Involvement of MAPK signaling molecules and Runx2 in the NELL1-induced osteoblastic differentiation

Nobuyuki Bokui\textsuperscript{a}, Takayuki Otani\textsuperscript{b}, Koichi Igarashi\textsuperscript{b}, Junichiro Kaku\textsuperscript{c}, Mitsuo Oda\textsuperscript{d}, Tadahiro Nagaoka\textsuperscript{e}, Masaharu Seno\textsuperscript{e}, Kenji Tatematsu\textsuperscript{a}, Toshihide Okajima\textsuperscript{a}, Takashi Matsuzaki\textsuperscript{a}, Kang Ting\textsuperscript{f}, Katsuyuki Tanizawa\textsuperscript{a}, Shun’ichi Kuroda\textsuperscript{a,*}

\textsuperscript{a} Department of Structural Molecular Biology, The Institute of Scientific and Industrial Research, Osaka University, Osaka 567-0047, Japan
\textsuperscript{b} Katayama Chemical Industries Co., Ltd., Osaka 562-0015, Japan
\textsuperscript{c} Hirai Dental Clinic, Tokyo 132-0035, Japan
\textsuperscript{d} School of Dentistry, Showa University, Tokyo 142-8555, Japan
\textsuperscript{e} Graduate School of Natural Science and Technology, Okayama University, Okayama 700-8530, Japan
\textsuperscript{f} Section of Orthodontics, UCLA School of Dentistry & Department of Biomedical Engineering, UCLA School of Engineering, Los Angeles, CA 90095, USA

Received 26 September 2007; revised 4 December 2007; accepted 4 December 2007

Available online 17 December 2007

Edited by Angel Nebreda

Abstract NELL1 is an extracellular protein inducing osteogenic differentiation and bone formation of osteoblastic cells. To elucidate the intracellular signaling cascade evoked by NELL1, we have shown that NELL1 protein transiently activates the MAPK signaling cascade, induces the phosphorylation of Runx2, and promotes the rapid intracellular accumulation of Tyr-phosphorylated proteins. Unlike BMP2, NELL1 protein does not activate the Smad signaling cascade. These findings suggest that upon binding to a specific receptor NELL1 transduces an osteogenic signal through activation of certain Tyr-kinases associated with the Ras-MAPK cascade, and finally leads to the osteogenic differentiation.

Keywords: MAP kinase; Osteoblast; Differentiation; Bone; Tyrosine phosphorylation

1. Introduction

Osteogenic differentiation of mesenchymal pluripotent cells is regulated by various soluble proteinous factors \cite{1}. Especially, BMP (bone morphogenetic protein) was originally identified as a molecule that promotes the differentiation of mesenchymal cells into an osteoblastic lineage, as suggested from its abilities not only to induce the expression of osteoblastic markers alkaline phosphatase (ALP) and osteocalcin (OCN) but also to stimulate mineralization \cite{2}. In the BMP signaling pathways, the Smad proteins play a major role in osteoblastic differentiation through activation of the target genes. Additionally, the runt-related transcription factor 2/core binding factor \textsuperscript{a} (Runx2/Cbfa1) was shown to be indispensable for osteoblast differentiation and bone formation \cite{3}.

We previously identified a gene excessively expressed at the sutures of unilateral coronal synostosis, causing the premature fusion of the growing cranial bone fronts, and designated NELL1 [novel epidermal growth factor (EGF)-like protein \textsuperscript{b}] \cite{4}. NELL1 protein consists of about 810 amino acid residues (aa) and harbors an about 20-aa signal peptide, an about 128-aa laminin G domain [previously known as an N-terminal thrombospondin (TSP)-like module], five von Willebrand factor C domains, and six EGF-like domains including three Ca\textsuperscript{2+}-binding types \cite{5}, as shown in Fig. 1. Concerning physiological functions, the NELL1 gene was shown to induce osteogenic differentiation of rat fetal calvarial (RFC) cells, and the transgenic mice overexpressing the NELL1 gene exhibited a craniosynostosis (CS)-like phenotype \cite{6}. On the other hand, the NELL1-deficient mice had reduced levels of extracellular matrix (ECM) proteins, thereby causing skeletal defects in the vertebral column and ribcage \cite{7}. These observations have facilitated recent attempts to examine the therapeutic effects of NELL1 protein on bone defects in animal models \cite{8,9}. However, it has not been elucidated how NELL1 protein evokes the intracellular signaling cascade in osteoblasts.

In this study, we have obtained highly purified recombinant human novel EGF-like protein 1 (hNELL1) and examined the direct effects of hNELL1 protein on various osteoblastic cells. We here show that hNELL1 protein added at a markedly low molar concentration is able to transiently activate the MAPK signaling cascade in various osteoblastic cells and transduces its signals through the Smad-independent signaling cascade. Furthermore, addition of hNELL1 protein resulted in the Runx2 phosphorylation and rapid accumulation of Tyr-phosphorylated proteins.

2. Materials and methods

2.1. Production and purification of hNELL1 protein

The hNELL1 cDNA was subcloned into the expression vector pIZT/V5-His contained in the InsectSelect Glow System (Invitrogen, Carlsbad, CA), and introduced into the Trichoplusia ni-derived High Five cells (Invitrogen). The hNELL1 protein secreted in the medium was collected and purified as described in Supplementary materials and methods.

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2.2. Cells
RFC cells, s mouse osteoblast cell line MC3T3-E1, a human osteosarcoma cell line Saos-2, a mouse myoblast-like cell line C2C12, and a mouse mesenchymal stem cell line C3H10T1/2 were obtained and cultured as described in Supplementary materials and methods.

2.3. Small interfering RNA experiments
Ras small interfering RNA (siRNA) assays were performed using a commercially available Ras siRNA/siAb assay kit from Dharmaco (Upstate, Lake Placid, NY). After C3H10T1/2 cells were grown to 50% confluence, cells were transfected with SMARTpool Ras and non-specific Control Pool (negative control) siRNA oligo mixtures using Lipofectamine RNAi Max (Invitrogen), according to the manufacturer’s protocol. Transfection of Ras siRNA was confirmed by Western blotting using an anti-Ras antibody (clone RAS10). Seventy two hours after transfection, cells were subjected to Western blotting and ELISA according to the protocols described above.

2.4. Immunoprecipitation assay for Runx2
Runx2 protein in RFC cells were immunoprecipitated with anti-Runx2 antibody (Cat. R9403, Sigma), and then analyzed with a horseradish peroxidase-conjugated anti-phospho-Ser antibody (Cat. ab9334, abcam, Cambridge, UK), as described in Supplementary materials and methods.

2.5. Pull down assay using hNELL1-VH
Cell lysates of hNELL1-VH-treated RFC cells were immunoprecipitated with an anti-V5 tag antibody, and then analyzed with an anti-phospho-Tyr antibody (clone 4G10, Upstate, Billerica, MA), as described in Supplementary materials and methods.

3. Results and discussion

3.1. Induction of osteomarkers
RFC cells were treated directly with the purified hNELL1-VH protein (see Supplementary Results and Supplementary Fig. 1). As shown in Fig. 2A, in the cells cultured for 3 days after the treatment, the activity of ALP increased nearly in a dose dependent manner of hNELL1-VH protein added. Addition of 50–100 ng/ml hNELL1-VH protein was sufficient to increase the ALP activity by about 7-folds. Hence, we have added 100 ng/ml hNELL1-VH protein constantly in the subsequent experiments, unless otherwise described. The ALP activity induced after the treatment with 50–100 ng/ml hNELL1-VH protein was only about a half that induced after the treatment with 100 ng/ml hBMP2 protein, examined as a positive control. However, BMPs have a molecular mass of 20–25 kDa and usually act as a homodimer, whereas about 120-kDa hNELL1-VH protein is probably a homotrimer in solution [5]. Therefore, comparison of their concentrations in a molar basis (hBMP2, 100 ng/ml = 2.5 nM; hNELL1-VH, 100 ng/ml = 0.28 nM) suggests that hNELL1-VH protein has an ALP inducing activity at much lower concentrations than hBMP2 protein. Other osteomarkers, osteopontin (OPN) and OCN, which are expressed in the differentiated osteoblast following ALP, were also induced in the RFC cells at 6 and 9 days, respectively, after the treatment with 100 ng/ml hBMP2 protein, examined as a positive control. However, BMPs have a molecular mass of 20–25 kDa and usually act as a homodimer, whereas about 120-kDa hNELL1-VH protein is probably a homotrimer in solution [5]. Therefore, comparison of their concentrations in a molar basis (hBMP2, 100 ng/ml = 2.5 nM; hNELL1-VH, 100 ng/ml = 0.28 nM) suggests that hNELL1-VH protein has an ALP inducing activity at much lower concentrations than hBMP2 protein. Other osteomarkers, osteopontin (OPN) and OCN, which are expressed in the differentiated osteoblast following ALP, were also induced in the RFC cells at 6 and 9 days, respectively, after the treatment with 100 ng/ml hNELL1-VH protein, as effectively as hBMP2 protein (Fig. 2B and C). Similar results have been obtained with a mouse osteoblast cell line MC3T3-E1 and a human osteosarcoma cell line Saos-2 (data not shown). These results clearly show that hNELL1-VH protein indeed induces the expression of osteomarkers in RFC cells when added extracellularly even at a very low concentration.

3.2. Activation of MAPK
During the bone differentiation of osteogenic cells, the MAPK signaling cascade is activated along with the expression...
of various osteomarkers [10]. We therefore examined the effect of treatment of serum-starved RFC cells with the purified hNELL1-VH protein on the phosphorylation of proteins involved in the MAPK signaling cascade. As shown in Fig. 3A (left panels), ERK1/2 and JNK1/2/3 were phosphorylated significantly after 10 and 20 min, respectively, of the treatment with hNELL1-VH protein. Based on the densitometric intensities, the amounts of phosphorylated ERK1/2 and JNK1/2/3 were estimated to have increased by about 22-folds as compared to the untreated RFC cells. Weak phosphorylation of p38α/β/γ (about 5-folds that of untreated cells) was also observed after 10 min. These phosphorylated MAPK members were detected only within 30 min after the treatment of RFC cells, indicating that hNELL1-VH protein induces transient rather than sustained (>2 h) activation of the MAPK members. For comparison, the phosphorylated forms of ERK1/2, JNK1/2/3, and p38α/β/γ also increased by about 20, 21, and 17-folds, respectively, in a similar timing after the treatment of RFC cells with hBMP2 protein (middle panels). With a mouse mesenchymal stem cell line C3H10T1/2, both hNELL1-VH and hBMP2 proteins induced the phosphorylation of ERK1/2 and JNK1/2/3 at comparable levels after 10 min of the treatment (see Figs. 3B and 4A). With a human osteosarcoma cell line Saos-2, the two proteins similarly induced the phosphorylation of ERK1/2 after 10 min but sustained that of ERK2 even after 60 min (Fig. 3C) and several more hours (data not shown). In mouse myoblast-like C2C12 cells (see Supplementary Fig. 2), hNELL1-VH protein was found to activate only JNK1/2/3 very weakly (about 1.3-fold) within 10 min. As reported previously [9], both proteins synergistically but weakly activated JNK1/2/3 (about 1.9-fold) in C2C12 cells. Taken together, these results demonstrate that hNELL1-VH protein per se can activate the MAPK signaling cascade within 10 min in various osteoblastic cells, like hBMP2. The duration of the MAPK phosphorylation, however, varies from 30 min to at least several hours among the osteoblastic cells. It is noteworthy that a NELL1 homologue, NELL2 functions as a survival factor for primary cultured neurons also through the activation of ERK and JNK [11].

3.3. Effects of RAS siRNA on OPN expression

The OPN expression was induced by the 6 days treatment of hNELL1-VH protein in C3H10T1/2 (Fig. 4B) and RFC cells (see Supplementary Fig. 3), while not in the hNELL1-VH-treated C2C12 cells as reported previously [9], which may be caused by the weak activation of MAPK (i.e., JNK1/2/3) in the hNELL1-VH-treated C2C12 cells (see below and Supplementary Fig. 2). We next investigated the effects of Ras small interfering RNA (siRNA) on the OPN expression in the hNELL1-VH-treated C3H10T1/2 cells. As shown in Fig. 4A, Ras siRNA diminished the expression of Ras protein and suppressed significantly the hNELL1-VH-induced ERK1/2 activation and partially the hNELL1-VH-induced JNK1/2/3 activation, while not affected the phosphorylation state of p38α/β/γ. The hNELL1-VH-induced OPN expression was also suppressed by Ras siRNA (Fig. 4B). In the hNELL1-VH-treated RFC cells, similar inhibitory effects were observed by using both a MEK inhibitor PD98059 and a JNK inhibitor SP600125 (see Supplementary Fig. 3). Various osteogenic stimuli, including BMP2, are known to induce the expression of osteomarkers (ALP, OPN, OCN, and so on) along with the activation of MAPK signaling molecules [12]. The activation of ERK and JNK, but not of p38, is necessary for the hNELL1-VH-induced OPN expression.

3.4. Phosphorylation of smad proteins

The phosphorylation of Smad1/5/8, which is usually triggered by the BMP-dependent oligomerization of BMP receptors type I (BMP-R1) and type II (BMP-R2) [13], was examined in the hNELL1-VH protein-treated RFC and Saos-2 cells by Western blotting using an anti-phosphorylated Smad1/5/8 antibody (Fig. 5A and B). Although hBMP2 protein immediately induced the sustained phosphorylation of Smad1/5/8 in both cells (right panels), hNELL1-VH protein showed almost no effect on the phosphorylation state of Smad1/5/8. Smad4 is then translocated from the cytoplasm to the nucleus by forming an oligomer with phosphorylated Smad1/5/8 [13]. Immunocytochemical observation of hBMP2-treated C3H10T1/2 cells revealed rapid nuclear translocation of Smad4, whereas in the hNELL1-treated cells Smad4 remained in the cytoplasm even after 60 min of the treatment (Fig. 5C), confirming the absence of phosphorylation of Smad1/5/8 after the treatment with hNELL1-VH protein.

Next, the promoter region (from −985 to 0 bp) of the human Id1 gene, one of the Smad-dependent BMP target genes, was...
Fig. 3. Activation of MAPK by hNELL1-VH. RFC cells (A), C3H10T1/2 cells (B), and Saos-2 cells (C) were treated with 100 ng/ml hNELL1-VH protein (derived from 125 µl medium), 100 ng/ml hBMP2 protein, or mock sample (derived from 2 ml medium) for the indicated times. Cell lysates (15 µg protein) were analyzed by Western blotting either with anti-phospho-ERK1/2 (p-ERK1/2), anti-phospho-JNK1/2/3 (p-JNK1/2/3), and anti-phospho-p38α/β/γ (p-p38) antibodies. Anti-ERK1/2 (ERK1/2), anti-JNK1/2/3 (JNK1/2/3), anti-p38, and anti-β-actin (actin) antibodies were used as internal controls. All experiments were repeated more than three times, and representative data are shown.

Fig. 4. Effects of Ras siRNA on OPN expression. C3H10T1/2 cells were transfected with Ras siRNA on 1st day, and then treated with 100 ng/ml hNELL1-VH protein for 15 min on 2nd day. (A) Cell lysates (15 µg protein) were analyzed by Western blotting as described in Fig. 3. (B) Cells were cultured for 6 days in the presence of hNELL1-VH. Amounts of OPN secreted into the culture medium were measured by ELISA on 7th day. Each value represents the means ± S.E.M. of triplicate measurements. (*) indicate that the data are significantly different from the control (DMSO, P < 0.005, t-test).
inserted proximal to the upstream of the firefly luciferase gene (Id985-luc) [14]. RFC cells harboring the Id985-luc gene were treated with either hNELL1-VH or hBMP2 protein for 2 days, and the luciferase activity in each cell lysate was measured (Fig. 5D). While the hBMP2 treatment approximately doubled the activity of the control cells, the hNELL1-VH treatment showed essentially no effect on the activity. When the RFC cells harboring the Id985mutB-luc gene, an Id985-luc gene lacking the Smad4-binding sites [14], were treated with hNELL1-VH or hBMP2 protein, the luciferase activity was not affected. These data unequivocally show that the NELL1-induced signaling pathway does not involve the phosphorylation of Smad proteins at least in osteoblastic cells.

3.5. Phosphorylation of Runx2

The transcription factor Runx2 is a key master regulator of osteoblast differentiation, enhancing transcription of the genes for osteomarker proteins [15]. Indeed, Runx2-knockout mice
were deficient in osteoblasts and bones in their skeleton [16]. Upon stimulation of the osteoblastic cells with FGF2, IGF1, or various ECM proteins, the transcription factor Runx2 is phosphorylated (namely activated) by ERK1/2 and then plays an important role in the osteoblast differentiation [17–20]. In the present study, the effect of Ras siRNA on the phosphorylation of Runx2 was investigated in the hNELL1-VH-treated C3H10T1/2 cells. As revealed by Western blotting of the anti-Runx2 immunoprecipitates with an anti-phospho-Ser antibody (Fig. 6), Runx2 was significantly phosphorylated in the NELL1-treated cells. Based on the above results (Fig. 4A) and suppression of Runx2 phosphorylation with Ras siRNA, Runx2 is phosphorylated presumably by ERK1/2. In the hNELL1-VH-treated C3H10T1/2 cells, as revealed by Western blotting of the anti-Runx2 immunoprecipitates with an anti-phospho-Ser antibody (Fig. 6), Runx2 was significantly phosphorylated in the NELL1-treated cells. Based on the above results (Fig. 4A) and suppression of Runx2 phosphorylation with Ras siRNA, Runx2 is phosphorylated presumably by ERK1/2. In the hNELL1-VH-treated RFC cells, similar inhibitory effects were observed by using a MEK inhibitor PD98059 (see Supplementary Fig. 4). In summary, Runx2 is most likely a transcription factor located downstream of the NELL1 signaling cascade. The Smad-independent Ras-MAPK signaling cascade appears to be a major route in the NELL1-induced expression of osteomarkers, namely in the osteoblastic differentiation.

3.6. Immediate accumulation of Tyr-phosphorylated proteins

The purified NELL1 homolog, NELL2, did not interact with a soluble form of BMP-R1 in an in vitro surface plasmon resonance assay [21] (unpublished data). In addition, although NELL2 also contains 6 EGF-like domains (see Fig. 1), NELL2 did not interact with the ErbB superfamily (ErbB-1–4) in an immunoprecipitation assay (unpublished data). The results described so far have suggested that the NELL1 protein interacts with an unidentified specific receptor that is associated with the Ras-MAPK cascade. As a preliminary approach toward identification of the NELL1 receptor and other closely juxtaposed proteins, hNELL1-VH-interacting proteins were immunoprecipitated immediately after the treatment of RFC cells with hNELL1-VH protein and the immunoprecipitates were analyzed by Western blotting with an anti-phospho-Tyr antibody. As shown in Fig. 7B, about 45- and 55-kDa proteins were identified as those directly or indirectly interacting with hNELL1-VH protein and being Tyr-phosphorylated in a very early stage of NELL1-binding to the cells. A slightly Tyr-phosphorylated protein of about 200 kDa was also found after 5 min. Amounts of these proteins reached a plateau at 5 min after the hNELL1-VH treatment and remained unchanged even after 20 min. Similar results were obtained with C3H10T1/2 cells treated with hNELL1-VH protein (data not shown). Collectively, it is suggested that NELL1 forms an extracellular complex with its specific receptor and then the complex facilitates either activation of Tyr-kinases, inactivation of Tyr phosphatases, or intracellular recruitment of Tyr-phosphorylated proteins.

3.7. Putative NELL1 receptor

Recently, various ECM proteins (e.g., laminin 5, fibronectin, vitronectin, and TSP1/2) have been revealed to interact with the integrin family [22]. The ligand-bound integrin transduces the signals through activation of the focal adhesion kinase (FAK), followed by indirect activation of the Ras-MAPK cas-

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Fig. 6. Phosphorylation of Runx2. C3H10T1/2 cells were transfected with Ras siRNA on 1st day, and then treated with 100 ng/ml hNELL1-VH protein for 15 min on 2nd day. The anti-Runx2 immunoprecipitates were analyzed by Western blotting using an anti-phosphoserine antibody (anti-pSer) (upper panel) or an anti-Runx2 antibody (anti-Runx2) (lower panel). All experiments were repeated more than three times, and representative data are shown.

Fig. 7. Detection of Tyr-phosphorylated proteins interacting with hNELL1-VH. RFC cells were treated with 100 ng/ml hNELL1-VH protein for 0, 2, and 5 min. Cell lysates were subjected to immunoprecipitation with either mouse IgG (A) or an anti-V5 tag antibody (B) and analyzed by Western blotting using an anti-phosphotyrosine antibody 4G10. Protein G released from the affinity resin was detected as non-specific bands (white asterisks). Tyr-phosphorylated proteins are indicated by arrowheads.
cade, and then leads to the osteogenic differentiation through Runx2 [23]. The signals elicited by mechanical force also utilize a similar signaling cascade from integrin to Runx2 [24]. In the amino acid sequence comparison between hNELL1 and various ECM proteins (Fig. 1), the N-terminal domain of hNELL1 (aa 85–174) shows a high degree of similarity with the laminin G domain of ECM proteins [25]. In particular, human TSP1 shows the highest identity (22%) with the corresponding region of hNELL1. The laminin G domain serves in the interaction with integrins and a 67-kDa laminin receptor [26]. Mutational analyses of TSP1 and TSP2 have demonstrated that a Glu residue (Glu-90) between β-strands D and E is important for the interaction with integrin α6β1, which is conserved in the similar positions of hNELL1 [22] (Fig. 1). Taken together, the results reported in this paper show that NELL1 is a novel osteogenic factor that transduces the osteogenic signals through the pathway involving Ras-MAPK and Runx2, but not involving BMP receptors and Smads. It is highly likely that an integrin-related molecule and Tyr-kinases are involved in the NELL1-signaling cascade, triggered by binding to the NELL1-specific receptor(s), whose identification is under way.

Acknowledgements: We thank Prof. Ryutaro Kamijo (Showa University Dental School) for helpful discussion and Prof. Takeshi Katagiri (Saitama Medical University) for supplying the Id1l vector. This study was supported in part by a Grant-in-Aid from JSPS (to SK; No. 15300126).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.febslet.2007.12.006.

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