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The C-terminal region of NELL1 mediates osteoblastic cell adhesion through integrin α3β1

Ai Hasebe [a], Yoko Nakamura [a], Hiroki Tashima [a], Kaneyoshi Takahashi [a], Masumi Iijima [a], Nobuo Yoshimoto [a], Kang Ting [b], Shun'ichi Kuroda [a,*], Tomoaki Niimi [a,*]

[a] Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan
[b] Dental and Craniofacial Research Institute, University of California Los Angeles, Los Angeles, CA 90095, USA

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ABSTRACT

NELL1 is a secretory osteogenic protein containing several structural motifs that suggest that it functions as an extracellular matrix component. To determine the mechanisms underlying NELL1-induced osteoblast differentiation, we examined the cell-adhesive activity of NELL1 using a series of recombinant NELL1 proteins. We demonstrated that NELL1 promoted osteoblastic cell adhesion through at least three cell-binding domains located in the C-terminal region of NELL1. Adhesion of cells to NELL1 was strongly inhibited by function-blocking antibodies against integrin α3 and β1 subunits, suggesting that osteoblastic cells adhered to NELL1 through integrin α3β1. Further, focal adhesion kinase activation is involved in NELL1 signaling.

1. Introduction

Osteogenesis proceeds through three major phases: (i) cell proliferation, (ii) extracellular matrix (ECM) development and maturation, and (iii) mineralization [1]. During the first phase, mesenchymal progenitors are recruited to the bone-forming site where they proliferate and differentiate into bone matrix-producing osteoblasts. These events are regulated by Runx2 (runt-related transcription factor 2), a key transcriptional regulator of osteogenesis, and are followed by the secretion of specific bone matrix proteins such as type I collagen, bone sialoprotein, and osteocalcin. It has been shown that the differentiation and maturation of osteoblasts are regulated by their interaction with several other ECM proteins [2–5]. These proteins are divided into two groups: structural proteins such as laminin and fibronectin, and matricellular proteins such as tenascin-C and thrombospondin-1. Matricellular proteins do not contribute directly to the formation of the structural elements of the ECM, but rather serve to modulate cell-matrix interactions and cell function through their interaction with cell surface receptors such as integrins, and other components of the ECM [6,7].

NELL1 is a secretory protein that exhibits potent osteoinductive activity [8]. The *NELL1* gene was originally identified in craniosynostosis patients as being specifically upregulated within prematurely fusing sutures [9]. The human *NELL1* gene encodes a polypeptide of 810 amino acids with structural similarities to thrombospondin-1. It contains several structural motifs, including an N-terminal thrombospondin-1-like (TSPN) domain (overlapping with a laminin G domain), coiled-coil (CC) domain, four von Willebrand factor type C (VWC) domains, and six epidermal growth factor (EGF)-like domains (see Fig. 2A) [8]. Similar to thrombospondin-1, secreted NELL1 forms a homotrimer, presumably through a coiled-coil domain [10]. NELL1 transduces osteogenic signals through the Ras-mitogen-activated protein kinase (MAPK) pathway and Runx2 [11]. We recently showed that recombinant NELL1 was incorporated into the ECM following secretion from NELL1-expressing cells, suggesting that NELL1 acts as an ECM component [12]. However, the cell-binding activity of NELL1 and its cell surface receptors have not yet been characterized.

In the present study, we utilized a series of recombinant NELL1 proteins to investigate whether NELL1 has cell-adhesive activity. We demonstrated that NELL1 promoted osteoblastic cell adhesion through at least three cell-binding sites localized to the C-terminal region of NELL1. Function-blocking monoclonal antibodies identi-

Abbreviations: CC, coiled-coil; ECM, extracellular matrix; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; TSPN, N-terminal thrombospondin-1-like; VWC, von Willebrand factor type C

* Corresponding authors. Fax: +81 52 789 5227 (S. Kuroda), fax: +81 52 789 5237 (T. Niimi).

E-mail addresses: skuroda@agr.nagoya-u.ac.jp (S. Kuroda), tniimi@agr.nagoya-u.ac.jp (T. Niimi).

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fied integrin α3β1 as the major cellular receptor necessary for cell adhesion onto NELL1. Furthermore, the focal adhesion kinase (FAK)-MAPK pathway is involved in NELL1 signaling.

2. Materials and methods

2.1. Antibodies and peptides

Function-blocking monoclonal antibodies to the human α1–6 and αV integrins (Alpha Integrin Blocking and IHC Kit), human integrin β1 (6S6), human integrin β3 (25E11), and human integrin β4 (3E1) were purchased from Millipore (Billerica, MA, USA). Rabbit polyclonal antibodies against human FAK, phospho-FAK (Tyr^{397}), human extracellular signal-regulated kinases (ERK) 1/2, and phospho-ERK1/2 (Thr^{202}/Tyr^{204}) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibody against human β-actin was purchased from GeneTex (Irvine, CA, USA). The synthetic peptide GD-6 (KQNCLSSRASFRGCVRNLRLSR), derived from mouse laminin α1 chain, was purchased from Anaspec (Fremont, CA, USA). The synthetic peptide CS1 (EILDVPST), derived from type III homology connecting segment of fibronectin, was purchased from GenScript (Piscataway, NJ, USA).

2.2. Cell culture

The mouse pre-osteoblast cell line MC3T3-E1 (clone 4) was obtained from RIKEN Cell Bank (Tsukuba, Japan), and was maintained in α-minimum essential medium (MEM) containing 10% (v/v) fetal calf serum (FCS). The human osteosarcoma Saos-2 and U2OS cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and were maintained in McCoy's 5A medium containing 10% (v/v) FCS. Mouse mesenchymal C3H10T1/2 cells and the human osteosarcoma MG-63 cells were also obtained from the Japanese Collection of Research Bioresources Cell Bank, and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FCS. The human fibrosarcoma HT-1080 cell line was maintained in DMEM containing 10% (v/v) FCS. The human cervical carcinoma HeLa cell line was maintained in MEM containing 10% (v/v) FCS.

2.3. Plasmid construction

Mammalian expression vectors for human NELL1 with N-terminal FLAG- and C-terminal hexahistidine-tags were prepared as follows: cDNA fragments encoding the N-(residues 17–272) or C-(residues 273–810) terminal regions of human NELL1 were amplified by polymerase chain reaction (PCR) from full-length human NELL1 cDNA. The PCR products were digested with *Bam*HI and *Xho*I, and cloned into the pSecTag2-FLAG vector [13].

An expression vector for the C-terminal region of human NELL1 in *Leishmania tarentolae* was prepared as follows: a cDNA encoding the C-terminal region of human NELL1 (residues 273–810) was amplified by PCR from the mammalian expression vector described above. The PCR product was subcloned into the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, USA), and the resultant plasmid was digested with *Xba*I and *Not*I, and cloned into the pLEXSY-Neo2 vector (Jena Bioscience, Jena, Germany) with a C-terminal hexahistidine-tag.

Bacterial expression vectors for human NELL1 fragments were prepared as follows: cDNAs encoding the TSPN and CC domains (residues 17–272), the N-terminal VWC domains (residues 273–388), the EGF-like domains (residues 389–634), and the C-terminal VWC domains (residues 635–810) of human NELL1 were amplified by PCR from full-length human NELL1 cDNA. The PCR products were digested with *Hind*III and *Xba*I, and cloned into the pCold-TF vector (Takara Bio, Otsu, Japan). The resultant vectors express hexahistidine-tagged and trigger factor (TF)-fused proteins. All primer sequences used in this study are available upon request.

2.4. Protein expression and purification

Preparation of recombinant full-length NELL1 protein has been described previously [12]. The recombinant N-terminal region of NELL1 was produced using the FreeStyle MAX 293 Expression System (Invitrogen) according to the manufacturer's instructions. Briefly, 293-F cells were transfected with the pSecTag2-FLAG-hNELL1-N plasmid using the FreeStyle MAX Reagent (Invitrogen), and grown in serum-free FreeStyle 293 Expression medium (Invitrogen) for 96 h. Recombinant protein was purified from conditioned medium using a HisTrap HP column (GE Healthcare, Piscataway, NJ, USA) as described [12]. Purified proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized with Coomassie brilliant blue (CBB) R-250 staining.

The recombinant C-terminal region of NELL1 was produced using the LEXSYcon2 Expression kit (Jena Bioscience) according to the manufacturer's instructions. Briefly, the pLEXSY-Neo2-hNELL1-C plasmid was transfected into *L. tarentolae* by electroporation, and stable transfectants were selected with G418 (50 μg/ml). The recombinant strain was cultured in brain–heart-infusion based medium supplemented with hemin (5 μg/ml), penicillin (50 U/ml) and streptomycin (50 μg/ml) at 26 °C in the dark with shaking (140 rpm). Recombinant protein was purified from conditioned medium using an Ni Sepharose 6 Fast Flow column (GE Healthcare).

Recombinant NELL1 fragments were expressed in *Escherichia coli* Rosetta-gami 2 (DE3) pLysS strain (Merck, Darmstadt, Germany). *E. coli* cells carrying pCold-TF expression vectors were cultured at 37 °C until the OD_{600} reached 0.5, and expression of the recombinant proteins was then induced by cold shock at 15 °C for 24 h in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested and lysed with BugBuster HT reagent (Novagen, Madison, WI, USA). The extracted proteins were purified on an Ni Sepharose 6 Fast Flow column (GE Healthcare).

2.5. Cell adhesion assay

Cell adhesion assays were performed using 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark). Briefly, plates were coated with the indicated concentration of NELL1 or its deletion mutants at 4 °C overnight and then blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Cells were harvested with 1 mM ethylenediaminetetraacetic acid (EDTA) or TrypLE Select solution (Invitrogen), suspended in serum-free DMEM at a density of 3×10^5 cells/ml, and then plated at 3×10^4 cells/well. After incubation in a CO_2 incubator at 37 °C for 1 h, non-adherent cells were washed from wells, and adherent cells were fixed and stained for 30 min with 0.4% (w/v) crystal violet in 50% (v/v) methanol. After washing with distilled water, attached cells were counted by microscopic examination of random fields in two independent wells. For adhesion inhibition assays, the cells were pre-incubated with EDTA (5 mM), peptides (10–100 μM), or function-blocking anti-integrin antibodies (5 μg/ml) for 20 min at room temperature before inoculation.

2.6. Immunoblot analysis

Sub-confluent C3H10T1/2 cells were subjected to serum starvation for 18 h. The cells were treated with NELL1 (0–10 nM) in serum-free DMEM for 20 min at 37 °C, and lysed with CelLytic M Cell Lysis Reagent (Sigma, St. Louis, MO, USA) containing 1× PhosSTOP

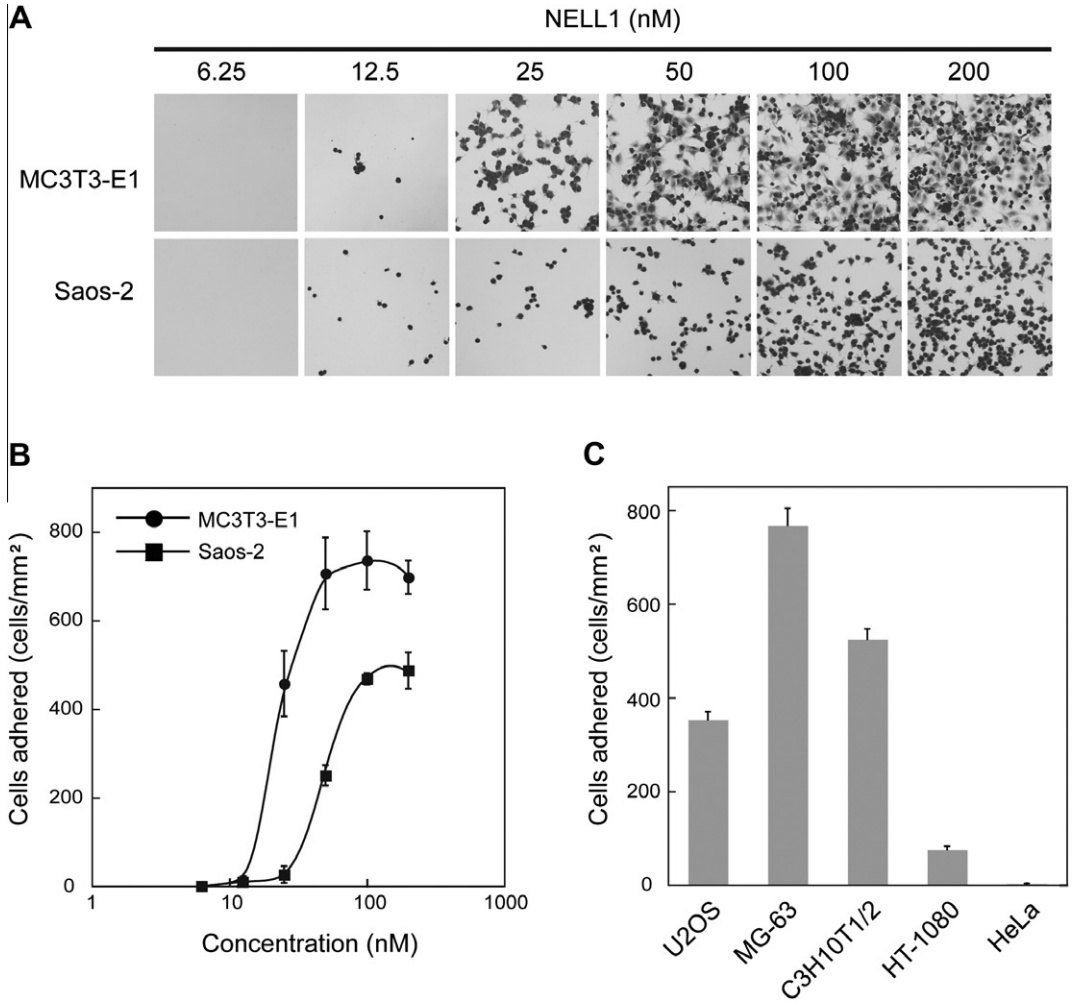

Fig. 1. Cell-adhesive activity of recombinant NELL1. (A and B) Dose-dependent adhesion of osteoblastic cells to NELL1. Purified NELL1 was coated onto 96-well plates at the indicated concentrations. MC3T3-E1 and Saos-2 cells were allowed to adhere for 1 h at 37 °C. Non-adherent cells were washed from the wells, and adherent cells were then fixed and stained with crystal violet. (A) Representative images of cells adhering to NELL1. (B) Cell number per area adhering to NELL1. (C) Cell-type specificity of adhesion to NELL1. NELL1 was coated at 100 nM and U2OS, MG-63, C3H10T1/2, HT-1080 and HeLa cells were allowed to adhere for 1 h at 37 °C. Each value represents the mean (±standard error) of triplicate results.

phosphatase inhibitor cocktail (Roche, Mannheim, Germany). Protein samples were separated on SDS–polyacrylamide gels and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare). The membranes were blocked with 2% (w/v) BSA in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20, followed by incubation with primary antibodies (1:1000) in Can Get Signal immunoreaction enhancer solution 1 (Toyobo, Osaka, Janan) at 4 °C overnight. The membranes were then washed with TBS containing 0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; GE Healthcare) in Can Get Signal solution 2 (Toyobo) at room temperature for 1 h. The membranes were developed using ECL Prime Western Blotting Detection Reagents (GE Healthcare) and imaged on an LAS-4000mini Luminescent Image Analyzer (GE Healthcare).

3. Results and discussion

3.1. Cell-adhesive activities of recombinant NELL1

Cell-adhesive activity of NELL1 was examined using the mouse MC3T3-E1 pre-osteoblast cell line and the human Saos-2 osteosarcoma cell line. Full-length NELL1 induced the adhesion of both cell lines in a dose-dependent manner, with maximal adhesion at a NELL1-coating concentration of approx. 100 nM (Fig. 1A & B). This cell-adhesive activity of NELL1 is comparable to that of thrombospondin-1 for several cell lines [14]. MC3T3-E1 cells displayed an elongated and well-spread morphology, while Saos-2 cells were less well spread (Fig. 1A). The human osteosarcoma cell lines, U2OS and MG-63, and the mouse C3H10T1/2 mesenchymal cell line, which can be induced to differentiate into osteoblast-like cells, also adhered well to NELL1, while human fibrosarcoma HT-1080 and human cervical carcinoma HeLa cells showed minimal or no adhesive activity (Fig. 1C). These results suggest that NELL1 promotes cell adhesion in a cell-type specific manner. Because the osteoblast is of mesenchymal origin, NELL1 may be an adhesion molecule for mesenchymal stem cells and osteoblastic cells.

3.2. The C-terminal region of NELL1 promotes cell adhesion

To determine which domain was responsible for promoting cell adhesion, the C-or N-terminally truncated NELL1 constructs, NELL1-N and NELL1-C, were generated (Fig. 2A). These truncated proteins were expressed in mammalian 293-F cells; however, the NELL1-C protein was degraded soon after its secretion into the culture medium. Therefore, the NELL1-C protein was expressed in cells of the lower eukaryote *L. tarentolae*, because the *L. tarentolae*

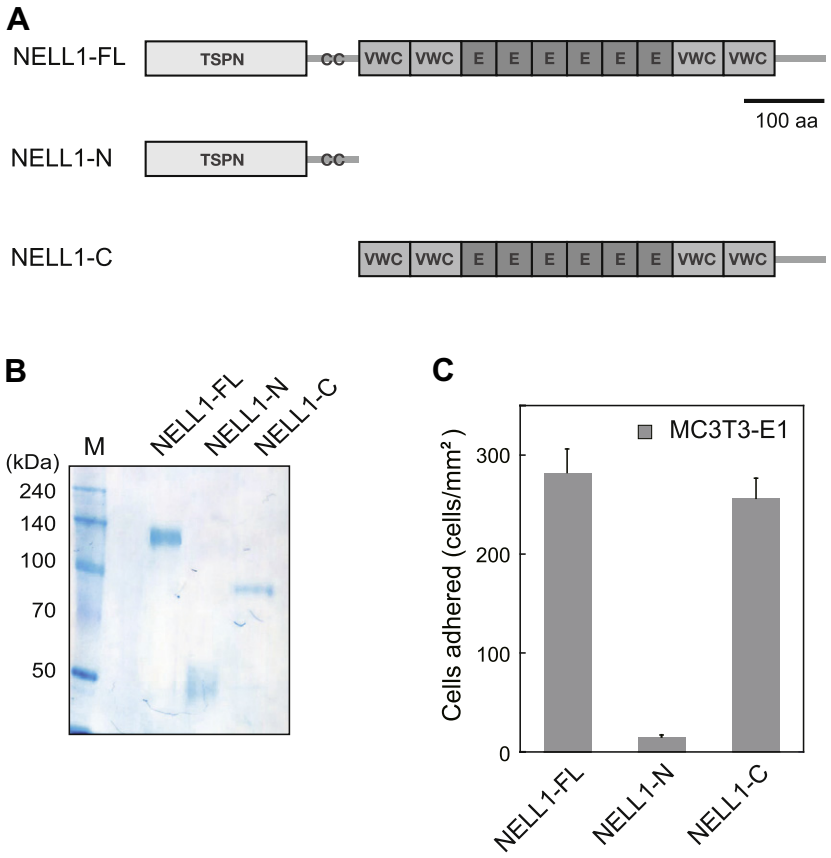

Fig. 2. C-terminal region of NELL1 promotes MC3T3-E1 cell adhesion. (A) Schematic representations of the NELL1 proteins. A full-length NELL1 protein (NELL1-FL) contains one TSPN domain, one coiled-coil (CC) domain, four von Willebrand factor type C (VWC) domains, and six epidermal growth factor (EGF)-like domains. The VWC and EGF-like domains were deleted to generate a C-terminally truncated protein (NELL1-N). The TSPN and CC domains were deleted to generate an N-terminally truncated protein (NELL1-C). (B) NELL1-FL and NELL1-N were expressed in 293-F cells, and NELL1-C was expressed in *L. tarentolae* cells. The purified proteins were resolved by SDS–PAGE and visualized by CBB staining. Molecular weight markers (M) are indicated to the *left*. (C) NELL1-FL and its deletion mutants were coated onto 96-well plates at a concentration of 50 nM, and MC3T3-E1 cells were allowed to adhere for 1 h at 37 °C. Each value represents the mean (± standard error) of triplicate results.

expression system enables proper protein folding and mammalian-type post-translational modification of recombinant eukaryotic proteins [15]. Full-length NELL1 (NELL1-FL) and its truncated proteins were purified to near homogeneity with nickel-chelate affinity chromatography as determined by CBB staining of SDS–polyacrylamide gels (Fig. 2B). The adhesive activity of these proteins was evaluated using MC3T3-E1 cells (Fig. 2C). NELL1-C displayed similar adhesive activity to NELL1-FL. In contrast, NELL1-N showed minimal adhesion activity even at higher coating concentrations up to 200 nM (data not shown). These results suggest that the cell-adhesive activity of NELL1 is encoded in the C-terminal region of NELL1 obtained from a *L. tarentolae* host.

To further localize the cell-binding region of the NELL1 protein, a series of NELL1 fragments were prepared as fusion proteins with a hexahistidine-tag and a trigger factor (TF) as a soluble tag (Fig. 3A). These NELL1 fragments were successfully expressed as soluble monomer proteins in *E. coli* and purified by nickel-chelate affinity chromatography (Fig. 3B). The adhesive activity of these proteins was evaluated using MC3T3-E1 cells (Fig. 3C). Although no fragments showed adhesive activity at a coating concentration of 50 nM (data not shown), three fragments within the C-terminal region of NELL1 – N-VWC, EGF, and C-VWC – displayed cell-adhesive activity at a coating concentration of 500 nM. The N-terminal region of NELL1 (TSPN-CC) again showed no adhesive activity. These results demonstrated that at least three cell-binding sites were localized to the C-terminal region of NELL1.

Recently, Nakamoto's group reported that NELL2, a NELL family protein predominantly expressed in the nervous system, binds to retinal axons through the TSPN and VWC domains independently [16]. NELL1 is also expressed in the nervous system, and it has been shown to have a weaker binding activity to retinal axons. Further studies will be required to identify the cell-binding sequence in NELL1 for other cell types as well as osteoblastic cells.

NELL1-deficient mice resulted in decreased bone mineralization along with reduced expression of genes for ECM proteins critical for chondrogenesis and osteogenesis [17]. In contrast, overexpression of *NELL1* gene in MC3T3-E1 cells resulted in increased osteoblast differentiation along with up-regulation of some sets of genes for ECM proteins [18]. The functional relationship between cell adhesion to NELL1 and osteogenic activity with the production of ECM proteins remains to be determined. Whether NELL1 fragments still have osteogenic activity is currently being investigated.

3.3. Integrin $\alpha 3\beta 1$ mediates the adhesion of osteoblastic cells to NELL1

Integrins represent the major family of adhesion receptors for ECM proteins [19]. Because integrin–ligand binding is divalent cation-dependent, we examined the effect of the divalent cation chelator, EDTA, on the adhesion of MC3T3-E1 and Saos-2 cells to NELL1 (Fig. 4A). Adhesion of both cell lines to NELL1 was completely inhibited in the presence of 5 mM EDTA, suggesting that adhesion of these cells to NELL1 is mediated by integrins.

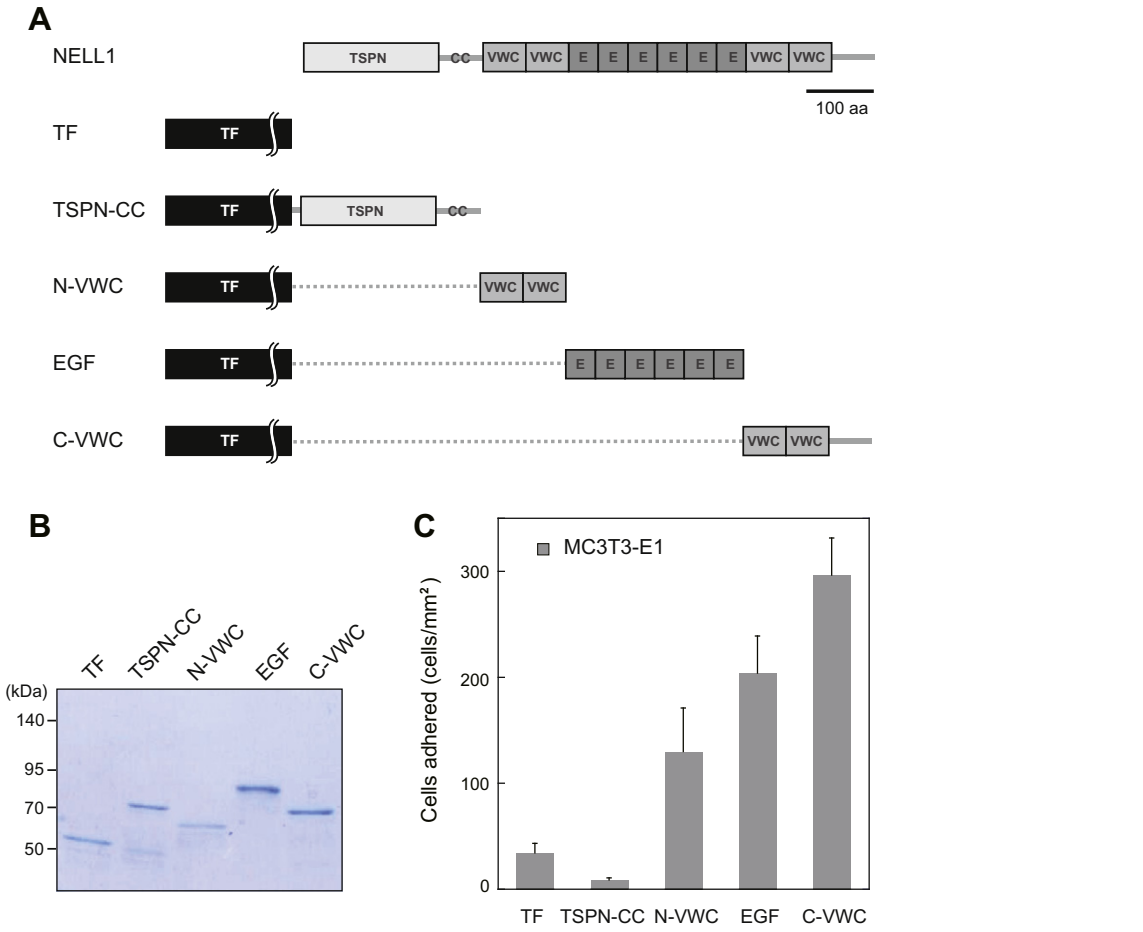

Fig. 3. C-terminal region of NELL1 contains several cell-binding sites. (A) Schematic representations of the trigger factor (TF)-fused NELL1 fragments. The TSPN and CC domains (TSPN-CC), the N-teminal VWC domains (N-VWC), EGF-like domains (EGF), and the C-terminal VWC domains (C-VWC) of NELL1 were expressed in *E. coli* as TF-fused proteins. (B) The purified proteins were resolved by SDS–PAGE and stained with CBB. Molecular weight markers (M) are indicated to the *left*. (C) The NELL1 fragments were coated onto 96-well plates at a concentration of 500 nM, and MC3T3-E1 cells were allowed to adhere for 1 h at 37 °C. Each value represents the mean (±standard error) of triplicate results.

To identify the integrin receptor(s) involved, a panel of function-blocking monoclonal antibodies against integrin subunits was tested for their ability to inhibit cell adhesion to NELL1. Because these antibodies were raised against human integrins, the inhibition assay was performed using human Saos-2 cells. Adhesion to NELL1 was significantly blocked by antibodies to the integrin α3 and β1 subunits, suggesting that integrin α3β1 is involved in the adhesion of Saos-2 cells to NELL1 (Fig. 4B). To confirm these results, cell adhesion to NELL1 was examined in the presence of the synthetic peptide GD-6 containing a recognition site for integrin α3β1 [20]. Adhesion of MC3T3-E1 cells to NELL1 was dose-dependently inhibited by GD-6 peptide but not by CS1 peptide containing a recognition site for integrin α4β1 (Fig. 4C) [21]. These results further indicated that integrin α3β1 is the major cellular receptor necessary for cell adhesion onto NELL1.

Integrin α3β1 is widely expressed on nearly all cell types including osteoblastic cells and mesenchymal stem cells, which displayed cell-adhesive activity to NELL1 (see Fig. 1C) [22–25]. However, HT-1080 and HeLa cells showed minimal or no adhesive activity in spite of their expression of integrin α3β1 [26]. Integrin α3β1 is known to interact with the tetraspanin family of transmembrane proteins [27]. For example, tetraspanin CD151 forms a stable complex with integrin α3β1 and modulates the ligand-binding activity of integrin α3β1 [28]. Some integrins associate with receptor tyrosine kinases to regulate signaling pathways cooperatively [29]. Taken together, our results suggest the existence of a specific co-receptor for NELL1 in osteoblastic cells and mesenchymal stem cells.

3.4. NELL1 induces phosphorylation of FAK and ERK1/2

Integrin-mediated cell adhesion activates various protein tyrosine kinases including FAK, which is directly activated by β1-integrin signaling and leads to the association of integrins with the cytoskeleton [30]. The FAK signaling pathway can subsequently activate MAPK cascades to elicit specific biological responses. The FAK-MAPK signaling pathway has been shown to be an important mediator of laminin-332-induced osteogenic differentiation of mesenchymal stem cells via integrin α3β1 [5]. Because NELL1 transduces osteogenic signals through MAPK cascades [11], we investigated the FAK-MAPK pathway as the possible mechanism of NELL1 signaling. To determine whether NELL1 induces tyrosine phosphorylation of FAK, C3H10T1/2 cells were treated with NELL1 for 20 min. As shown in Fig. 5, NELL1 treatment resulted in increased FAK phosphorylation levels relative to total FAK proteins. Incubation of NELL1 with C3H10T1/2 cells also resulted in a similar activation of the MAPK family member, ERK1/2. Based on the densitometric intensities of the bands, the amounts of phosphorylated FAK and ERK1/2 were estimated to have increased by 2.5- and 5.2-folds, respectively, as compared to the untreated control cells. These results indicate that NELL1 can induce FAK and ERK1/2 activation, probably via integrin receptors. Neither MC3T3-E1 nor

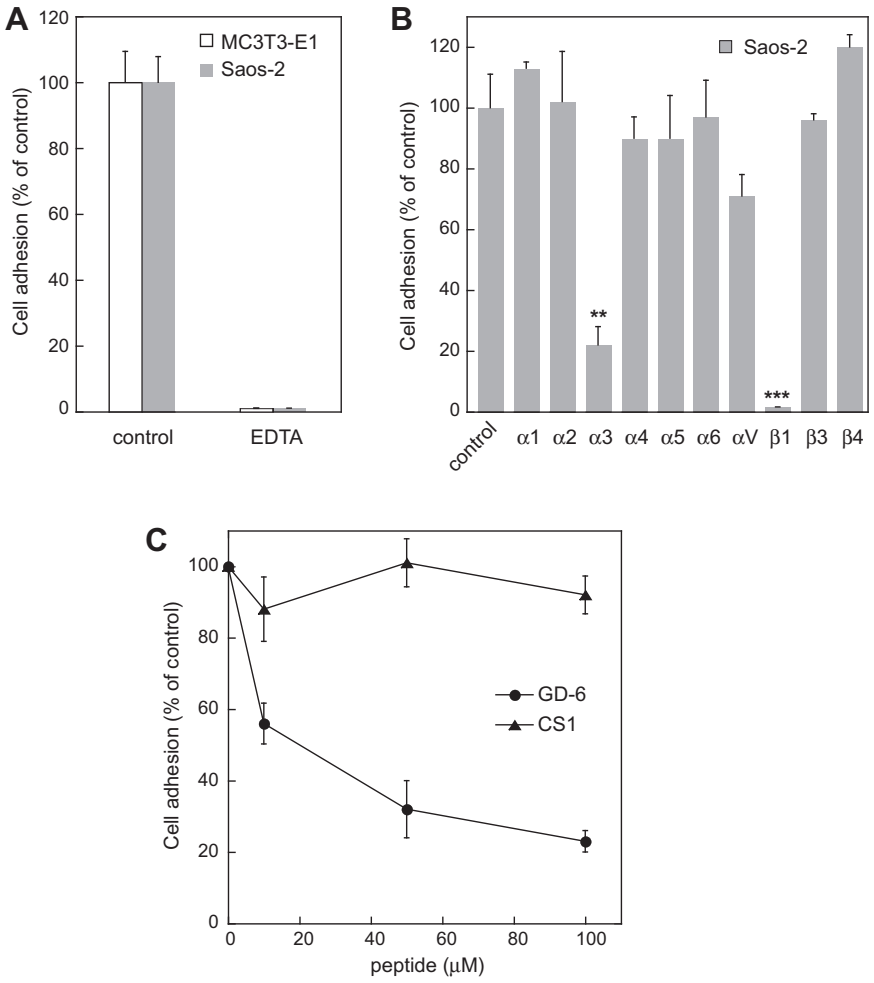

Fig. 4. Cell-adhesive activity of NELL1 is integrin-dependent. (A) The effect of EDTA on adhesion of cells to NELL1. NELL1-FL was coated onto 96-well plates at a concentration of 50 nM for MC3T3-E1 cells or 100 nM for Saos-2 cells. Cells were pre-incubated with EDTA (5 mM) for 20 min and allowed to adhere for 1 h at 37 °C. (B) Integrin α3β1-mediated cell adhesion to NELL1. NELL1-FL was coated onto 96-well plates at a concentration of 100 nM. Saos-2 cells were pre-incubated with function-blocking anti-integrin antibodies (5 μg/ml) for 20 min, and allowed to adhere for 1 h at 37 °C. Each value represents the mean percentage (±standard error) of triplicate results relative to untreated cells set to 100%. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test) (C) The effect of α3β1 integrin-binding GD-6 peptide on adhesion of cells to NELL1. NELL1-FL was coated onto 96-well plates at a concentration of 50 nM. MC3T3-E1 cells were pre-incubated with GD-6 or CS1 peptide for 20 min and allowed to adhere for 1 h at 37 °C.

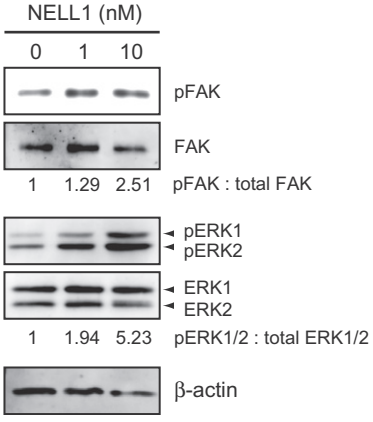

Fig. 5. Activation of FAK and ERK1/2 by NELL1. C3H10T1/2 cells were treated with NELL1-FL for 20 min at 37 °C. Cell lysates were analyzed by immunoblotting with anti-phosphorylated-FAK and anti-phosphorylated-ERK1/2 antibodies. Anti-FAK, anti-ERK1/2, and anti-β-actin antibodies were used as internal controls. The relative fold induction of FAK and ERK1/2 phosphorylation is shown. Values were normalized to total FAK and ERK1/2 proteins, respectively.

Saos-2 cells showed activation of FAK under the conditions investigated in this study (data not shown). This may be due to the osteoblastic differentiation state of these cell lines.

In summary, we have demonstrated that NELL1 promoted osteoblastic cell adhesion via at least three cell-binding sites within its C-terminal region. One of the cell surface receptors for NELL1 was determined to be integrin α3β1. Collectively, our results suggest that NELL1 is a matricellular protein that modulates osteoblast differentiation via the integrin-FAK-MAPK pathway.

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