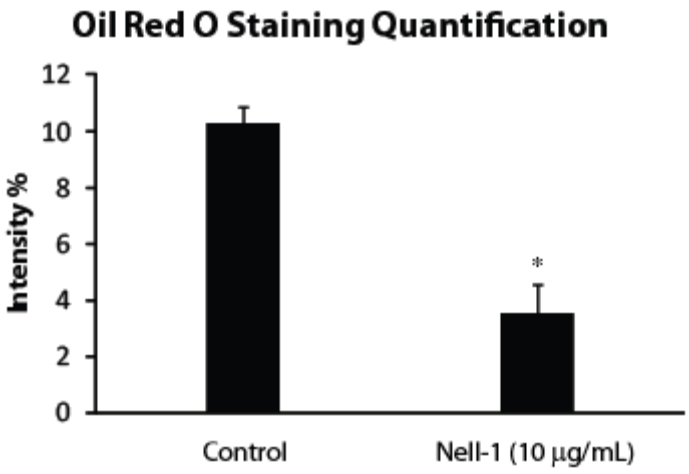


Figure 11. NELL-1 Inhibits Adipogenic Differentiation in C3H10T(1/2) Cells. a) After C3H10T(1/2) cells were seeded on 24-well plates coated with PBS or NELL-1 (10 μg/ml) and cultured in adipogenic differentiation medium for 21 days, cells were stained with Oil Red O stain. Each group had n=3. **b)** Oli Red O staining was quantified using Image-Pro Plus 6.0 software on 8 separate random fields. * $P<0.01$ compared to control group at corresponding time point.

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