Original Paper

Nell-1 Enhances Osteogenic Differentiation of Pre-Osteoblasts on Titanium Surfaces via the MAPK-ERK Signaling Pathway

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Key Words

Titanium (Ti) surface • Pre-osteoblasts • Nell-1 • MAPK/ERK signaling pathway

Abstract

Background/Aims: This study aimed to investigate the effect of Nell-1 on the osteogenic behaviors of pre-osteoblasts on titanium (Ti) surfaces and to identify the underlying signaling pathway. **Methods:** Nell-1 at different concentrations was added to culture medium to stimulate MC3T3-E1 subclone 14 on Ti surfaces. A CCK-8 colorimetric assay was used to detect cell proliferation. Alkaline phosphatase activity (ALP) assay and enzyme-linked immunosorbent assay (ELISA) were used to evaluate ALP activity and the osteocalcin (OCN) secretion, respectively. Indicators of osteoblastic differentiation were assessed using real-time polymerase chain reaction analysis (RT-PCR). Western blot (WB) assay was used to analyze the expression changes of the osteogenic proteins and the mitogen-activated protein kinase (MAPK) pathway. **Results:** Nell-1 significantly increased the osteogenic gene and protein expression levels of ALP, OCN, Runx2, osteoprotegerin (OPG), collagen type I (Col-I), and Osterix (Osx) in pre-osteoblasts on Ti surfaces. The optimal concentration of Nell-1 was 100 ng/ml. In addition, Nell-1 activated ERK and JNK, but not P38, in MC3T3-E1 cells on the Ti surface. Except for ALP and Col-I, the promotive effects of Nell-1 on the expression of osteogenic markers were suppressed by ERK inhibitor U0126. **Conclusion:** Certain concentrations of Nell-1 can promote the osteogenic differentiation of pre-osteoblasts on Ti surfaces by activating the MAPK/ERK signaling pathway.

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Introduction

Titanium (Ti) is one of the most commonly used materials for dental implants. Surface modification plays an important role in Ti implants' special physical morphology, chemical composition, and biochemical modifications, which promote the adhesion, proliferation and differentiation of osteoblasts on Ti surfaces; as a result, the bone integration cycle can be

shortened, and bone-bonding strength is enhanced [1-5]. Esposito M *et al.* analyzed various factors associated with the success of osseointegrated dental implants based on the findings of clinical trials and concluded that the initial adhesion of cells determines the final fate of osteoblasts on the implant surface [6]. The early adhesion of osteoblasts to the tissue-implant interface is of great significance for the osteointegration and final success of the implants because it largely determines whether the bond between a dental implant and bone tissue will be long lasting.

Cytokine-based therapy for the osteointegration of implants has attracted considerable attention from researchers due to its strong ability to regulate cellular functions. Nell-1, a unique Nel-like secreted protein consisting of 810 amino acids, was first isolated and characterized in craniosynostosis patients, and it was found to be upregulated within prematurely fusing sutures [7]. Transgenic mice overexpressing the Nell-1 gene showed a phenotype similar to that of human craniosynostosis, while Nell-1-deficient mice exhibited cranial and vertebral bone defects, suggesting an important role of Nell-1 in skeletal development [8, 9]. Mechanistically, Nell-1 binds to the cell membrane receptor integrin b1[10] and regulates the activity of the master osteogenic transcription factor, Runt-related transcription factor 2 (Runx2) [11]. In recent years, the role of Nell-1 in the potent induction of bone development has been confirmed in *in vivo* models [12, 13]. As an effective growth factor, Nell-1 is very specifically associated with the osteochondral lineage; thus, it has higher bio-safety and accuracy for treating bone defects and enhancing bone regeneration compared with bone morphogenetic proteins (BMPs) [14, 15]. Studies have demonstrated that overexpression of Nell-1 via adenovirus infection led to upregulated expression of osteogenic genes, including osteopontin (OPN) and osteocalcin (OCN), in MC3T3-E1 pre-osteoblasts [16]. Although the osteogenic effects of Nell-1 have been clearly elucidated [16-19], the effects of Nell-1 on the growth, function and mechanism of MC3T3-E1 pre-osteoblasts, particularly MC3T3-E1 cells cultured on Ti, remain unclear.

There are four main molecular pathways involved in osteogenesis, i.e., the BMP-Smads pathway, the Wnt/ β -catenin pathway, the mitogen-activated protein kinase (MAPK) pathway and the Notch pathway. They coordinate and interact with one another and play an important role in the proliferation and differentiation of osteoblasts. The MAPK signaling pathway is involved in controlling cell growth, development, division, death and other physiological sub-activity processes by influencing nuclear transcription factors [20]; additionally, the MAPK signaling pathway plays a pivotal role in the activation and signaling of various growth factors [21-24]. One of the most thoroughly studied MAPK family members, extracellular signal-regulated kinase (ERK), is extensively expressed in cells and involved in the regulation of a series of life-cycle processes, including meiosis, cell differentiation, mitosis, and energy metabolism [25]. The study by Bokui N *et al.* revealed that the ERK signaling pathway may play a role in the Nell-1-induced osteoblastic differentiation of primary rat embryonic cranial cells (RFC) [26]. This prompted us to further explore the role of the MAPK-ERK signaling pathway in the Nell-1-induced osteoblastic differentiation of MC3T3-E1 pre-osteoblasts on Ti surfaces.

Here, we explore the roles of Nell-1 in activating pre-osteoblasts on Ti surfaces and seek to clarify the regulatory role of ERK-MAPK in the Nell-1-induced osteogenic behavior of MC3T3-E1 pre-osteoblasts on Ti surfaces.

Materials and Methods

Biomaterials

Pure Ti disks with dimensions of 22 mm and 34 mm were machined to fit into 12-well plates and 6-well plates, respectively. They were polished using #400, #600, #800 and #1200 sandpaper consecutively; blasted with white corundum sand at 0.8 pressure; cleaned ultrasonically in acetone, absolute ethyl alcohol and ultrapure water; passivated with 40% nitric acid for 30 min; and then ultrasonically cleaned with

distilled water and dried at room temperature. Prior to use, all disks were disinfected with ultraviolet irradiation for 30 min.

Bioactive recombinant human Nell-1 protein (rhNell-1) was produced and purified from Chinese hamster ovary cells (R&D Systems, USA).

Cell osteoblast culture and cell seeding

The mouse osteoblastic MC3T3-E1 subclone 14 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China (ATCC CRL-2594). MC3T3-E1 cells were cultured in α -MEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% penicillin/streptomycin (Sigma-Aldrich Corp, USA), and 2 mM L-glutamine (Gibco, USA) and incubated in a 5% CO₂ atmosphere at 37°C.

The cells were seeded at a density of 20,000 cells per ml in the 12-well plates and 50,000 cells per ml in the 6-well plates. The Ti disks with treated and disinfected surfaces were placed at the bottom of the plates. Osteoblastic differentiation was induced in α -MEM containing 10 mM β -glycerophosphate, 0.1 μ M dexamethasone and 0.05 mM ascorbic acid.

Inhibition of the MAPK/ERK signaling pathway

U0126, an ERK/MAPK inhibitor, was purchased from MedChem Express (MCE, USA). MC3T3-E1 pre-osteoblasts were pre-incubated with U0126 at a concentration of 5 μ M for 1 h and then incubated in basal medium or differentiation medium with the inhibitor for 6 to 18 days according to the requirements of each experiment.

Cell viability assay

MC3T3-E1 pre-osteoblasts were seeded on the Ti disks in a 12-well plate. After 24 h, the cells were divided into five groups and incubated with Nell-1 at concentrations of 0 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, or 500 ng/ml at 37°C. On day 1, 3 or 5, the culture medium in each well was replaced with 10% CCK-8 buffer; the cells were incubated for another 2 h and then subjected to a CCK-8 colorimetric assay for cell viability assessment. The solution sample (200 μ l) from each well was transferred in triplicate into a 96-well plate, and its absorbance was measured at a wavelength of 450 nm by a spectrophotometer. The mean reading of each triplication was used for the analysis, and the experiment was repeated three times.

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) activity was measured using an ALP activity assay kit (Wako Pure Chemical Industries, Ltd., 1-2, Doshomachi 3-Chome, Chuo-Ku, Osaka, Japan). The MC3T3-E1 pre-osteoblasts cultured in 6-well plates were lysed with 80 μ l of cell lysis reagent (Sigma-Aldrich, St Louis, MO, USA) and then the lysate was normalized for total cellular protein content using a BCA protein assay kit (Thermo, Rockford, IL, USA). After reaction with p-nitrophenol phosphate (pNPP) substrate, the OD values of the standards and samples were measured at a wavelength of 405 nm according to the manufacturer's instructions to evaluate the ALP activity level. The experiment was repeated three times in quadruplicate wells.

Enzyme-linked immunosorbent assay (ELISA) for OCN expression

The supernatant in each well of the 6-well plates was collected and stored in a refrigerator at 80°C prior to OCN quantification. The OCN levels were determined using commercially available mouse-specific ELISA kits (R&D Systems, USA). The absorbance at a wavelength of 450 nm was measured using a microplate reader. The results were normalized according to the total cellular protein content determined with a BCA protein assay kit and expressed as ng/mg protein.

Table 1. Primer sequences for real-time PCR

Gene	Sequences
Runx2	forward: 5-CCCGTGGCCTTCAAGGT-3
	reverse: 5-CGTTACCCGCCATGACAGTA-3
COL-1	forward: 5-ATGCCGCGACCTCAAGATG-3
	reverse: 5-TGAGGCACAGACGGCTGAGTA-3
OCN	forward: 5-AGCAGCTTGGCCAGACCTA-3
	reverse: 5-TAGCGCCGGAGTCTGTTCACCTAC-3
OSX	forward: 5-TGGTACACGGCAGGCATCCA-3
	reverse: 5-GGAGCAACGTCAGATGGGTAACT-3
ALP	forward: 5-TGCATGAATTCCTGCCTTACCAACTCATTT-3
	reverse: 5-TGCATAAGCTTGAGAGCCACAAAGGGA-3
OPG	forward: 5-CAGAGAAGCCACGCAAAAGTG-3
	reverse: 5-AGCTGTGTCTCCGTTTATCCT-3
GAPDH	forward: 5-TGTGTCCGTCTGGATCTGA-3
	reverse: 5-TTGCTGTTGAAGTCGAGGAG-3

Western blot (WB) assay

The MC3T3-E1 pre-osteoblasts cultured in 6-well plates to subconfluence were stimulated with Nell-1 at concentrations of 0 g/m, 1 ng/ml, 10 ng/ml, 100 ng/ml, or 500 ng/ml. Cellular proteins were extracted using RIPA lysis buffer (Sigma-Aldrich) and quantified using a BCA protein assay kit. After protein separation on a 10% SDS-PAGE gel, the sample was transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). After blocking with 5% fat-free milk, the membrane was incubated overnight at 4°C with the following primary antibodies: osteoprotegerin (Cat.ab9986, Abcam), Collagen I (Cat.ab34710, Abcam), Runx2 (Cat.8486, Cell Signaling), Phospho-ERK1/2 (Cat.4370, Cell Signaling), ERK1/2 (Cat.4695, Cell Signaling), Phospho-SAPK/JNK (Cat.4668, Cell Signaling), SAPK/JNK (Cat.9252, Cell Signaling), Phospho-P38 (Cat.4511, Cell Signaling), P38 (Cat.8690, Cell Signaling), or GAPDH. Except for anti-Phospho-ERK1/2 antibody, which was used at a dilution of 1:2000, all primary antibodies were used at 1:1000 dilution. The following morning, the membrane was incubated with the corresponding secondary HRP-conjugated antibodies for 1 h at room temperature and then exposed to an ECL reagent (Super Signal, Pierce, Rockford, IL, USA). The signals were viewed and analyzed using the ChemiDoc™ XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA, USA), and the band densities were quantified with Image Lab software (BioRad). Each test was repeated at least three times.

Real-time PCR

MC3T3-E1 pre-osteoblasts in 6-well plates were washed three times with PBS and lysed with TRIzol reagent (TriPure Isolation Reagent, Roche Applied Science). Total RNA was extracted according to the manufacturer's protocol. The extracted RNA was quantified with UV spectrophotometry, and only samples with a ratio of absorbance at 260 and 280 nm (the 260/280 ratio) greater than 1.8 were used in the subsequent steps. Total RNA (0.5 µg) was reverse transcribed in duplicate, and another RNA sample incubated without reverse transcriptase was used as the control (noRT control). The PrimeScript RT reagent kit was used for cDNA synthesis, and a MyCycler™ thermocycler (Bio-Rad, USA) was used for polymerase chain reaction (PCR). All primers used are listed in Table 1. Samples were run in parallel with each primer set in real-time PCR with bio-radiQ SYBR® Green Super Mix. Each step was repeated at least three times.

Statistical analysis

The results were analyzed using SPSS software (SPSS 21, Chicago, IL, USA). The data are presented as means ± SD; at least three independent experiments were performed for each test. Differences between groups were assessed with Student's *t*-test and one-way analysis of variance. *P* values < 0.05 were considered statistically significant.

Results

Ti surface characteristics and growth of MC3T3-E1 pre-osteoblasts

The processed Ti surface presented an uneven and rough morphology in SEM images (Fig. 1A). At 24 h after seeding, MC3T3-E1 cells were attached to the Ti surface and well dispersed. Moreover, the cells developed polygonal membranes and showed the morphological characteristics of pre-osteoblasts (Fig. 1B).

Fig. 1. Representative scanning electron microscope (SEM) images at a magnification of ×2000. A: Surface morphology of the Ti disks. B: Pre-osteoblasts attached to a Ti surface after 24 h.

Fig. 2. Effect of Nell-1 on the proliferation of MC3T3-E1 pre-osteoblasts cultured on Ti surfaces. Treatment with Nell-1 at all concentrations (0-500 ng/ml) decreased the proliferation of MC3T3-E1 cells on Ti surfaces from day 1 to day 5 in a time-dependent manner. Data represent the mean $\pm$ SD (bars) of three independent experiments; * P <0.05, ** P <0.01 compared with the 0 ng/ml Nell-1 treatment group (control group).

Fig. 3. Effect of Nell-1 on the ALP activity of MC3T3-E1 pre-osteoblasts cultured on Ti surfaces. Cells cultured in basal medium or differentiation medium containing different concentrations of Nell-1 (0-500 ng/ml) were monitored for ALP activity. The results at days 4, 8 and 12 showed that ALP activity in the cells was enhanced by Nell-1 and was highest in the 100 ng/ml group on the 8th day. Data represent the mean $\pm$ SD (bars) of three independent experiments; * P <0.05, ** P <0.01 compared with the cells cultured in differentiation medium containing 0 ng/ml Nell-1.

Fig. 4. Effect of Nell-1 on osteocalcin expression in MC3T3-E1 pre-osteoblasts cultured on Ti surfaces. Cells cultured in basal medium or differentiation medium containing different concentrations of Nell-1 (0-500 ng/ml) were monitored for OCN expression. The results at days 6, 12, 18, and 24 showed that OCN expression in the cells was enhanced by Nell-1, and expression was highest in the 100 ng/ml group on the 18th day. Data represent the mean $\pm$ SD (bars) of three independent experiments; * P <0.05, ** P <0.01 compared with cells cultured in differentiation medium containing 0 ng/ml Nell-1.

Nell-1 suppressed the proliferation but enhanced the osteogenesis of MC3T3-E1 pre-osteoblasts on the Ti surface

The proliferation of MC3T3-E1 pre-osteoblasts was assessed with a CCK-8 assay. The proliferation activity of the cells treated with 10, 100 and 500 ng/ml Nell-1 was significantly lower than that of the control group on the Ti surfaces, suggesting that Nell-1 suppressed the proliferation of MC3T3-E1 pre-osteoblasts in a dose-dependent manner (Fig. 2). Early osteogenesis is characterized by increased ALP activity. In this study, after Nell-1 treatment, the ALP activity of MC3T3-E1 cells attached to the Ti surface increased remarkably from day 4 to day 8 and then decreased between days 8 and 12. In particular, Nell-1 at concentrations of 10 and 100 ng/ml significantly enhanced the ALP activity of the cells. The most prominent change occurred on post-treatment day 8 in the cells treated with 100 ng/ml Nell-1 (P < 0.05) (Fig. 3). Next, the expression of OCN, an osteogenic marker of later stages of osteogenesis, was tested. The results showed that OCN expression increased from day 6 to day 18 and then declined from day 18 to day 24. On days 12 and 18, OCN expression in the cells treated with 10 and 100 ng/ml Nell-1 was significantly higher than that of the control group in differentiation media (P < 0.05), with the highest expression occurring on day 18 in the 100 ng/ml Nell-1 treatment group (Fig. 4). Additionally, after 6 days of Nell-1 stimulation, the expression of Runx2, osteoprotegerin (OPG), and collagen type I (Col-I) significantly

Fig. 5. Nell-1-induced increases in osteogenic protein expression in MC3T3-E1 pre-osteoblasts cultured on Ti surfaces. Representative Western blots (A) showed that the expression levels of Runx2, OPG, and Col-I in the pre-osteoblasts increased after treatment with Nell-1 for six days. Western blot analysis showed similar patterns of change for Runx2 (B), OPG (C) and Col-I (D) protein expressions with the increased dose of Nell-1. Data represent the mean $\pm$ SD (bars) of three independent experiments; * P <0.05, ** P <0.01 compared with cells cultured in differentiation medium containing 0 ng/ml Nell-1.

Fig. 6. Nell-1-induced changes in the mRNA expression levels of osteogenic markers in MC3T3-E1 pre-osteoblasts cultured on Ti surfaces. Real-time PCR analysis demonstrated similar patterns of change in ALP (A), OCN (B), Runx2 (C), Col-I (D), OPG (E) and Osx (F) gene expression with the increased dose of Nell-1 at day 6, indicating that 100 ng/ml was the most effective dose. Data represent the mean $\pm$ SD (bars) of three independent experiments; * P <0.05, ** P <0.01 compared with cells cultured in differentiation medium containing 0 ng/ml Nell-1.

increased in cells treated with 10, 100 and 500 ng/ml Nell-1 compared with the control group, with the most prominent increase occurring in the 100 ng/ml Nell-1 treatment group (P < 0.01) (Fig. 5). Consistent with these results, real-time PCR analysis demonstrated that the mRNA expression levels of all the detected osteogenic markers, including ALP, OCN, Runx2, OPG, Col-I and Osterix (Osx), in MC3T3-E1 pre-osteoblasts were upregulated after Nell-1 treatment and were highest in the 100 ng/ml Nell-1 group compared with the control group (Fig. 6).

Fig. 7. Increase in ERK and JNK phosphorylation in pre-osteoblasts treated with Nell-1. MC3T3-E1 cells were cultured in serum-free medium on Ti surfaces for 4 h and then treated with Nell-1 at different concentrations for 1 h. Panel (A) shows the representative Western blots of p-JNK, JNK, p-ERK, ERK, p-P38 and P38 in the pre-osteoblasts after treatment with Nell-1 at concentrations of 0–500 ng/ml for six days. Western blot analysis of p-JNK and p-ERK shows that Nell-1 enhanced the phosphorylation of JNK (B) and ERK (C) but suppressed P38 phosphorylation. GAPDH was used as an internal control, and the expression levels of phosphorylated

JNK, ERK and P38 were normalized to those of total JNK, ERK and P38, respectively. Data represent the mean $\pm$ SD (bars) of three independent experiments; * $P < 0.05$, ** $P < 0.01$ compared with cells cultured in differentiation medium containing 0 ng/ml Nell-1.

Fig. 8. A: Promotive effect of U0126 on Nell-1-induced change in the ALP activity of MC3T3-E1 pre-osteoblasts on Ti surfaces. Cells were cultured in basal medium or differentiation medium containing 100 ng/ml Nell-1 with or without the addition of 5 μ M U0126 on day 8. B: Suppression of Nell-1-enhanced OCN expression in MC3T3-E1 pre-osteoblasts on Ti surfaces. Cells

were cultured in basal medium or differentiation medium containing 100 ng/ml Nell-1 with or without the addition of 5 μ M U0126 on day 18. Data represent the mean $\pm$ SD (bars) of three independent experiments; * $P < 0.05$, ** $P < 0.01$ compared with cells cultured in differentiation medium containing 0 ng/ml Nell-1.

Nell-1 activated ERK and JNK in MC3T3-E1 pre-osteoblasts on the Ti surface

The activation of ERK, p38, and JNK in MC3T3-E1 cells cultured in serum-free α -MEM on the Ti surface were examined after 1 h of Nell-1 stimulation. WB assays showed that while no remarkable change in P38 phosphorylation was detected, the phosphorylation of JNK and ERK in MC3T3-E1 cells was significantly enhanced by Nell-1, particularly by 100 ng/ml Nell-1, compared with the control groups ($P < 0.05$) (Fig. 7).

U0126 inhibited Nell-1-promoted osteoblast differentiation

To further verify the relationship between Nell-1 and ERK activation and elucidate the role of Nell-1 in promoting ALP activity in pre-osteoblasts, U0126 (5 μ M), an ERK inhibitor, was used to pretreat the MC3T3-E1 cells 8 days before treatment with Nell-1 at the concentration of 100 ng/ml. The results showed that ALP activity in the cells that were pretreated with U0126 and then treated with 100 ng/ml Nell-1 increased compared to the untreated control cells (Fig. 8B). However, the MC3T3-E1 cells treated with U0126 alone also showed significantly upregulated ALP activity; consequently, the evidence regarding

Fig. 9. Effect of U0126 on Nell-1-induced changes in p-ERK, Runx2, OPG and Col-I protein expression in MC3T3-E1 pre-osteoblasts on Ti surfaces. Cells were treated with or without 100 ng/ml Nell-1 plus 5 μ M U0126 for six days. A: Representative Western blots. B-E: Western blot analysis of p-ERK (B), Runx2 (C), OPG (D) and Col-I (E) protein expression. The expression level of phosphorylated ERK was normalized to total ERK, and other osteogenic markers were normalized to GAPDH. Data represent the mean $\pm$ SD (bars) of three independent experiments; * P <0.05, ** P <0.01 compared with cells cultured in differentiation medium containing 0 ng/ml Nell-1.

Fig. 10. Real-time PCR analysis of OCN (A), Runx2 (B), OPG (C), Osx (D), ALP (E) and Col-I (F) transcriptions in MC3T3-E1 pre-osteoblasts treated with 100 ng/ml Nell-1 and 5 μ M U0126 for 6 days. Data represent the mean $\pm$ SD (bars) of three independent experiments; * P <0.05, ** P <0.01 compared with cells cultured in differentiation medium containing 0 ng/ml Nell-1.

whether U0126 can inhibit Nell-1-promoted ALP activity remained inconclusive (Fig. 8A).

The effect of U0126 pretreatment on OCN expression in MC3T3-E1 cells stimulated by Nell-1 for 18 days was then evaluated. Nell-1 significantly increased OCN expression, and U0126 effectively inhibited this effect, suggesting that U0126 can suppress Nell-1-promoted OCN expression by inhibiting ERK1/2 (Fig. 8B). For the differentiation markers Runx2, OPG and Col-I, WB assays revealed an upregulation of their expression levels in the MC3T3-E1 cells treated with Nell-1 for 6 days, and the upregulation of all markers except Col-I was suppressed by U0126 pretreatment. Similar to the findings regarding ALP activity, Col-I upregulation induced by Nell-1 was further enhanced by U0126 (Fig. 9).

Finally, the mRNA expression of osteogenic markers in MC3T3-E1 cells treated with Nell-1 for 6 days were tested. Consistent with the WB results, the mRNA expression of all these markers was upregulated after Nell-1 treatment, and except for ALP and Col-I, transcriptional promotion by Nell-1 was inhibited by U0126 (Fig. 10).

Discussion

Titanium implants have been widely used in clinical settings due to their excellent biological properties and reliable and predictable performance [27, 28]. An ideal dental implant surface enhances the abilities of osteoblasts to secrete matrix proteins and deposit bone mineral for osteogenesis and facilitates osteointegration to withstand occlusal loading, which is pivotal for the implant's success [3]. In addition, a number of exogenous growth factors that can promote the early adhesion and differentiation of osteoblasts have gained increasing attention in the field of oral implantation [29]. Primary considerations in the development of dental implants include optimizing cell-tissue interactions, enhancing the bone response to the endosseous implant, and promoting the favorable behavior of bone tissue at the interface zone. For this purpose, pure Ti plates that have been polished, sandblasted and passivated can serve as ideal dental implants. B Groessner-Schreiber *et al.* proved that that porous or rough Ti implant surfaces may act as "natural" substrata that allow bone tissue to grow in direct contact with an implant and thereby strengthen clinical implant fixation [30]. In this study, scanning electron microscopy (SEM) showed that the Ti surface was rough and uneven and that pre-osteoblasts attached to the Ti surface for 24 h were completely spread out.

Nell-1 is osteochondral specific for inducing premature suture closure in human craniosynostosis and chondrogenic or osteoblastic differentiation [7, 8, 31-33]. When adenovirus-mediated (Ad-) Nell-1 and Ad-BMP2 were transferred to bone marrow stromal cells (BMSCs) from adult goats or injected into the intermuscular spaces of nude mice, Nell-1 had a promotive effect on the osteogenic differentiation of BMSCs similar to that of bone morphogenetic protein-2 (BMP2) [34]. In addition, the co-expression of Nell-1 and apoptosis-related protein 3 (APR3) enhanced OCN and BSP expression and matrix mineralization when Nell-1 and APR3 were assembled in the nuclear envelope of the human osteoblast cell lines Saos2 and U2OS [35]. In particular, MC3T3-E1 pre-osteoblasts carrying the Nell-1 gene that overexpresses Nell-1 protein also have a higher expression of osteogenic markers compared with normal cells [16]. Matrix mineralization is enhanced in the MC3T3-E1 cells cultured in the presence of rhNell-1 or rhBMP-2 [36]. However, whether the addition of Nell-1 to culture medium can promote the osteogenic differentiation of pre-osteoblasts on the surface of Ti disks remains to be clarified.

Osteoblast differentiation includes two main processes: osteogenic commitment and osteogenic differentiation [37]. Osteogenic commitment is defined by the upregulation of a key transcription factor, Runx2 [37], while osteogenic differentiation is characterized by the expression of specific osteogenic proteins and transcription factors, such as ALP, OCN, Col-I, osteoprotegerin (OPG) and Osterix (Osx) [38-44]. In this study, ALP activity and OCN expression increased significantly in the Nell-1-treated cells on Ti surfaces compared with

cells cultured in basal medium or Nell-1-free differentiation medium. The highest ALP activity and OCN expression were observed in the 100 ng/ml Nell-1 groups. The gene expressions of ALP and OCN were also improved in these groups. In addition, the expression of Runx2, Col-I, OPG and Osx was augmented by Nell-1 at both the gene and protein levels. Consistently, the expression of these osteogenic proteins and genes was highest in cells treated with Nell-1 at the concentration of 100 ng/ml.

However, this study found that cell proliferation in the Nell-1-treated groups decreased over time compared with the control group, suggesting Nell-1 inhibited the proliferation of MC3T3-E1 cells on Ti surfaces. Nell-1 positively promoted osteogenesis but negatively affected the proliferation of pre-osteoblasts, indicating that Nell-1 has a stronger and more specific effect for eliciting osteoblast differentiation.

Osteogenic differentiation is a complex process involving numerous signaling cascades. Most of the exogenous growth factors involved in osteogenesis, such as BMP-2 and FGF, act on osteoblasts by binding to their cell-surface receptors to activate intracellular signaling pathways [45]. MAPKs, which are a group of serine/threonine-specific protein kinases expressed in all cell types, regulate various cellular processes, including proliferation, differentiation, migration, survival and gene expression [46]. The MAPK pathway is the most active pathway, and it can be activated by various growth factors [21]. The findings that MAPK induces the phosphorylation and stabilization of Runx2 suggest that MAPK-ERK signaling might have a positive effect on osteogenesis [22-24]. Therefore, to further elucidate the mechanisms underlying the effects of Nell-1, the MAPK-ERK signaling pathway was comprehensively investigated in the present study. The results showed that Nell-1 effectively activated the ERK and JNK pathways rather than the P38 pathway in pre-osteoblasts on Ti surfaces. Nakamura Y *et al.* found that Nell-1 mediated cell adhesion by binding to integrin receptors to activate ERK in MC3T3-E1 cells [47], confirming that Nell-1 can activate the ERK pathway in pre-osteoblasts. Interestingly, the selective activation of different MAPK pathways by Nell-1 depends on cell type [26]. Nell-1 effectively activates ERK in Saos-2 cells and all three major MAPKs in RFC cells, but activates ERK and JNK transiently in C3H10T1/2 cells and only JNK in C2C12 cells [14, 26]. In addition, compared with BMP2, Nell-1 does not affect the Smad1/5/8 pathway in any cell type [26]. It is noteworthy that OPN production could be reduced by the siRNA-mediated degradation of Ras through the inhibition of the ERK pathway [26]. Moreover, the JNK inhibitor SP600125 suppresses Nell-1-induced mineralization and intracellular phosphate accumulation in Saos-2 cells, while ERK1/2 and P38 inhibitors have no effect [48]. In this study, the expression of a series of osteogenic proteins and genes in pre-osteoblasts was significantly decreased by co-treatment with the ERK inhibitor U0126 and Nell-1. Nell-1 elevated the expression levels of OCN, Runx2, OPG and Osx, and its promotive effect could be reversed by U0126. The results suggest that the MAPK/ERK signaling pathways may be involved in the Nell-1-induced osteogenic differentiation of pre-osteoblasts on Ti surfaces.

Conversely, the results showed that ALP activity and the protein and gene expressions of Col-I were much higher in the cells treated with ERK inhibitor than in untreated cells. We speculated that U0126 may cause other effects in addition to suppressing the ERK signaling pathway, leading to an increase in ALP and Col-I. Previous research found that U0126 can activate the phosphorylation of P38 in BMSCs and increase ALP activity [49]. Consistently, this study confirmed that the expression of p-P38 in MC3T3-E1 cells could be up-regulated by U0126 treatment for 1 h (Fig. 11). P38 kinase is essential for the regulation of ALP expression during osteogenic differentiation [50]. Moreover, previous research found that p38 may be involved in the control of type I collagen expression [50]. Thus, it is very possible that U0126 may activate P38 or other pathways, leading to the upregulation of ALP and Col-I. Nevertheless, we could not rule out the possibility that the inhibition of a signaling pathway will lead to the inhibition or activation of other pathways due to the complex relationships of these pathways, and this possibility needs to be further explored.

Conclusion

The results suggest that Nell-1 promoted the osteogenic differentiation of pre-osteoblasts on a Ti surface. One of the mechanisms of Nell-1-mediated osteoblastic differentiation may be the activation of MAPK-ERK signaling, but the exact regulatory mechanisms need to be further explored. Nell-1 exerted a positive effect on osteogenic differentiation, which makes it a good candidate growth factor for clinical application to promote the osteointegration of dental implants.

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Disclosure Statement

The authors declare no potential conflicts of interest with respect to the authorship and or publication of this article.

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Fig. 11. Promotive effect of U0126 on P38 phosphorylation in MC3T3-E1 pre-osteoblasts. Cells on Ti surfaces were treated with U0126 (0–10 μM) for 1 h. A: Representative Western blots of p-P38 and P38. B: Western blot analysis of p-P38 expression. The expression level of phosphorylated P38 was normalized to that of total P38. Data represent the mean ± SD (bars) of three independent experiments; *P<0.05, **P<0.01* compared with cells cultured in differentiation medium containing 0 ng/ml U0126.

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