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NELL-1 Promotes Cell Adhesion and Differentiation via Integrinβ1

Jia Shen,[1] Aaron W. James,[1,2] Jonguk Chung,[1] Kiho Lee,[1] James B. Zhang,[1] Stephanie Ho,[1] Kevin S. Lee,[1] Toyong M. Kim,[1] Tomoaki Niimi,[3] Shun'ichi Kuroda,[3] Kang Ting,[1,2*] and Chia Soo[2**]

[1]*Dental and Craniofacial Research Institute and Section of Orthodontics, School of Dentistry, University of California, Los Angeles, Los Angeles, California 90095*

[2]*UCLA and Orthopaedic Hospital, Department of Orthopaedic Surgery and the Orthopaedic Hospital Research Center, University of California, Los Angeles, Los Angeles, California 90095*

[3]*Department of Bioengineering Sciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furocho, Chikusa-ku, Nagoya 464-8601, Japan*

ABSTRACT

NELL-1 (Nel-like molecule-1) is a secreted osteogenic growth factor first identified in human craniosynostosis (CS) patients. NELL-1 protein has been observed to promote bone and cartilage differentiation and to suppress adipogenesis in both in vitro and in vivo models. Despite these findings, the cell surface receptors of NELL-1 have remained unknown. In this study, we observed for the first time that NELL-1 promotes cell adherence in multiple cell lines, including ST2, C3H10T1/2, M2-10B4, ATDC5, and MC3T3 cells. Additionally, we found that NELL-1 binds to extracellular Integrinβ1 and induces cell focal adhesion. By utilizing siRNA methods, we determined that NELL-1 cell surface binding and enhanced cell attachment were dependent on Integrinβ1 expression. Finally, we observed that pre-coating of culture dishes or PLGA (polylactic-*co*-glycolic acid) scaffold with NELL-1 resulted in a significant increase in both cell attachment and osteogenic differentiation. Our results identify for the first time a cell surface target of NELL-1, Integrinβ1, and elucidate new functions of NELL-1 in promoting cell adherence and osteogenic differentiation. J. Cell. Biochem. 113: 3620–3628, 2012.

KEY WORDS: NELL-1; ST2; OSTEOGENIC DIFFERENTIATION; CELL ADHERENCE; FAK; INTEGRINβ1

NELL-1 (Nel-like molecule-1) is an osteogenic growth and differentiation factor first identified in human craniosynostosis (CS) patients [Ting et al., 1999]. Transgenic *Nell-1* overexpressing mice exhibit gross bone overgrowth and a CS-like phenotype [Zhang et al., 2002]. In contrast, *Nell-1* deficient mice result in major skeletal anomalies with reduced bone formation in the craniofacial complex, spine, and long bones [Zhang et al., 2012]. Previous studies have found that recombinant NELL-1 protein effectively induces bone and cartilage formation and suppresses adipogenesis in various in vitro and in vivo models [James et al., 2011; Li et al., 2010; Siu et al., 2011; Siu et al., 2012]. Mechanistically, NELL-1 is known to regulate Runt-related transcription factor-2 (Runx2) activity and phosphorylation [Zhang et al., 2011b], MAPK signaling [Bokui et al., 2008], and Hedgehog signaling pathways [James et al., 2011]. Recently, the intracellular molecule, apoptosis-related protein 3 (APR3), was identified to

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*Correspondence to: Kang Ting, DMD, DMedSci, School of Dentistry, University of California, Box 951668, CHS 30-113, Los Angeles, CA 90095-1668. E-mail: kting@dentistry.ucla.edu

**Correspondence to: Chia Soo, MD, FACS, 200 UCLA Medical Plaza Suite 445, Los Angeles, California 90095. E-mail: bsoo@ucla.edu

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interact with NELL-1 [Zou et al., 2011]. Despite such accumulating data, the cell surface receptors of NELL-1 have been completely unknown to this date.

NELL-1 is a secreted protein comprised of 810 amino acids with a molecular weight of about 90 kDa before *N*-glycosylation and oligomerization. Recombinant human NELL-1 has a molecular weight of about 140 kDa under reducing conditions and has been reported to be secreted as either trimers or pentamers [Zhang et al., 2010]. NELL-1 contains several structural motifs, including a secretory signal peptide, a thrombospondin-1 (TSP-1)-like module (TSPN, overlapping with a laminin G domain), four chordin-like, cysteine-rich (CR) domains and six epidermal growth factor (EGF)-like domains.

Integrins are cell surface receptors that mediate cellular adhesion to extracellular matrix structures and to other cells. Integrin-mediated adhesive events are shown to be important for cell proliferation, function, and survival [Martin et al., 2002]. The binding of ligands to Integrin receptors leads to clustering of Integrin proteins, promoting the formation of focal adhesion structures, which play important roles in modulating cell adhesion and cell shape changes [Kim et al., 2011]. Furthermore, it has been observed that Integrinβ1-specific collagen-mimetic surface supports osteoblastic differentiation [Reyes and Garcia, 2004]. NELL-1 is essential in developing proper extracellular matrix proteins for bone and cartilage cell differentiation [Chen et al., 2011]. Most recently, NELL-1 was verified to promote cell adhesion and osteogenic differentiation without an understanding of the underlying mechanisms [Hasebe et al., 2012]. It has been reported that Integrinβ1, one of the β subunits of Integrins, interacts with the LDVP (Leu-Asp-Val-Pro) sequence in the N-terminal region of TSP-1 [Li et al., 2002]. NELL-1 shows high sequence homology to TSP-1 (33.2% homology in the TSPN domain) and contains the conserved LDVP sequence at the corresponding position in its TSPN domain modules [Tan et al., 2006]. Therefore, NELL-1 may also interact with cell surface Integrinβ1 through its TSPN module. In this study, we have shown NELL-1 indeed promotes cell adhesion through binding Integrinβ1 by multiple approaches, which opens a new avenue for mechanistic investigation of NELL-1's osteoinductivity.

MATERIALS AND METHODS

CELL CULTURE AND CELL ADHESION ASSAY

The ST2 cell line, a clone derived from mouse bone marrow was purchased from American Type Culture Collection (ATCC, Manassas, VA). The 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). Cell adhesion assay was performed as previously described [Humphries, 2009]. NELL-1 protein, in PBS solution at indicated concentration was added to 96-well plates (100 μl/well). The plates were then placed on a shaker for 10 min prior to incubation overnight at 4°C. Then, 1% BSA was used as a blocking reagent before the cells were seeded. ST2 cells in RPMI medium, C3H10T1/2 cells in DMEM medium, MC3T3 and M2-10B4 cells in RPMI medium, and ATDC5 cells in DMEM/F-12 (1:1) medium at the concentration of 5×10^5 cells/ml (100 μl/well) were added to each well for 15 min. Attached cells were stained with crystal violet for 60 min and quantified in acetic acid at 570 nm.

WESTERN BLOT AND REAL-TIME PCR

Western blot and real-time PCR were performed as previously described [Chen et al., 2011]. Anti-phosphorylated FAK (Cell Signaling), anti-FAK (Cell Signaling), anti-Integrinβ1 (Abcam), and GAPDH (Santa Cruz) primary antibodies were used at 1:1,000 dilution. The real-time PCR was performed using the 7300 Real-Time PCR System instrument (Applied Biosystems). The reactions were incubated in 96-well optical plates at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and at 60° for 10 s. The threshold cycle (C_t) data were determined using default threshold settings. The C_t was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. For gene expression studies of osteogenesis, SYBR Gene Expression Master Mix primer sequences of *Osteocalcin* (*Ocn*) were sense: GCAATAAGGTAGT-GAACAGACTCC and anti-sense: AGCAGGGTTAAGCTCACACTG, and those of *Osteopontin* (*Opn*) were sense: CAGCCTGCAA-GATCCTA and anti-sense: GCGCAAGGAGATTCTGCTTCT. The primer sequences of *GAPDH* control were sense: TGCACCACC-AACTGCTTAGC and anti-sense: CCACCACCCTGTTGCTGTAG.

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 h. Attached cells were fixed with ice-cold acetone for 10 min and blocked with 1% BSA. Anti-active Integrinβ1 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 Integrinβ1 staining was quantified by the mean optical density of staining signal × per percent area positively stained × 100 [Allen, 2002]. For immuno-fluorescent 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.

IMMUNOPRECIPITATION

Sulfo-SBED Biotin Label Transfer Reagent (Thermo Scientific) was used to crosslink NELL-1 and cell surface Integrinβ1. 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 min to allow NELL-1 to 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 Integrinβ1 was performed by using a long-wave UV lamp (365 nm). Then, the interacting complex was pulled down by Streptavidin-conjugated sepharose beads (Cell Signaling), and Integrinβ1 (Abcam) was detected by the Western

blot from elute of the sepharose beads upon reduction of the disulfide bonds in SBED.

NELL-1 CELL SURFACE BINDING ASSAY

ST2 cells (5×10^5) were incubated in 100 μl of his-tagged NELL-1 (1 μg/ml) for 30 min on ice. The cells 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 × His tag (Thermo Scientific), and incubated for 30 min on ice in the dark. After two washes with 1 ml of PBS, samples were analyzed by flow cytometry (LSRII; BD Biosciences).

OSTEOGENIC DIFFERENTIATION AND ALKALINE PHOSPHATASE (ALP) STAINING OF ST2 CELLS

For osteogenic differentiation, ST2 cells were seeded on 24-well plates at a density of 5×10^4 cells/well. ALP assay were performed in triplicate wells. After attachment, cells were treated with osteogenic differentiation medium consisting of RPMI 1640, 10% FBS, 50 μg/ml ascorbic acid, and 3 mM β-glycerophosphate for 9 days. ALP staining was performed as previously described [James et al., 2009].

SMALL INTERFERING RNA EXPERIMENTS

RNA knockdown experiments were performed using chemically synthesized and annealed small-interfering RNA (siRNA) specific to Integrinβ1 (Santa Cruz). When ST2 cells reached 30% confluence, cells were transfected with 50 nM Integrinβ1 siRNA or non-target negative control siRNA (Santa Cruz) using Lipofectamine RNAiMax (Invitrogen). Efficiency of knockdown was validated using Western blot.

PLGA SCAFFOLD FABRICATION AND NELL-1 PROTEIN COATING

PLGA scaffold was fabricated as previously described [Cowan et al., 2007]. One hundred nanogram NELL-1 protein or PBS was applied to the scaffold and then allowed to be absorbed before 1.5×10^5 MC3T3 cells were seeded. After 1 h of cell attachment, scaffolds were cultured in osteogenic differentiation media containing 50 μg/ml ascorbic acid, 10 mM β-glycerolphosphate, and 10^{-8} M dexamethasone. After 4 weeks, the MC3T3 cells cultured on scaffolds (n = 4 for each conditions) were fixed in formalin, embedded in OCT medium, and sectioned at 5 μm with cryogenic microtome. Micro-CT scanning was performed by μCT40 (Scanco) with threshold of 80.

STATISTICAL ANALYSIS

Statistical means and standard deviations were calculated from numerical data. To allow multiple comparisons between groups, statistical analyses were performed using an appropriate ANOVA for single-variable comparisons followed by post hoc Tukey's range test to directly compare two groups.

RESULTS

NELL-1 PROTEIN PROMOTES CELL ADHESION

Integrins are a family of transmembrane glycoproteins. The best-known function of Integrins is to mediate adhesion of cells to the surrounding tissue and extracellular molecules [Martin et al., 2002]. We first studied if pre-coating of NELL-1 on cell culture plates (a novel method to apply NELL-1) could increase cell adhesion. ST2 cells were primarily used for this study because the clone of mouse bone marrow stromal cells (BMSCs) has been used in previously reported cell adhesion assays [Ichida et al., 2011]. NELL-1 in a dose-dependent manner increased ST2 cell attachment when pre-coated onto culture plates overnight, and a significant increase of adhesion was observed at concentrations exceeding 1 μg/ml (Fig. 1A). The cell adhesive activity of NELL-1 was also observed with several other cell lines, including C3H10T1/2 multipotential mesenchymal cells, M2-10B4 BMSCs, ATDC5 chondroprogenitor cells, and MC3T3 pre-osteoblasts (Fig. 1B). We next examined a time-lapse sequence of ST2 cell attachment from 0 to 3 h post-cell seeding on NELL-1 pre-coated plates (Fig. 1C). ST2 cells exhibited faster adhesion and clear differences in morphology (including greater cell spreading) when placed on NELL-1 pre-coated plates. In summary, NELL-1 promoted cell adhesion in culture across a number of different cell lines and concentrations.

NELL-1 PROTEIN PROMOTES ST2 CELL PROLIFERATION

After observing that NELL-1 significantly increased cell attachment, we next examined the effects of NELL-1 pre-coating on ST2 cell proliferation. Proliferation was evaluated with a standard MTS assay. Increasing concentrations of NELL-1 significantly increased cellular proliferation of ST2 cells from Days 1 to 3 (Fig. 1D). Interestingly, even the lowest dose of NELL-1 pre-coating that was tested (0.5 μg/ml) significantly promoted ST2 cell proliferation. Thus, NELL-1 pre-coating significantly induced ST2 cell proliferation, in a fashion similar to previously observed proliferation of other cell types, including rabbit chondrocytes [Lee et al., 2010] and purified human perivascular stem cells [Zhang et al., 2011a].

NELL-1 PROTEIN STIMULATES ST2 CELL ADHESION SIGNALS

Cell adhesion is mediated by the binding of Integrin extracellular domains to diverse protein ligands. The activation of Integrins is achieved by rapid, reversible changes in the conformation of the extracellular domains of the Integrin heterodimers [Woodside et al., 2001] followed by activation of focal adhesion kinase (FAK) and regulation of cytoskeleton organization [Parsons et al., 2010]. Since NELL-1 promoted cell adhesion, we next inquired if NELL-1 could result in Integrinβ1 activation. Our results suggested ST2 cells seeded on NELL-1 pre-coated cell culture plates exhibited not only faster adhesion, but also significantly stronger staining for active Integrinβ1 when compared to PBS control at 2 h (Fig. 2A). Quantification of the intensity of active Integrinβ1 per high-powered field confirmed a significant increase with NELL-1 (Fig. 2B).

FAK is a cytoplasmic tyrosine kinase that is phosphorylated upon Integrin activation at the sites of focal adhesions [Schaller, 2010]. FAK phosphorylation was next investigated by the Western blot after ST2 cells were seeded on NELL-1 pre-coated plates (10 μg/ml NELL-1). Results showed that NELL-1 increased FAK phosphorylation, observed at all time points from 1 to 8 h after cell seeding (Fig. 2C). In contrast, there was no difference detected in the total FAK between NELL-1 and control groups (Fig. 2C). Quantification of the Western blot confirmed a significant induction of FAK

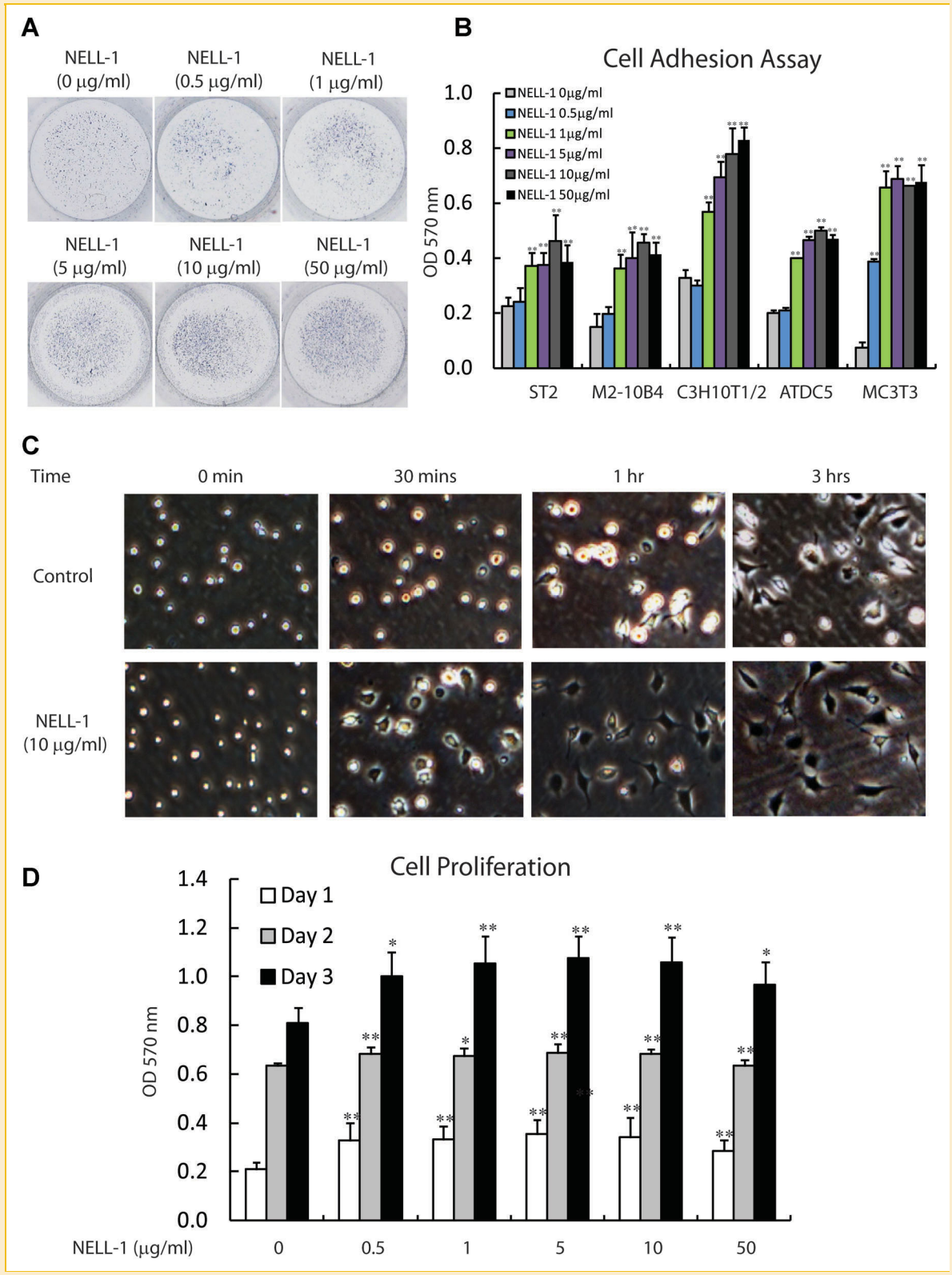

Fig. 1. NELL-1 protein stimulated cell adhesion and proliferation. A: Crystal violet staining of adherent ST2 cells. NELL-1 protein (0.5–50 μg/ml) increased ST2 cell adhesion in a dose-dependent manner. Cells were attached to the plates for 15 min. Representative image of (n = 4 wells per treatment). B: NELL-1 promoted adhesion of ST2, C3H10T1/2, M2-10B4, ATDC5, and MC3T3 cells. Cells were attached to the plates for 15 min. Photometric quantification of crystal violet staining was done by leaching the dye in 10% (v/v) acetic acid. Absorbance was measured at 570 nm (n = 4 wells per treatment). C: NELL-1 protein (10 μg/ml) stimulated faster ST2 adhesion compared to PBS control at 0, 30 min, 1 and 3 h (n = 3 wells per treatment per time point). D: NELL-1 protein coated on cell culture plates promoted cell proliferation. Cell proliferation was measured by the MTS assay. Absorbance was measured at 570 nm (n = 4 wells per treatment per time point). $^{*}P < 0.05$, $^{**}P < 0.01$ compared to 0 μg/ml NELL-1 group at corresponding time point.

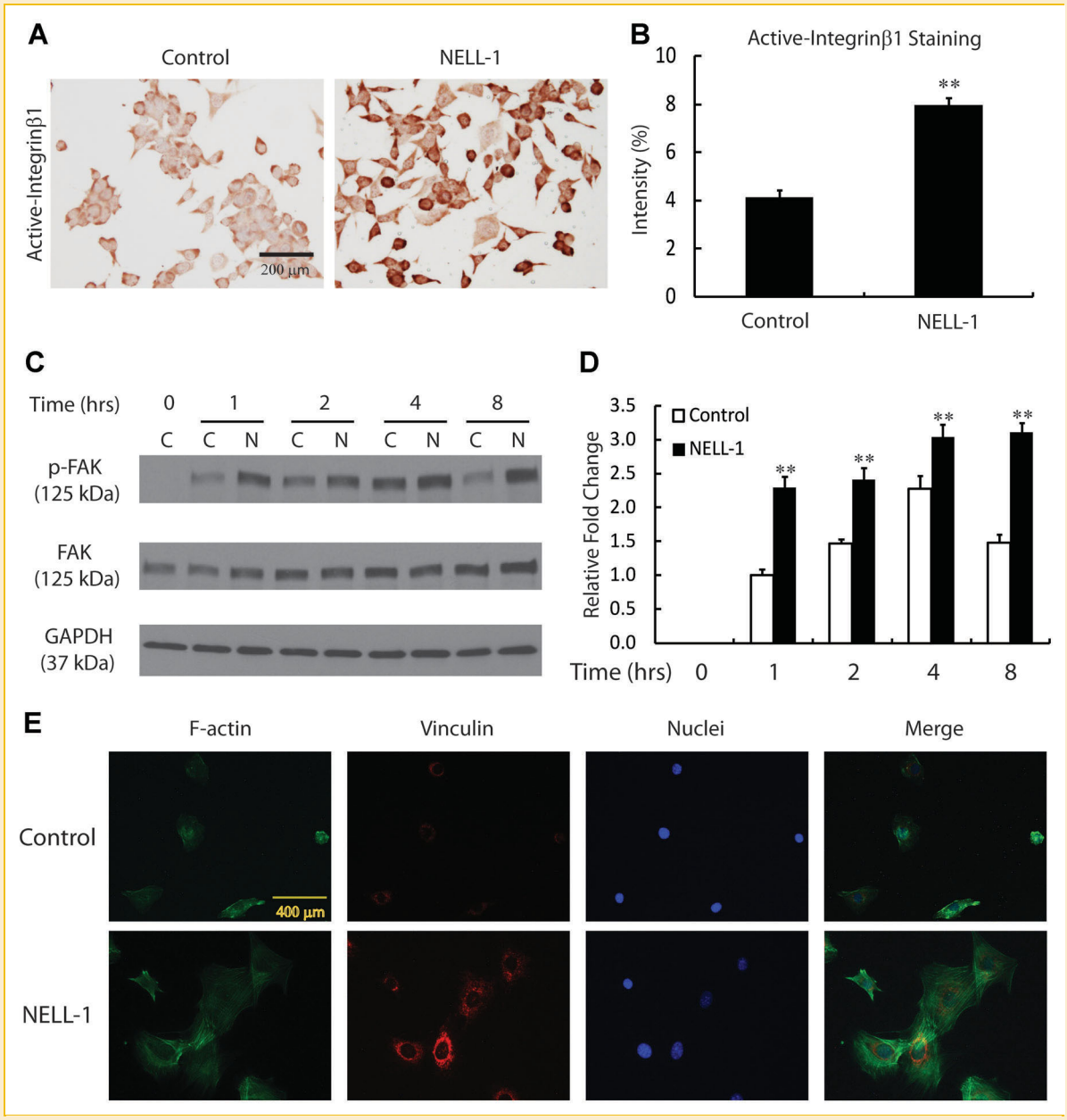

Fig. 2. NELL-1 protein stimulated cell adhesion signals. A: NELL-1 protein (10 μg/ml) was coated on Millicell EX 8 well chamber slides, and the activation of Integrinβ1 was assessed by immunocytochemistry 2 h after ST2 cell were seeded. B: Quantification of (A) using Image-Pro Plus software on eight separate random fields. C: NELL-1 protein (N, 10 μg/ml) coated on 24-well plates activated the phosphorylation of FAK at 0, 1, 2, 4, and 8 h. NELL-1 protein (C, 0 μg/ml) was used as control. Experiments were repeated in triplicate, n = 4 wells per experiment. Representative data were shown. D: Quantification of the level phosphorylation of FAK in (C) on three replicates using Image J software. E: ST2 cells on control or NELL-1 protein (10 μg/ml)-coated chamber slides, showing larger cytoskeleton assembly and adhesion size. Fluorescent staining: F-actin (green); Vinculin (red); Nucleus (blue). $^{**}P < 0.01$ compared to control at corresponding time points.

phosphorylation by NELL-1, observed from 1 to 8 h after cell seeding (Fig. 2D).

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 increased F-actin and Vinculin staining in comparison to control at 2 h after seeding (Fig. 2E). In summary, NELL-1 pre-coating resulted in Integrinβ1 activation, FAK phosphorylation, and increased cytoskeleton assembly and adhesion size in ST2 cells in vitro.

NELL-1 DIRECTLY INTERACTS WITH INTEGRINβ1; KNOCKDOWN OF INTEGRINβ1 BLOCKS NELL-1 BINDING AND INDUCTION OF CELL ATTACHMENT

Similar to TSP-1, NELL-1 contained the LDVP sequence in the TSPN domain (Fig. 3A), suggesting its binding to Integrinβ1. In order to determine if NELL-1 directly or indirectly binds to Integrinβ1, we next employed the Sulfo-SBED biotin label transfer reagent to crosslink NELL-1 with its cell surface-binding proteins. These cell surface-binding proteins were then pulled down by Streptavidin-

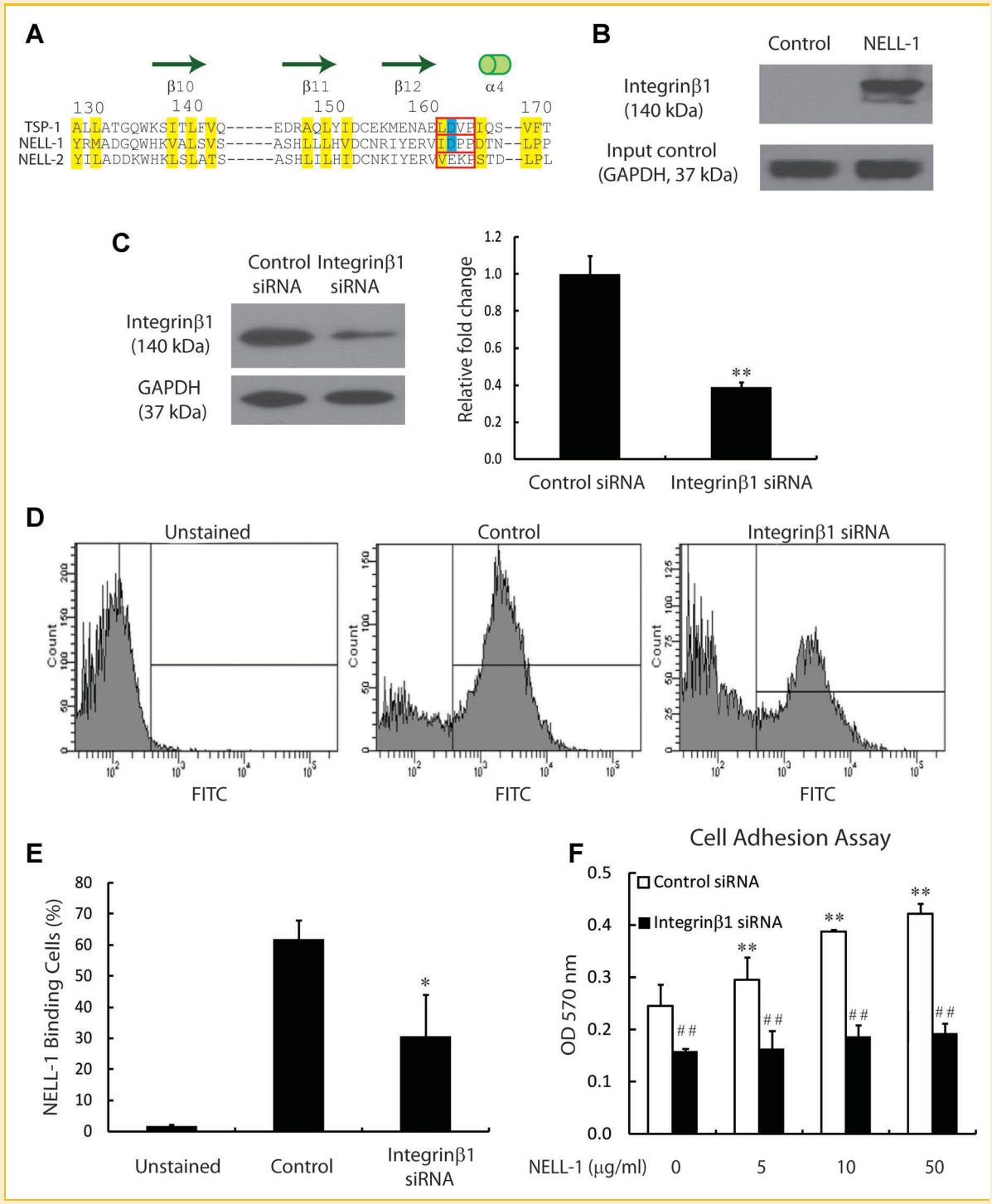

Fig. 3. NELL-1 protein promoted ST2 cell adhesion mediated by Integrinβ1. A: The sequence alignment of the human NELL-1, NELL-2 and TSP-1 of the LDVP sequence (highlighted in red) in the TSPN domain. B: NELL-1 protein directly bound to the extracellular domain of Integrinβ1. Sulfo-SBED biotin label transfer reagent was used to crosslink NELL-1 and cell surface Integrinβ1. Biotinylated interacting complex was pulled down by Streptavidin-conjugated sepharose beads and detected for the existence of Integrinβ1 by the Western blot. PBS without NELL-1 was used as negative control. No Integrinβ1 band was seen in control. The level of GAPDH before pulldown was used as input control. C: The Western blot showed the efficiency of Integrinβ1 siRNA knockdown (top) with the quantification of the Western blot (bottom) on three replicates. D: Knockdown of Integrinβ1 blocked NELL-1 binding on ST2 cells. ST2 cells were incubated with His-tagged NELL-1 protein and stained with FITC conjugated anti-His-tag antibody. Flow cytometry was performed to analyze the percentage of cells with NELL-1 binding. The percentages of NELL-1 binding cells in control and Integrinβ1 siRNA groups were shown in (E). n = 4. $^{*}P < 0.05$ compared to control group. F: Knockdown of Integrinβ1 blocked NELL-1 mediated ST2 cell adhesion. Cell adhesion assay was performed as previously described. n = 4 wells per treatment. $^{**}P < 0.01$ compared groups of different doses of NELL-1 treatment to NELL-1 (0 μg/ml) group. $^{\#\#}P < 0.01$ compared control siRNA and Integrinβ1 siRNA groups at the same dose of NELL-1 treatment.

conjugated sepharose beads. Integrinβ1 was identified as one of the biotinylated proteins by the Western blot, indicating NELL-1 directly bound to the extracellular domain of Integrinβ1 (Fig. 3B).

Having demonstrated that NELL-1 bound to the extracellular domain of Integrinβ1, we next examined whether NELL-1 binding could be altered with knockdown of Integrinβ1. To test this possibility, we treated ST2 cells with siRNA for Integrinβ1. Western blot and quantification confirmed the knockdown of Integrinβ1 (Fig. 3C). Next, flow cytometry was performed to analyze the percentage of NELL-1 binding cells (Fig. 3D,E). Our results showed a reduction in the number of NELL-1 bound cells from 61% to 32% with Integrinβ1 siRNA. Thus, Integrinβ1 siRNA significantly attenuated, but did not eliminate, NELL-1 cell binding. We next inquired whether Integrinβ1 expression was necessary for NELL-1's promotion of cell adhesion. Integrinβ1 knockdown inhibited ST2 cell adhesion under control conditions (left, Fig. 3F). More importantly, Integrinβ1 siRNA also blocked NELL-1's induction of ST2 cell adhesion at all examined concentrations (Fig. 3F). In summary, NELL-1 bound to Integrinβ1, and this NELL-1/Integrinβ1 binding was required for NELL-1's induction of cell attachment.

NELL-1 PRE-COATING PROMOTES OSTEOGENIC DIFFERENTIATION

NELL-1 protein has previously been shown to positively regulate osteogenic differentiation of BMSCs [Aghaloo et al., 2007]. However, in all cases, NELL-1 protein was added to the supernatant in cell culture. Next, we instead cultured ST2 cells on plates pre-coated with NELL-1. ST2 cells were induced toward osteogenic differentiation as previously reported, by culturing in RPMI medium supplemented with ascorbic acid and β-glycerophosphate [Otsuka et al., 1999]. NELL-1 pre-coating was observed to enhance ST2 cell osteogenic differentiation. This was observed by alkaline phosphatase (ALP) staining at 9 days (Fig. 4A), as well as photographic ALP quantification (Fig. 4B). The pro-osteogenic effect of NELL-1 pre-coating was confirmed by quantitative RT-PCR for *Osteocalcin* (*Ocn*) and *Osteopontin* (*Opn*), the markers of late-term osteogenic differentiation (Fig. 4C,D). Thus, NELL-1 pre-coating of culture plates was observed to be an effective method for increasing ST2 cell osteogenic differentiation.

To further verify the effects of NELL-1 coating, we next turned to a 3D model of NELL-1 protein pre-coating, using PLGA (polylactic-*co*-glycolic acid) scaffolds. This 3D scenario has increased clinical relevance, as a similar scaffold has been used by our research group for calvarial defect healing [Aghaloo et al., 2006]. MC3T3 pre-osteoblast cells were seeded on either control or NELL-1-coated scaffolds. Osteogenic differentiation was assessed after 4 weeks. MC3T3 cells on NELL-1-coated scaffolds were observed to have increased cell numbers as exhibited by H&E staining (Fig. 4E) and bone formation and mineralization as evaluated by 3D micro-CT reconstruction and quantification (Fig. 4F,G).

DISCUSSION

Integrins are cell surface receptors that mediate cell adhesion. Integrins integrate signaling from extracellular growth factors and cytokines, organize the cytoskeleton, stimulate downstream cascades, and regulate genes expression. Most recently, NELL-1 protein has been experimentally verified to be capable of promoting cell adhesion and osteogenic differentiation while the underlying mechanisms remained not well understood [Hasebe et al., 2012]. Here, we have, for the first time, identified that NELL-1 directly bound to and activated Integrinβ1 as well as enhanced phosphorylation of FAK in ST2 murine marrow cells, thereby promoting ST2 cell adhesion. These findings suggested Integrinβ1 to be the most reasonable cell surface receptor candidate for NELL-1 protein binding.

A characteristic feature of cell surface-adhesion proteins is their ability to bind to multiple Integrins via different motifs. Among them, the RGD (Arg-Gly-Asp)-containing amino acid sequence is the most recognized motif by Integrins [Meyer et al., 2006]. However, there is no RGD sequence found in NELL-1. It has been reported that TSP-1 interacts with Integrinα4β1 by LDVP sequence in its TSPN domain, with the Asp residue as the most important binding site [Li et al., 2002]. Sequence alignment has demonstrated that NELL-1 contains a similar LDVP sequence on the TSPN domain and Asp residue at the same position [Tan et al., 2006]. Therefore NELL-1 binding to Integrinβ1 may be accomplished through its TSPN domain (Laminin G domain). Additionally, there are reports of the interaction of EGF-like domain and Integrin receptors [Ieguchi et al., 2010]. NELL-1 contains six EGF-like domains, which may also mediate the interaction between NELL-1 and Integrinβ1. In this study, we have identified Integrinβ1 as the NELL-1 cell surface receptor, while the specificity of NELL-1 binding to Integrinα subunits has yet to be determined.

BMSCs have the potential to differentiate into multiple lineages, providing a potential source for tissue regeneration. Extracellular protein and Integrin interactions result in not only cell binding but also cell spreading, which lead to changes in cytoskeletal structures. The nature of cell adhesion and the degree of cytoskeletal tension have been widely accepted as affecting stem cell behaviors, such as cell migration, proliferation, and differentiation [Ode et al., 2010]. In addition, a vast number of signaling pathways have been identified to be stimulated by activation of Integrins, including the MAPK pathway amongst others. Indeed, NELL-1 has been shown to be able to activate ERK and JNK signaling in multiple cells types [Bokui et al., 2008; Zhang et al., 2011b]. Thus, the physical binding of NELL-1 to cell surface Integrinβ1 may initiate the activation of the MAPK signaling pathway.

Bioengineered scaffolds have been widely used in orthopedic applications [Lu et al., 2010]. It has been recommended that extracellular molecules should be included on orthopedic scaffolds to mimic the cellular environment and to maintain a stronger interaction with the biomaterial and adjacent cells [Hidalgo-Bastida and Cartmell, 2010]. NELL-1, as a secreted osteogenic protein, has been successfully applied to diverse animal models to promote bone formation. The current findings will expand the methods of NELL-1 delivery in tissue engineering settings of bone formation and regeneration. NELL-1 accelerated bone formation in repair scenarios perhaps not only by up regulating osteogenic signaling pathways (as previously shown), but also by promoting osteoprogenitor cells' attachment and proliferation via direct adhesion to Integrinβ1. NELL-1 pre-coating has resulted in larger cytoskeleton assembly and adhesion size of the cells on culture plates, which may come from the surface tensional force change. It has been widely

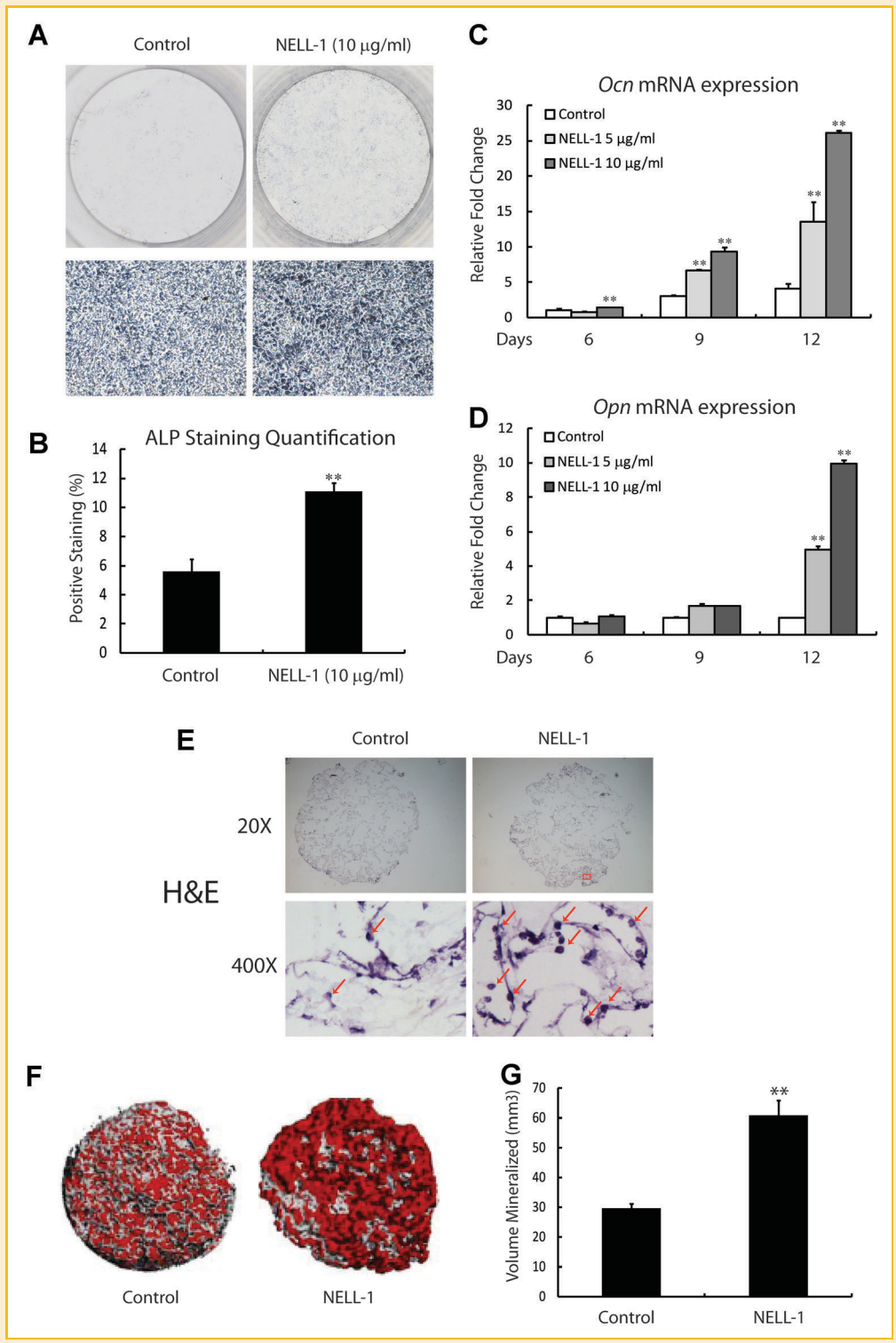

Fig. 4. NELL-1 protein coated on the cell culture plates promoted osteogenic differentiation. A: ALP staining of ST2 cells. ST2 cells were seeded on 24-well plates coated with PBS or NELL-1 (10 μg/ml) and cultured in osteogenic differentiation medium for 9 days (n = 3 wells per treatment). B: Quantification of ALP staining by Image-Pro Plus 6.0 software on eight separate random fields. C,D: Real-time PCR analysis for the osteogenic differentiation markers, *Ocn* and *Opn*. ST2 cells were seeded on 24-well plates coated with PBS or the indicated concentration of NELL-1 protein. Cells were cultured in osteogenic differentiation medium for different time periods (6, 9, and 12 days). Medium, with or without, protein was changed every 3 days (n = 3 wells per treatment per time point). $^{**}P < 0.01$ compared to control group at corresponding time point. E: H&E staining of PBS- (control) or NELL-1-coated PLGA scaffold seeded with MC3T3 cells after 4 weeks of osteogenic differentiation. Arrow: MT3T3 cells. F,G: 3D-microCT reconstruction images and quantification of mineralization of scaffolds as in (E). Mineralized areas (higher than threshold of 80) were highlighted in red and overlaid onto the scaffold background. n = 4. $^{**}P < 0.01$ compared to control group.

understood that anisotropic distribution of tensional forces along the cell periphery can regulate cell shape, junction remodeling, and cell spatial repositioning within tissues [Tseng et al., 2012]. Our results suggest that NELL-1 can be used to modulate the orthopedic scaffold surface tensional force distribution and therefore to shape tissue morphogenesis.

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