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NELL-1 increases pre-osteoblast mineralization using both phosphate transporter Pit1 and Pit2

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ABSTRACT

NELL-1 is a potent osteoinductive molecule that enhances bone formation in multiple animal models through currently unidentified pathways. In the present manuscript, we hypothesized that NELL-1 may regulate osteogenic differentiation accompanied by alteration of inorganic phosphate (Pi) entry into the osteoblast via sodium dependent phosphate (NaPi) transporters. To determine this, MC3T3-E1 pre-osteoblasts were cultured in the presence of recombinant human (rh)NELL-1 or rhBMP-2. Analysis was performed for intracellular Pi levels through malachite green staining, Pit-1 and Pit-2 expression, and forced upregulation of Pit-1 and Pit-2. Results showed rhNELL-1 to increase MC3T3-E1 matrix mineralization and Pi influx associated with activation of both Pit-1 and Pit-2 channels, with significantly increased Pit-2 production. In contrast, Pi transport elicited by rhBMP-2 showed to be associated with increased Pit-1 production only. Next, neutralizing antibodies against Pit-1 and Pit-2 completely abrogated the Pi influx effect of rhNELL-1, suggesting rhNELL-1 is dependent on both transporters. These results identify one potential mechanism of action for rhNELL-1 induced osteogenesis and highlight a fundamental difference between NELL-1 and BMP-2 signaling.

1. Introduction

NELL-1 [NEL-like molecule-1] is a novel osteoinductive growth factor first isolated and characterized in craniosynostosis patients as locally upregulated within prematurely fusing sutures [1]. Since this time, NELL-1 delivered by adenoviral vector or recombinant human (rh)NELL-1 has been successfully used in numerous skeletal tissue engineering applications, across mammalian species and in the calvarial, axial and appendicular skeleton [2–7]. A number of studies have investigated the *in vitro* effects of NELL-1 on osteoblasts and osteoprogenitor cell types [8–11]. Overall, addition of NELL-1 enhances osteogenic differentiation, while antagonism of NELL-1 signaling blocks differentiation. Specifically in MC3T3-E1 cells, infection with an adenovirus overexpressing *NELL-1* (*AdNELL-1*) induces MC3T3-E1 pre-osteoblasts to upregulate the expression of intermediate and late expressing genes including *Osteopontin* (*Opn*) and *Osteocalcin* (*Ocn*) [3]. Moreover, we demonstrated that in myoblasts, NELL-1 can stimulate the non-BMP-2 associated c-Jun N-terminal kinases (JNK), but not the extracellular signal-related kinases/p42/44 (ERK1/2) or p38, mitogen-activated protein kinase (MAPK) pathway [12]. Again, despite this accumulating data, the precise mechanism of action of NELL-1 in enhancing osteoblast differentiation has not yet been determined.

Classically, the stages of osteogenic differentiation are characterized by proliferation, matrix maturation, and mineralization resulting in hydroxyapatite formation through the accumulation of calcium and phosphate in matrix vesicles that bud from the plasma membrane [13] and/or direct nucleation of extracellular collagen by associated non-collagenous matrix proteins [14]. Inorganic phosphate (Pi) is a necessary component of hydroxyapatite, known to regulate osteoblast function during differentiation by temporally coordinating the cellular and molecular events preceding mineralization [15,16]. Elevated intracellular Pi is required for the initiation of mineralization, but not its maintenance [16]. The primary mechanism for Pi entry through the cell membrane is via a family of sodium dependent phosphate (NaPi) transporters. The type III transporters are found in all mammalian cells including osteoblasts [17,18]. Several studies have already demonstrated the ability of various growth factors such as PTH (Parathyroid hormone), IGF-I (Insulin-like Growth Factor-I), FGF-2 (Fibroblast Growth

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Factor-2), and BMP-2 (Bone Morphogenetic Protein-2) to increase Pi transport in osteoblastic or chondrocytic cells through type III NaPi transporters before and during mineralization [15,18–21].

In order to improve our understanding of the osteoinductive factor NELL-1, we investigated the ability of rhNELL-1 to accelerate osteogenesis accompanied by regulation of Pi transport. We also compared this to the well studied growth factor BMP-2. Such findings will bring insight to the myriad mechanisms whereby NELL-1 signaling may enhance osteogenic differentiation.

2. Methods

2.1. Alizarin red staining and quantitation

MC3T3-E1 pre-osteoblasts were obtained from Sigma Aldrich (Catalog #99072810) and were seeded 2×10^5 cells/well into 24-well culture plates. Cells were treated with 0–500 ng/mL rhNELL-1 or 100 ng/ml rhBMP-2 in the presence of osteogenic media containing DMEM, 10% fetal bovine serum (Gemini, Woodland, CA), 50 μg/ml ascorbic acid, 10 mM β-glycerol phosphate, 10^{-8} μM dexamethasone. Media was replenished every 3 days for 2 weeks. Cells were fixed with 10% formalin, and stained with 1% alizarin red. Images were captured with a Leica DMLB microscope. Alizarin red stain was leached with 10% acetic acid at 85 °C. Supernatant was quantified at 450 nm. Experimental conditions were performed in quadruplicate, repeated three times.

2.2. Intracellular phosphate detection

MC3T3-E1 pre-osteoblasts (2×10^4 cells/well) were seeded at subconfluence into 96-well culture plates. Cells were treated with 0–500 ng/mL rhNELL-1 or 100 ng/mL rhBMP-2 in the presence of osteogenic media. Additional conditions included 2.5 μg/ml actinomycin D, 5 μM cycloheximide, 300 μM foscarnet, 1:10 diluted anti-Pit-1 and anti-Pit-2 antibodies. Cells were cultured from 1 day to 4 weeks. At the appropriate time point, cells were washed with PBS and lysed in 100 μl lysis buffer with 0.2% NP-40 on ice for 10 min. Intracellular Pi levels were quantified when exposed to 0.2% (w/v) malachite green, a coloring method for determining subnanomolar amounts of phosphate in cells according to previously published protocols [23]. Briefly, 50 μl cell lysate were incubated with 10 μl 28 mM ammonium molybdate for 10 min at room temperature. Lysate was then incubated for an additional 30 min after adding 10 μl 0.2%/0.76 mM malachite green. Absorbance was then measured at 610 nm. Experimental conditions were repeated four times in triplicate and normalized with the concentrations of total cellular proteins as determined by BCA Protein Assay Kit (Pierce Biotechnology).

2.3. Proliferation

MC3T3-E1 pre-osteoblasts were seeded to subconfluence in a 96-well plate. The following morning, rhNELL-1 (0–500 ng/ml) was added to the basal culture media (DMEM, 10% serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin). On days 1, 3, and 7 after stimulation, cells were incubated with the CellTiter 96-well plate One Solution Reagent for 2 h according to the manufacturer's instructions.

2.4. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was determined with the use of *p*-nitrophenol phosphate as a substrate at an absorbance at 405 nm, according to the manufacturer's instructions. Cell lysate was harvested as described above. Experimental conditions were repeated three times in quadruplicate and normalized with the concentrations of total cellular proteins as determined by BCA Protein Assay Kit.

2.5. Real-time PCR

Total RNA was extracted by Trizol reagent and DNase treated RNA was tested for its integrity by agarose gel electrophoresis. Two micrograms of DNase I treated total RNA was used for reverse transcription as previously described. The product of reverse transcription was used for real time PCR (RT-PCR). RT-PCR analysis of *Pit-1*, *Pit-2*, and *Gapdh* gene expression was performed with the ABI Prism 7300 real time PCR system and the primers and probes were purchased as TaqMan primer-probe sets. Analysis was based upon calculating the relative expression level of the gene of interest compared to *Gapdh*, and then normalized to the expression induced by control wells at day 0.

2.6. Western blot analysis

Subconfluent MC3T3-E1 pre-osteoblasts in 6-well plates were stimulated with 100 ng/mL rhNELL-1 in osteogenic media for up to 6 days. Cell lysate was harvested in RIPA buffer and quantified using the BCA Protein Assay Kit. Forty micrograms of total proteins was separated on a 12% SDS gel and transferred overnight onto a nitrocellulose membrane. The following morning, membranes were blocked in blocking buffer for 1 h then incubated with primary antibodies against Pit-1 and Pit-2 overnight. After washing with PBS containing Tween 20, anti-Rabbit HPR conjugated secondary antibodies were applied to membranes for 1 h. Membranes were exposed to Super Signal ECL detection reagents and then visualized on film. Protein loading and transfer efficiency was assessed using the MemCode reversible protein stain kit according to manufacturer's instructions. Bands were quantitated using BioQuant Software.

2.7. Statistical analysis

Statistical analysis was performed using unpaired student *t*-test when two values were being compared. A one-way ANOVA was performed when more than two groups were compared, followed by a post hoc Tukey's test was used to compare two groups. $^*p < 0.05$ was considered to be significant.

3. Results

3.1. NELL-1 accelerates extracellular matrix mineralization

First, recombinant human (rh)NELL-1 protein was added to standard osteogenic differentiation medium (ODM) treated MC3T3-E1 pre-osteoblasts. RhNELL-1 significantly increased mineralization at a dose of 100 ng/ml after 2 weeks differentiation (Fig. 1A). As phosphate is one of the main constituents of biological apatite and a prerequisite to mineralization, we investigated rhNELL-1 and rhBMP-2 induced osteogenic differentiation in the presence of foscarnet, a chemical that decreases phosphate availability [23] (Fig. 1B). For ease of comparison, all groups were normalized to control (left), so as to directly address the relative effects of foscarnet with growth factor addition. The presence of foscarnet significantly reduced growth factor-induced mineralization with either NELL-1 or BMP-2. These data suggested an important role of phosphate transport in both NELL-1 and BMP-2 induced MC3T3-E1 differentiation.

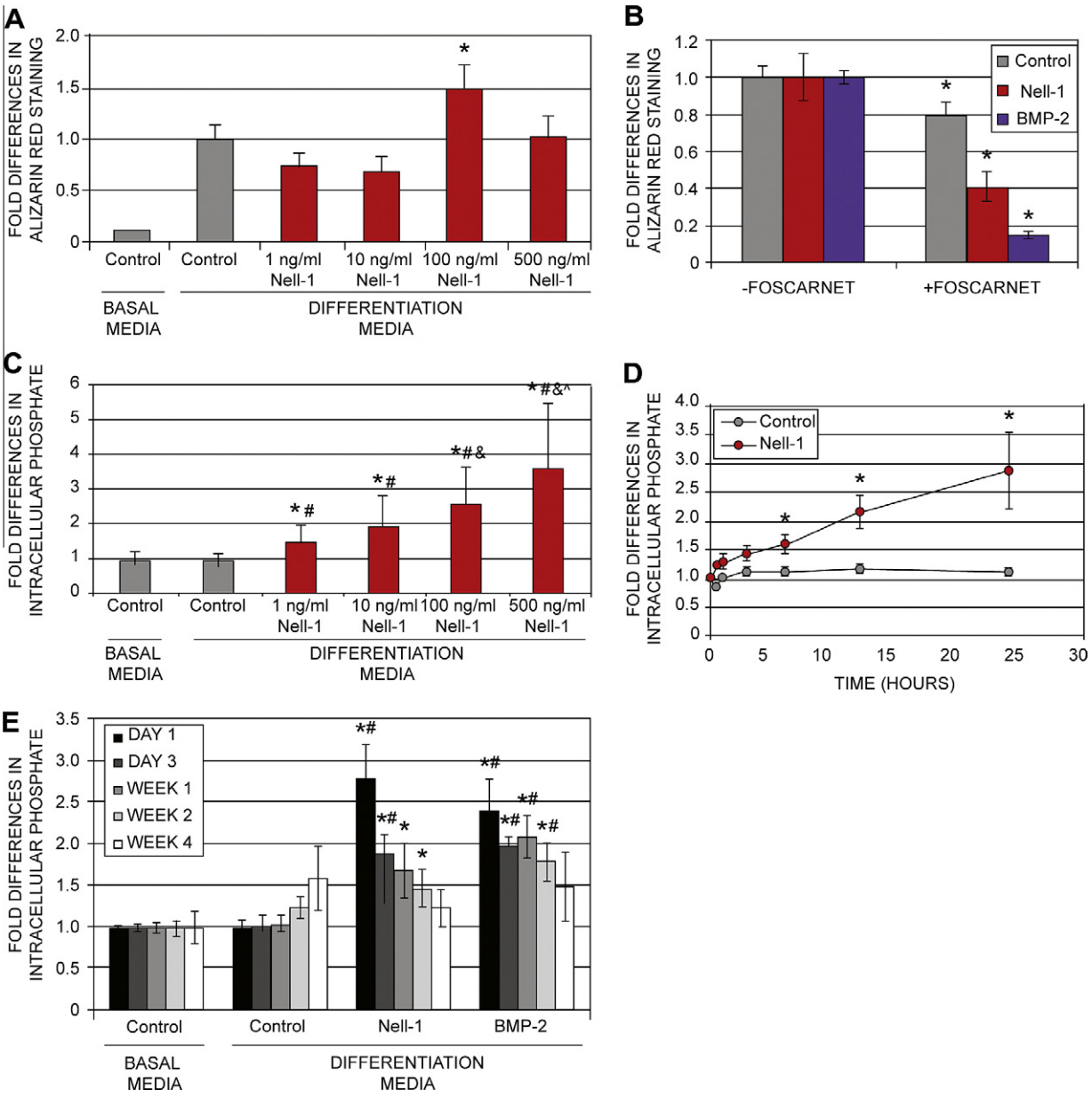

Fig. 1. RhNELL-1 induced MC3T3-E1 mineralization is antagonized by foscarnet, and effects on rhNELL-1 on Pi transport. (A) Fold increase bone nodule formation as assessed by leaching and photometric quantitation of alizarin red staining after 2 weeks in the presence of 0–500 ng/ml rhNELL-1; $^{*}p \leqslant 0.05$ in comparison to control samples in basal media. (B) Fold increase (±SE) in Alizarin red staining after 2 weeks differentiation in the presence of 100 ng/ml rhNELL-1, 100 ng/ml rhBMP-2 with or without 300 μM foscarnet. Data displayed as means ± 1 SE. $^{*}p \leqslant 0.05$ in comparison to corresponding samples in the absence of inhibitors. (C) Fold increase in intracellular Pi levels after 1 day in the presence of 0–500 ng/ml rhNELL-1. Data displayed as means ± 1 SE. (*) Significant differences from control samples in basal media, (#) control samples in differentiation media, (&) differentiation media with 1 ng/ml rhNELL-1, or (^) 10 ng/ml rhNELL-1; $p \leqslant 0.05$. (D) Fold increase in intracellular Pi levels over 24 h in the absence or presence of 100 ng/ml rhNELL-1; $^{*}p \leqslant 0.05$ at corresponding time point. (E) Fold increase in intracellular Pi levels in cells cultured in the presence of 100 ng/ml rhNELL-1 or rhBMP-2 for 1 day to 4 weeks; $^{*}p \leqslant 0.05$ in comparison to basal media at corresponding time point; $^{\#}p \leqslant 0.05$ in comparison to differentiation media at corresponding time point.

3.2. NELL-1 regulates Pi transport

The specific effects of rhNELL-1 on intracellular Pi levels were next investigated. RhNELL-1 (1–500 ng/ml) increased intracellular phosphate levels in a dose dependent manner after 1 day of stimulation (Fig. 1C). All tested concentrations of rhNELL-1 significantly increased Pi levels above control (Fig. 1C). Additionally, Pi transport steadily increased over the first 24 h of rhNELL-1 stimulation (100 ng/ml) (Fig. 1D). Intracellular Pi levels peaked at 1 day and remained elevated through 2 weeks of culture in the presence of either rhNELL-1 or rhBMP-2 (100 ng/ml each) (Fig. 1E). Thus, similar to BMP-2, rhNELL-1 significantly and persistently increased intracellular Pi levels in MC3T3-E1 pre-osteoblasts.

3.3. NELL-1 increases ALP activity, but not by proliferation

Increases in ALP activity characteristically occurs early in the osteogenic differentiation process. Results showed that rhNELL-1 generally increased ALP activity over control, with the highest in 1 ng/ml NELL-1 (1–100 ng/ml reaching statistical significance) (Fig. 2A). RhBMP-2 (100 ng/ml), on the other hand, induced significantly elevated ALP activity levels to a higher degree than equivalent concentrations rhNELL-1 (Fig. 2B). These results suggested one potential difference in NELL-1 and BMP-2 signaling.

A converse relationship between growth factor induced proliferation and differentiation is frequently observed. In line with this, rhNELL-1 reduced in proliferation in comparison to control

(Fig. 2C). Thus, while NELL-1 positively regulates osteogenesis, it had negative effects on proliferation of MC3T3-E1 cells.

3.4. NELL-1 regulates Pit phosphate transporter activity

Next, the regulation of Pi transport by NELL-1 signaling was investigated. Type III NaPi transporters, including Pit-1 and Pit-2, are found in osteoblasts and increase intracellular directed Pi transport. Transporter specificity was examined using anti-Pit-1 and anti-Pit-2 neutralizing antibodies. Interestingly, while both anti-Pit-1 and anti-Pit-2 antibodies reduced rhNELL-1 mediated effects on Pi transport, rhBMP-2 mediated effects were not reduced by anti-Pit-1 and anti-Pit-2 antibodies. (Fig. 3A).

We next inquired as to the effects of osteogenic media, as well as new gene or protein production for the effects of NELL-1 on Pi transport. First, rhNELL-1 increased Pi transport irrespective of whether basal or differentiation media was used (Fig. 3B). In addition, intracellular phosphate levels were equivalently elevated in cells cultured in the presence or absence of Actinomycin D (Actino). On the other hand, intracellular phosphate levels were no longer elevated in the presence of Cycloheximide (Cyclo).

Next, the effects of rhNELL-1 on Pit-1 and Pit-2 gene and protein expression were further investigated. As expected, rhNELL-1 did not increase either *Pit-1* (Fig. 3C) or *Pit-2* (Fig. 3D) gene transcript abundance. In contrast, BMP-2 stimulation significantly increased *Pit-1* expression after 6 days of treatment, but not *Pit-2*. Western blot demonstrated rhNELL-1 increased protein levels of Pit-2 (Fig. 3E), while rhBMP-2 increased the production of Pit-1. Notably, significance in Western blot densitometry analysis was appreciated at one hour post stimulation (Fig. 3F).

Next, cells were cultured in the presence of excessive Pi (10 mM NaPO4) (Fig. 4A). As expected, Pi transport in the absence of growth factor stimulation was significantly increased. As compared to the previous culture conditions, rhNELL-1 and rhBMP-2 induced Pi transport was relatively unchanged at 1 day, suggesting transporter saturation. In contrast, by week 4 both rhNELL-1 and rhBMP-2 exposure resulted in increased intracellular Pi even in the presence of excess Pi, possibly due to the production of new Pi transporters.

Finally, we examined changes in Pi transport by rhNELL-1 and rhBMP-2 after cellular transfection with an empty vector (pcDNA3.1), Pit-1 overexpression plasmid (pcPit-1), or Pit-2 overexpression plasmid (pcPit-2) (Fig. 4B). As previously seen, both rhNELL-1 and rhBMP-2 significantly increased Pi transport upon empty vector transfection. Of note, rhBMP-2 induced Pi transport was relatively unchanged upon excessive Pit-2 production, while rhNELL-1 induced Pi transport was significantly elevated in cells producing excessive Pit-1 or Pit-2.

4. Discussion

The current study demonstrated that rhNELL-1 increased intracellular phosphate levels in a dose dependent manner in pre-osteoblast MC3T3-E1 cells, a critical step in mineralization. It further revealed that NELL-1 induced alkaline phosphatase activity and matrix mineralization in MC3T3-E1 pre-osteoblasts. Interestingly, the maximally effective dose for upregulation of alkaline phosphatase differed from doses most effective for matrix mineralization and for intracellular Pi increase. Alkaline phosphatase activity was not dose-dependently upregulated by rhNELL-1, whereas intracellular phosphate was dose-dependently increased by rhNELL-1. These data suggest that rhNELL-1 has important dose-dependent and potentially biphasic biological effects, similar to our previous observations [11].

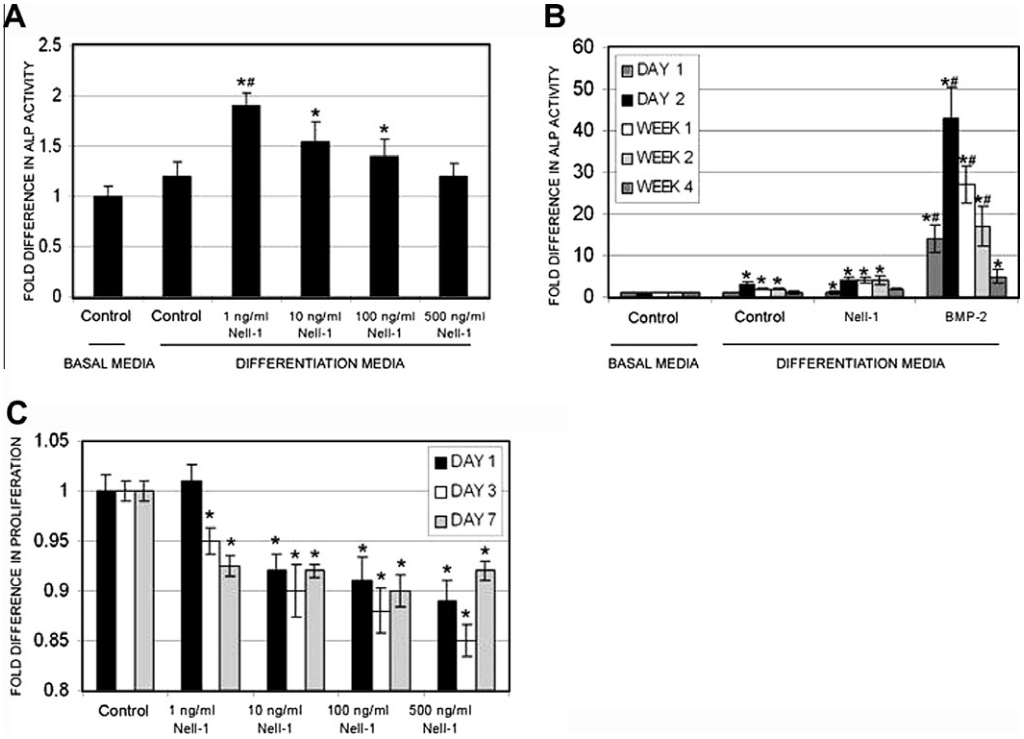

Fig. 2. RhNELL-1 increases ALP activity, but not proliferation of MC3T3-E1. (A) Fold increase in ALP activity after 1 day of culture in the presence of 0–500 ng/ml rhNELL-1, as compared basal media; (*) Significant differences from control samples in basal media, (#) control samples in differentiation media; $^{*}p \leqslant 0.05$. (B) Fold increase in ALP activity in the presence of 100 ng/ml rhNELL-1 or rhBMP-2 on day 1 to week 4; $^{*}p \leqslant 0.05$ in comparison to control samples in basal media; # $p \leqslant 0.05$ in comparison to control samples in differentiation media. (C) Fold increase in proliferation after 1, 3, and 7 days of stimulation with 0–500 ng/ml rhNELL-1; $^{*}p \leqslant 0.05$ in comparison to control at corresponding time point. Data displayed as means ± 1 SE.

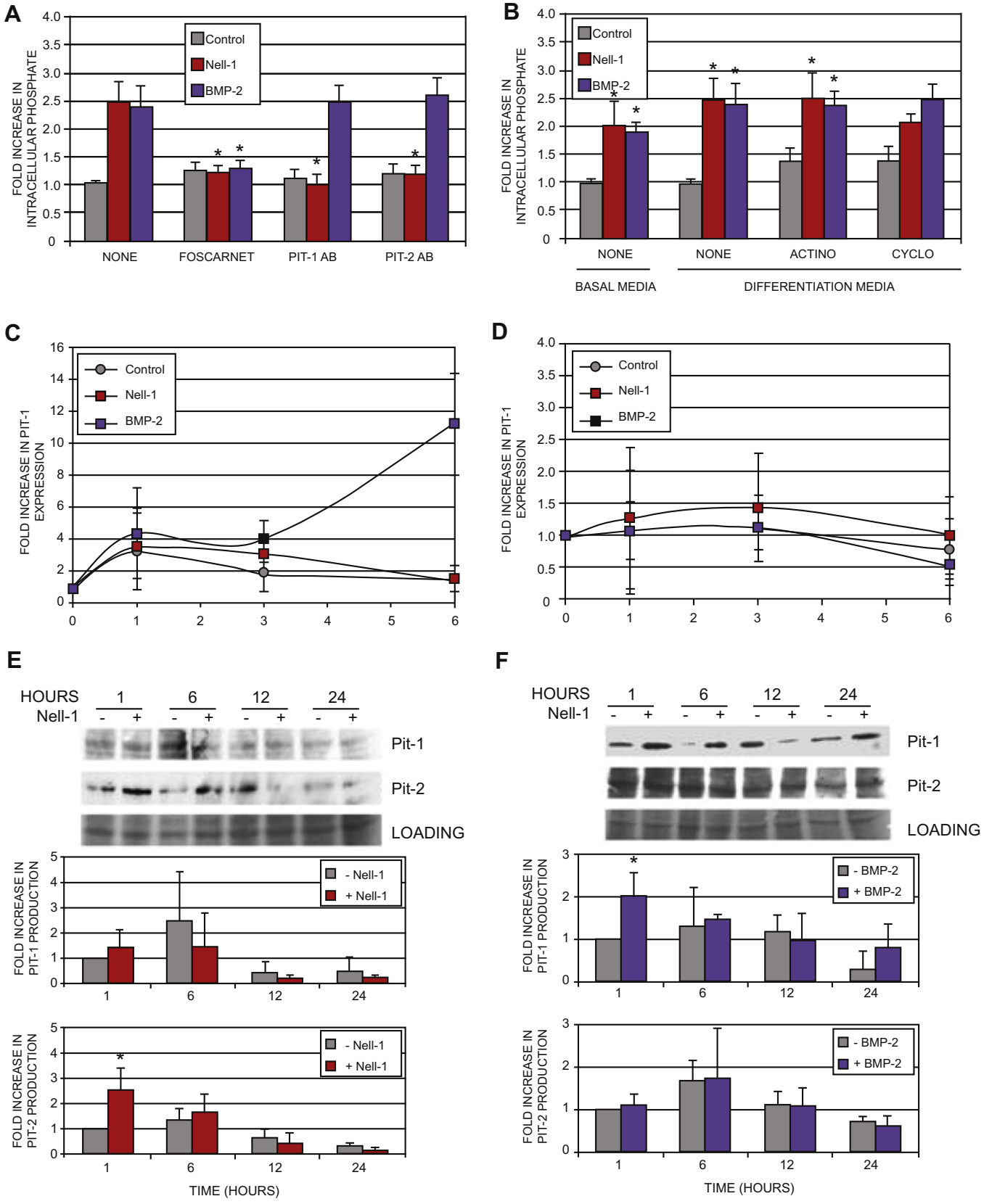

Fig. 3. RhNELL-1 regulates Pit transporters. (A) Fold increase in intracellular Pi levels after 1 day of culture in the presence of 100 ng/ml rhNELL-1 or rhBMP-2 with or without 300 μM foscarnet, anti-Pit-1 antibody, or anti-Pit-2 antibody; (*) Significant differences from corresponding samples in the absence of inhibitors $^{*}p \leqslant 0.05$. (B) Fold increase in intracellular Pi levels in cells cultured in the presence of basal culture media, differentiation culture media, 2.5 μg/ml actinomycin D (Actino) or 5 μM cycloheximide (Cyclo); $^{*}p \leqslant 0.05$ in comparison to control samples in basal media. Gene expression of (C) *Pit-1* and (D) *Pit-2* in cells stimulated with 100 ng/ml rhNELL-1 or rhBMP-2 for 1, 3, or 6 days. Images displaying (E) rhNELL-1-induced and (F) BMP-2-induced Western blot bands for Pit-1, Pit-2, and protein dye for loading efficiency, while the graphs quantitate the Western bands normalized to loading efficiency; $^{*}p \leqslant 0.05$ in comparison to controls at corresponding time point. Experiments were repeated three times and data are displayed as means ± SE with significant differences calculated; $^{*}p \leqslant 0.05$.

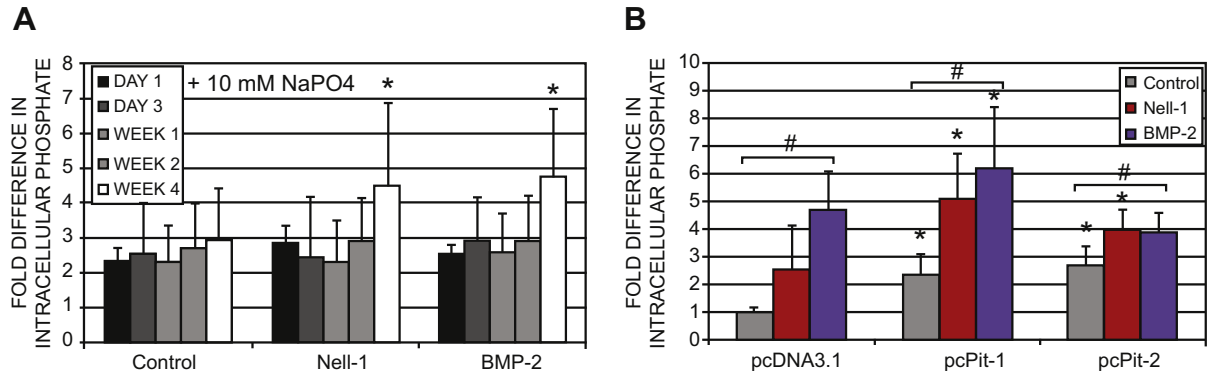

Fig. 4. Effects of excess phosphate or transporters on Pi Transport. (A) Fold increase in intracellular Pi levels after being cultured in the presence of 10 mM NaPO4 along with 100 ng/ml rhNELL-1 or rhBMP-2; $^{*}p \leqslant 0.05$ in comparison to control at corresponding time point. (B) Fold increase in intracellular Pi levels after transfection with pcDNA3.1, pcPit-1, or pcPit-2 for 1 day and cultured in the presence of 100 ng/ml rhNELL-1 or rhBMP-2 for 1 additional day. Significant differences from (*) pcDNA3.1 transfected cells or (#) the no growth factor condition are noted; $p < 0.05$.

Osteogenic cells utilize Pi for basic metabolic processes, but also for the initiation events of bone matrix calcification in matrix vesicles [15,16]. ALP is an enzyme that cleaves a phosphate from an organic source, such as β-GP, to increase the extracellular concentration of Pi. RhBMP-2 is known to significantly increases ALP activity and Pi transport into cells to initiate mineralization [15]. Osteoblasts regulate inward Pi transport via a Na gradient through type III NaPi transports. The loss of type III mediated transport does not decrease the viability of cells, indicating the presence of additional housekeeping Pi transporters [24]. Pi transport is low in proliferative cells, but increases during differentiation [25] as a result of increased phosphate transporters or transporter activity [15]. Our findings suggested that increased Pi transport in response to rhNELL-1 was primarily due to increased transporter activity and secondarily due to increased transporter protein production. The use of foscarnet, a general NaPi transport inhibitor, effectively eliminated rhNELL-1 induced Pi transport and matrix mineralization. Thus, NaPi transporter activity appears to play a significant role in rhNELL-1 induced MC3T3-E1 osteogenesis.

Pit-1 expression has been detected in hypertrophic chondrocytes during endochondral bone formation [26] and Pit-1 overexpression results in pathological calcification [27]. Unlike rhBMP-2, rhNELL-1 did not upregulate the transcript abundance of either *Pit-1* or *Pit-2*; however, rhNELL-1 did upregulate Pit-2, but not Pit-1, protein concentration at 1 h after stimulation. Furthermore, the loss of Pit-1 and Pit-2 activity eliminated rhNELL-1 induced Pi transport suggesting their significant role in NELL-1 activities.

Pi has been shown to regulate numerous skeletal processes distinct from osteoblast differentiation as shown in the present manuscript. For example, Pi regulates bone resorption, where Pi depletion is well characterized to stimulate osteoclastic bone resorption [30,31]. Conversely, Pi inhibits osteoclast differentiation as well as other bone resorption processes [32]. These studies have shown that Pi modulates the RANK/RANKL/OPG axis to inhibit osteoclast differentiation [33,34]. In addition, chondrocyte differentiation has been shown to also be regulated by Pi influx. Using the growth plate as a model, low levels of Pi are present in pre-mineralized chondrocytes [35]. In contrast, abundant Pi is present as chondrocytes begin to mineralize. Interestingly, NELL-1 signaling is important in both bone maintenance and homeostasis (manuscript in submission), as well as chondrocytic differentiation [36]. NELL-1 may also mediate some of its effects on osteoclasts and chondrocytes via regulation of Pi.

In summary, rhNELL-1 induces and/or enhances the osteogenic differentiation of MC3T3-E1 cells accompanied by increased intracellular phosphate levels. This occurs, in distinction to rhBMP-2, accompanied by activation of both Pit-1 and Pit-2 transporters, as well as increased Pit-2 production. Such studies elucidating the basic mechanisms of action of NELL-1 may be of benefit in future skeletal tissue engineering applications.

Conflict of interest

Drs. X.Z, K.T, and C.S. are inventors of Nell-1 related patents. Drs. X.Z, B.W., K.T, and C.S are founders of Bone Biologics Inc. which sublicenses Nell-1 patents from the UC Regents.

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