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Original Article

Effect of NAMPT on the proliferation and apoptosis in odontoblast-like MDPC-23 cell

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Abstract

This study aimed to evaluate the physiological role of NAMPT associated with MDPC-23 odontoblast cell proliferation. Cell viability was measured using the (DAPI) staining, caspase activation analysis and immunoblotting were performed. Visfatin promoted MDPC-23 odontoblast cell growth in a dose-dependent manner. Furthermore, the up-regulation of Visfatin promoted odontogenic differentiation and accelerated mineralization through an increase in representative odontoblastic biomarkers in MDPC-23 cells. However, FK-866 cell growth in a dose-dependent manner induced nuclear condensation and fragmentation. FK-866-treated cells showed H&E staining and increased apoptosis compared to control cells. The expression of anti-apoptotic factors components of the mitochondria-dependent intrinsic apoptotic pathway significantly decreased following FK-866 treatment. The expression of pro-apoptotic increased upon FK-866 treatment. In addition, FK-866 activated caspase-3 and PARP to induce cell death. In addition, after treating FK-866 for 72 h, the 3/7 activity of MDPC-23 cells increased in a concentration-dependent manner, and the IHC results also confirmed that Caspase-3 increased in a concentration-dependent. Therefore, the presence or absence of NAMPT expression in dentin cells was closely related to cell proliferation and formation of extracellular substrates.

Keywords: Nicotinamide phosphoribosyl transferase, Visfatin, FK-866, Proliferation.

1. Introduction

Teeth formation is accomplished through precise developmental processes regulated by epithelial-intermediate leaf interactions between the dentin epithelium and the outer mesenchymal cells [1]. The dentin epithelium forms a dental organ that secretes enamel proteins, while the outer mesenchymal cells form a dental papilla, which differentiates into dentin cells and pulp cells to produce crown dentin and pulp [2]. After the crown formation is completed, the inner and outer epithelium proliferates under the crown to form a Hertwig epithelial muscle coating, and the shape of this epithelial muscle is determined to induce differentiation of dental hair cells to form a root image [3]. The dentin is the most important component of the tooth and makes up most of the crown and root. Ivory cell differentiation is a highly organized and complex process involving various cytoplasmic molecules, signaling transporters, growth factors, ions, and receptors [1, 4, 5].

In the development of multicellular organisms, cellular responses to cellular homeostasis and stimuli, cellular cycle arrest, and apoptosis play an essential role. Many molecules control tooth development through the cell cycle [6]. Cell cycle arrest can protect DPCs from catastrophic thermal shock following recovery procedures [7] and irradiation-induced damage [8]. Recent interest in dentin regeneration has prompted numerous studies, but due to the specificity of dentin and various experimental limitations, much of the data is minimal [9]. To solve these problems, the development of dentin regeneration inducer using molecular biology, biotechnology, and tissue engineering is considered a major priority in future dentin regeneration research [9]. Therefore, a clear understanding of the differentiation mechanism of dentin cells is essential.

Nicotinamide phosphoribosyltransferase (NAMPT), also known as visfatin or pre-B cell colony enhancing factor, is a rate-limiting enzyme of the mammalian lifting pathway for NAD+ biosynthesis from Nicotinamide [10].

Intracellular levels of NAD+ and nicotinamide have been linked to recent bone formation, suggesting a pos-

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sible mechanism for developing senile osteoporosis [11]. Recently, studies on the effectiveness of NAMPT on osteoblasts have also been reported [12]. However, few data have been reported on the effectiveness of NAMPT on dental cell survival.

The MDPC-23 cell line, widely used in dentin cell research, comes from the healing head of the CD-1 mouse molars [13]. Therefore, we investigated the molecular mechanism by which NAMPT regulates cell survival in MDPC-23 dentin cells derived from mouse dental papilla cells.

2. Materials and methods

2.1. Cell culture

MDPC-23 odontoblast-like cells were supplied by Dr. J. E. Nör (University of Michigan, Ann Arbor, MI, USA) and cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Welgene, Daegu, Korea), supplemented with 10% fetal bovine serum (FBS; Welgene) and penicillin (100 U mL^{-1})-streptomycin (100 μg mL^{-1}) in a 5% CO_2-humidified atmosphere. Furthermore, 50 μg mL^{-1} ascorbic acid and 10 mM β-glycerophosphate were added for cell differentiation.

2.2. Cell viability test

MDPC-23 cells were seeded at a concentration of 2 × 10^4 cells per well in 48-well plates. After 24 h of growth at 37 °C, the cells were treated with specific NAMPT inhibitors, FK866 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or Visfatin (Sigma-Aldrich, St. Louis, MO, USA) at various concentrations for 48 h and 72 h, respectively, after which cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) colorimetric assay. At least three separate experiments were performed for each concentration combination.

2.3. Live/Dead cell assay

The MDPC-23 cells (2 X 10^4 cells/well) were cultured in an 8-well chamber slide and allowed to attach to the bottom of the chamber slide overnight. Thereafter, the cells were treated with FK-866 and Visfatin for 72 h at 37°C and stained using the Live/Dead cell viability assay kit. The cells were imaged using a fluorescence microscope (Eclipse TE2000, Nikon Instruments, Melville, NY, USA). The Live/Dead cell viability assay kit uses green calcein AM to stain the live cells (green fluorescence) and ethidium homodimer-1 to stain the dead cells (red fluorescence).

2.4. DAPI staining

MDPC-23 cells (2 X 10^4 cells/well) were cultured in an 8-well chamber slide and allowed to attach to the bottom of the chamber slide overnight. After 72 h growth, the cells were treated with 0, 5 or 10 μM FK-866 for 72 h. DAPI staining was done according to the previously described method [14]. The stained cells examined by fluorescent inverted microscopy (Eclipse TE2000, Nikon Instruments, Melville, NY, USA).

2.5. H&E Staining

Morphological alteration as cell shrinkage is a representative characteristic of apoptosis. Hence to observe the morphological alteration, MDPC-23 cells (2 × 10^4 cells/well) were cultured in 8-well chamber slides and allowed to adhere for 24 h. The cultured MDPC-23 cells were then treated with 0, 5, or 10 μM FK-866 for 72 h at 37 °C. After cultivation, the cells were rinsed with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 1 h at 4 °C. The morphology of MDPC-23 cells incubated with FK-866 was observed by H&E staining. Images of these cells were captured by a microscope (Leica DM750; Leica Microsystems, Heerbrugg, Switzerland).

2.6. Caspase-3/-7 activity assay

The activity of the apoptosis executioner caspase-3/-7 was investigated using the cell-permeable fluorogenic substrate PhiPhiLux-G1D2 according to the manufacturer's instructions and was, and cells were imaged using a fluorescence microscope (Eclipse TE2000, Nikon Instruments, Melville, NY, USA).

2.7. Alizarin Red S staining

The MDPC-23 cells were fixed with 70% ethanol for 30 min and stained with 1% alizarin Red S (Sigma-Aldrich) in 0.1% NH_4OH at pH 4.2–4.4. Mineralization assays were performed by staining MDPC-23 cells with alizarin Red S solution. The density of stained nodules was measured using colorimetric spectrophotometry to quantify the mineralization intensity. The stained cells were collected by centrifugation at 10,500 g for 15 min at 4 °C. The cells were solubilized with 5% sodium dodecyl sulfate in 0.5 N HCl for 30 min at 23 °C. An aliquot of 200 μL of the solubilized solution was transferred to a 96-well plate, and the absorbance was measured at 405 nm using a spectrophotometer.

2.8. Immunoblotting

Protein extraction was performed to determine the level of odontoblastic biomarkers in the MDPC-23 cells. The total protein concentration extracted from primary rat chondrocytes was determined using a bicinchoninic acid protein assay kit (Thermo Scientific) according to the manufacturer's instructions. Equal amounts of proteins were loaded onto an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system and subsequently transferred onto nitrocellulose membranes. Immunoblotting was performed using primary antibodies against Fas (1:1000), Cleaved caspase-3 (1:1000), PARP (1:1000), Bcl-2 (1:1000), Bcl-xl (1:1000), Bax (1:1000), p-ERK1/2(1:1000), p-AKT (1:1000), p-NF-κB (1:1000), SIRT-1 (1:1000), p53 (1:1000), and β-actin (1:2500), all purchased from Santa Cruz Biotechnology. Immunoreactive bands were visualized using an enhanced chemiluminescence system (Thermo Scientific) according to the manufacturer's instructions and then imaged using a Microchemi device (DNR Bioimaging Systems, Jerusalem, Israel).

2.9. Immunocytochemistry

MDPC-23 cells were cultured in chamber slides at a seeding density of 2×10^4 cells per well for 24 h, then treated with FK-866 for 72. Subsequently, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed with PBS. Immunocytochemistry was performed using Vetastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Fixed cells were incubated with Caspase-3 antibody for 1 h and peroxidase-conjugated goat anti-mouse antibody for 1 h. Cells were observed and

imaged using Leica DM 750 microscope (Leica Microsystems, Heerbrugg, Switzerland).

2.10. Statistical analysis

All experiments were performed at least in triplicate. The results are presented as mean ± SEM. Statistical significance was analyzed using Student's *t*-test for two groups and a one-way analysis of variance for multi-group comparisons. All statistical analyses were performed using SPSS (version 12.0; SPSS Inc., Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Visfatin promotes cell survival

To verify the cytotoxic effects of Visfatin on MDPC-23, cells were treated with the following various concentration of Visfatin for 48h and 72h. After incubation, the cell viability was assessed using an MTT assay. As shown in Fig. 1 (A), the viability of MDPC-23 cells treated with 10, 25, and 50 ng/ml Visfatin were 110 ± 3.37, 119 ± 3.47, and 100 ± 3.48, respectively, compared to non-treated control (100 ± 1.84). These data indicate that Visfatin promotes MDPC-23 cell growth in small doses (Fig.1A). Also, when treated for 72 h, the viability of MDPC-23 cells treated with 10, 25, and 50 ng/ml Visfatin were 104 ± 1.89, 114 ± 3.12, and 100 ± 3.47, respectively, compared to non-treated control (100 ± 1.84).

To confirm the Visfatin-induced cell viability in MDPC-23 cells, microscopy was used to visualize the live and dead cells stained with calcein-AM (green fluorescence) and ethidium homodimer 1 (red fluorescence), respectively. As shown in Fig. 1(B), the MDPC-23 cells incubated with the following concentration Visfatin for 72 h were stained green due to the cleavage of the membrane permeable calcein-AM by the cytosolic esterase in living cells. No dead cells were found stained by ethidium bromide homodimer 1. These data indicated that Visfatin promotes cell survival.

3.2. Visfatin induces MDPC-23 cell proliferation

Visfatin was treated in MDPC-23 cells for 72 h, and immunoblotting was performed to determine how Visfatin grows MDPC-23 cells. The expression levels of Bcl-2 and Bcl-xL, pro-survival factors associated with the intrinsic mitochondria-dependent apoptosis pathway, were upregulated by Visfatin in MDPC-23 cells. Along with this, while those of mitochondria-dependent pro-apoptosis factors such as Bax and Bad were downregulated by Visfatin in MDPC-23 cells (Fig. 2(A)). Furthermore, the expression levels of p-ERK1/2, p-AKT, p-NF-κB and SIRT1 cell survival factors were upregulated by Visfatin in MDPC-23 cells (Fig. 2(B)). On the other hand, the expression level of p53 that stop the cell cycle has decreased. These data indicated that Visfatin induces MDPC-23 cells.

3.3. Visfatin upregulates MDPC-23 cell Diffentiation

To confirm the role of Visfatin during MDPC-23 odontoblast differentiation, ascorbic acid (50 µL/ mL) and β-glycerophosphate (10 mM) were added to MDPC-23 cells to induce differentiation. In the differentiation induction medium, the degree of mineralized nodules in the group treated with Visfatin tended to increase compared to the control group after 72 h of culture, and this difference was shown in Fig 3 (A) and (B). Western blot was

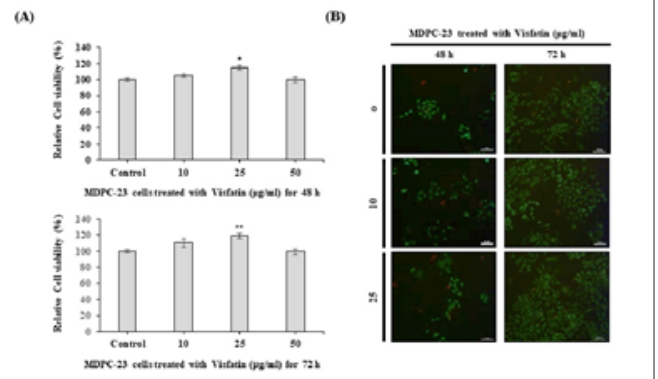

Fig. 1. Effect of Visfatin on cell viability in MDPC-23 cells. The effect of Visfatin on the viability of MDPC-23 cells (A) was accessed by the MTT assay. The percentage of cell viability was calculated as a ratio of A570nms of Visfatin treated with cells and untreated control cells. Each data point represents the mean ± SEM of four experiments. *p<0.05 vs. control and **p<0.01 vs. control (the control cells measured in the absence of Arctigenin). Induction of cell death by Visfatin. (B) MDPC-23 cell death by Visfatin. The cells were treated with 0, 10 or 25 µg/ml Visfatin for 72 h. MDPC-23 cells emitting green fluorescence are live cells stained by green calcein AM, whereas cells emitting red fluorescence are dead cells stained by ethidium homodimer-1.

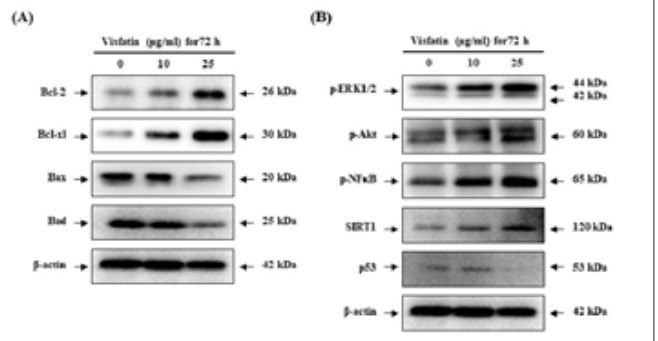

Fig. 2. Visfatin induces MDPC-23 cell proliferation. Visfatin was treated in MDPC-23 cells for 72 h, and immunoblotting was performed to determine how Visfatin grows MDPC-23 cells. (A) The expression levels of Bcl-2 and Bcl-xL, pro-survival factors associated with the intrinsic mitochondria-dependent apoptosis pathway, were upregulated by Visfatin in MDPC-23 cells. (B) The expression levels of p-ERK1/2, p-AKT, p-NF-κB and SIRT1, cell survival factors were upregulated by Visfatin in MDPC-23 cells.

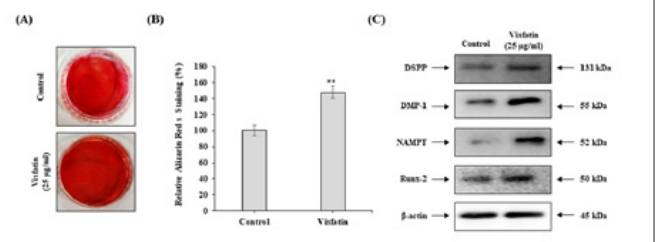

Fig. 3. The effects of Visfatin on mineralization and differentiation in MDPC-23 cells (A) Alizarin Red S stain showing mineralized nodule formation in MDPC-23 cells, cultured in differentiation media and treated with Visfatin for up to 72 h. The mineralization was quantified using colorimetric spectrophotometry (B). Each data point represents the mean ± SEM of three experiments. ** $P < 0.01$. (C) Treatment with Visfatin at 25 µg/mL according to the defined conditions. The expression level of odontoblast biomarkers was assayed by western blotting according to standard methods.

performed to evaluate changes in the expression of dentin-differentiated biomarkers. DSPP, DMP-1, and NAMPT expression increased in the Visfatin treatment group, respectively. (Fig. 2(c)). These results indicate that Visfatin promoted MDPC-23 cell differentiation.

3.4. FK-866 induces MDPC-23 cell death

To determine the cytotoxicity of Visfatin to MDPC-23 cells, the cells were treated along different concentrations of FK-866 for 48 h and 72 h. After incubation, the cell viability was assessed using an MTT assay. As shown in Fig. 4 (A), when treated for 48h, the viability of MDPC-23 cells treated with 1, 2.5, 5 and 10 nM FK-866 were 98 ± 1.48, 93 ± 2.49, 87 ± 2.18, and 76 ± 1.18, respectively, compared to non-treated control (100 ± 0.84). Also, when treated for 72 h, the viability of MDPC-23 cells treated with 1, 2.5, 5 and 10 nM FK-866 were 94 ± 4.11, 89 ± 1.12, 73 ± 1.29, and 47 ± 1.17, respectively, compared to non-treated control (100 ± 1.36). These data indicate that FK-866 induces MDPC-23 cell death in small doses (Fig.4A).

To confirm the FK-866-induced cell cytotoxicity in MDPC-23 cells, microscopy was used to visualize the live and dead cells stained with calcein-AM (green fluorescence) and ethidium homodimer 1 (red fluorescence), respectively. As shown in Figure 4(B), when MDPC-23 cells were treated with FK-866 72h, the number of cells decreased in a concentration-dependent manner, and the number of dead cells stained red increased. To investigate the mechanism underlying FK-866-induced MDPC-23 apoptosis, DAPI staining was performed to determine chromatin condensation in MDPC-23 cells following treatment with 5 and 10 nM FK-866 for 72 h. The number of MDPC-23 cells with condensed nuclei was significantly increased following FK-866 treatment (Fig. 4(B)). The results of cell staining through H&E also confirmed that the number of MDPC-23 cells decreased, and the condition deteriorated after FK-866 treatment. (Fig. 4(B)). These results suggest that FK-866 induces cell death through apoptosis signaling pathways in MDPC-23 cells.

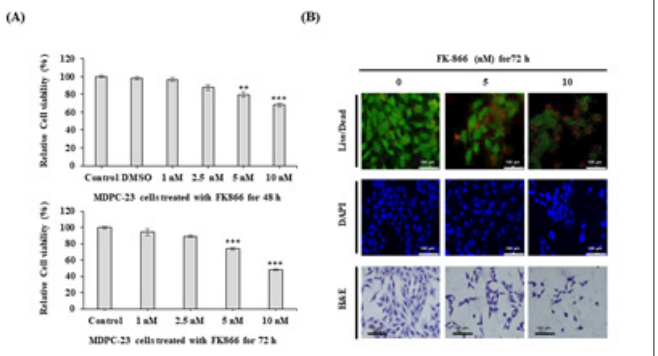

Fig. 4. Effect of FK-866 on cell viability in MDPC-23 cells. The effect of Visfatin on the viability of MDPC-23 cells (A) was accessed by the MTT assay. The percentage of cell viability was calculated as a ratio of A570nms of FK-866 in treated and untreated control cells. Each data point represents the mean ± SEM of four experiments. **p<0.01 vs. control and ***p<0.001 vs. control (the control cells measured in the absence of FK-866). Induction of cell death by FK-866. (B) DAPI Stain and (C) H&E Stain.

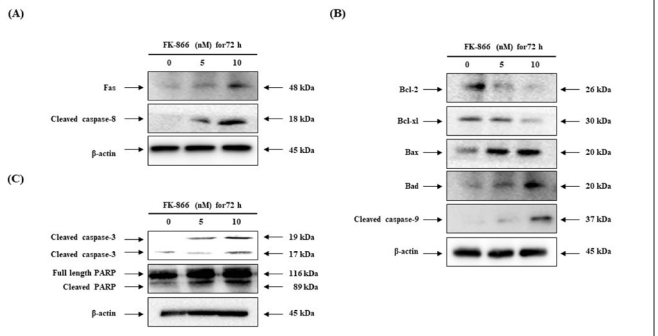

Fig. 5. FK-866-induced MDPC-23 cell death is mediated by both intrinsic and extrinsic pathways**.** (A) Extrinsic death receptor-mediated apoptotic signaling pathway induced by FK-866. FK-866 upregulated the expression level of the death receptor ligand Fas and subsequently activated the extrinsic death receptor-mediated apoptotic signaling pathway through the cleavage of caspase-8 in MDPC-23 cells. (B) Intrinsic mitochondria-dependent apoptotic signaling pathway induced by FK-866. FK-866 downregulated anti-apoptotic factors Bcl-2 and Bcl-xL associated with the intrinsic mitochondria-dependent apoptotic pathway and upregulated the mitochondria-dependent pro-apoptotic factors Bax and Bad in MDPC-23 cells. (C) Extrinsic death receptor-mediated and intrinsic mitochondria-dependent apoptosis signaling pathways via the activation of caspase-3 and PARP induced by Arctigenin. Cleaved caspase-8 and cleaved caspase-9 induced the activation of caspase-3 and PARP in MDPC-23 cells treated with FK-866.

3.5. FK-866-induced MDPC-23 cell death is mediated by extrinsic death receptor-dependent and intrinsic mitochondria-dependent apoptotic pathways

To determine the cellular apoptotic pathways associated with FK-866-induced MDPC-23 cell death, immunoblotting was performed. Fas, which is an apoptotic ligand that triggers the death receptor-dependent extrinsic apoptotic pathway in cells, was induced by FK-866 in MDPC-23 cells (Fig. 5A). As shown in Fig. 5(A), the expression level of cleaved caspase-8, the downstream target of pro-apoptotic factor Fas, increased following FK-866. These data suggest the involvement of the extrinsic death receptor-mediated apoptosis pathway in FK-866-induced MDPC-23 cell apoptosis.

The expression levels of Bcl-2 and Bcl-xL, anti-apoptotic factors associated with the intrinsic mitochondria-dependent apoptosis pathway, were downregulated by FK-866 in MDPC-23 cells, while those of mitochondria-dependent pro-apoptotic factors such as Bax and Bad were upregulated by FK-866 in MDPC-23 cells (Fig. 5(B)). And FK-866 treatment increased the expression level of cleaved caspase-9 in MDPC-23 cells (Fig. 5(B)). These data show that FK-866-induced MDPC-23 cell death involves the intrinsic mitochondria-dependent apoptosis pathway.

Both cleaved caspase-8 and caspase-9 acted in the extrinsic death receptor-mediated and intrinsic mitochondria-dependent apoptosis pathways in MDPC-23 cells after FK-866 treatment, following to expression of cleaved caspase-3 (Fig. 5(C)). These results suggest that FK-866 induces MDPC-23 cell apoptosis, which is mediated by death receptor-mediated extrinsic and mitochondria-dependent intrinsic apoptosis through activation of the caspase cascade in MDPC-23 cells.

3.6. FK-866 induces apoptosis through Caspase-3 in MDPC-23 cells

To verify whether FK-866-induced MDPC-23 cell

death is mediated by apoptosis, caspase-3/7 activity was measured using the cell-permeable PhiPhiLux-G1D2. As shown in Fig. 6(A), the activities of caspase-3/7 increased in MDPC-23 cells treated with FK-866. In addition, in ICC staining, when FK-866 was treated on MDPC-23 cells, Caspase-3 was increased in a concentration-dependent manner (Fig 6 (B)). These results suggest that FK-866 induces cell death through Caspase-3 in MDPC-23 cells.

4. Discussion

Stem cells replace damaged pulp tissue, so it is important to promote survival and induce them to adhere to dentin [15]. MDPC-23 cells originated from dental papilla cells in the mandible's first molar of the CD-1 mouse on fetal 18-19 days [13]. These cells exhibit unique characteristics, including the expression of markers for dental subcellular differentiation (i.e., dentin phosphoproteins and dentin cialoproteins) [16]. As the dentin matrix deposits, dentin blast cells form distal cytoplasmic protrusions buried in dentin tubules [17, 18]. However, little is known about the differentiation mechanisms of dentin cells, factors that regulate dentin formation, and related biomolecular mechanisms. The role of NAMPT in bone formation has recently attracted more attention [17]. NAMPT showed insulin-like activity as a growth factor in osteoblasts. NAMPT showed insulin-like activity as a growth factor in osteoblasts. Decrease in NAMPT has also been suggested to be linked to age-related fat production [11]. However, there are no published studies on the role of NAMPT in dentin cell survival and the molecular mechanisms by which NAMPT promotes tooth production.

Therefore, in this study, Visfatin (NAMPT) and FK866, a NAMPT inhibitor, were applied to MDPC-23 cells, a dental precursor cell line derived from mouse dental papilla cells, to reveal the effect of NAMPT on dental cell survival. The results indicated that Visfatin grows MDPC-23 cells (Fig. 1). When Visfatin was treated on MDPC-23 cells, as shown in Fig. 1, the MTT results tended to survive the cells, and the live/dead assay results also tended to increase the cell viability.

Members of the Bcl-2 family are proteins that play an essential role in regulating apoptosis, an important process in the development and normal physiology of multicellular organisms [19]. The essential mechanism of this family of proteins is given by the role of proteins in survival, which inhibits apoptosis by directly binding to the relative protein, the effector protein of apoptosis [20]. Due to the role of BCL-xL in the regulation of cell survival and proliferation, it has been of great interest in its study [21]. Visfatin was treated in MDPC-23 cells for 72 h, and immunoblotting was performed to determine how Visfatin grows in MDPC-23 cells. The expression levels of Bcl-2 and Bcl-xL, pro-survival factors associated with the intrinsic mitochondria-dependent apoptosis pathway, were upregulated by Visfatin in MDPC-23 cells (Fig.2 (A)). In response to death-inducing stimuli, pro-apoptotic members of the family (BIM, PUMA, BID, BMF, NOXA, BIK, BAD, and HRK; collectively termed BH3 [BCL-2 homology domain 3]-only proteins) inhibit the anti-apoptotic BCL-2 family members [22]. In addition, some BH3-only proteins (for example, BIM and PUMA) have been reported to activate BAX and BAK directly 4, 5. Once activated, BAX and BAK form pores within the mitochondrial outer membrane, which leads to mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome C and other apoptogenic factors from the mitochondria into the cytoplasm [23]. Additionally, those mitochondria-dependent pro-apoptosis factors such as Bax and Bad were downregulated by Visfatin in MDPC-23 cells (Fig. 2(A)). The PI3 K/AKT/mTOR signalling pathway is important in regulating signal transduction and biological processes such as cell proliferation, apoptosis, metabolism and angiogenesis [24]. In our results, the expression levels of p-ERK1/2, p-AKT, p-NF-κB and SIRT1 cell survival factors were upregulated by Visfatin in MDPC-23 cells (Fig. 2(B)). On the other hand, the expression level of p53 that stop the cell cycle has decreased. These data indicated that Visfatin induces MDPC-23 cells.

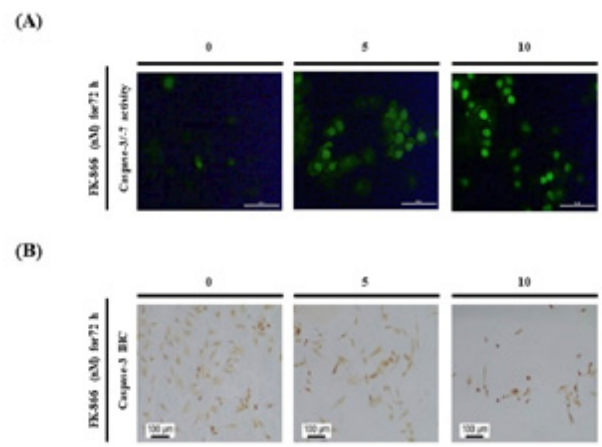

Fig. 6. FK-866 induces apoptosis through Caspase-3 in MDPC-23 cells**.** Apoptotic Phenomenon in MDPC-23 cells stimulated with FK-866. (A) FK-866 induces caspase-3/-7 activation in MDPC-23 cells. The caspase-3/-7 intracellular activity assay was performed using PhiPhiLux-caspase-3/-7 substrate. Images were observed by fluorescence microscopy (Eclipse TE 2000; Nikon Instruments, Melville, NY, USA). Immunocytochemistry (B) was performed to verify the expression of Caspase-3 in the MDPC-23 cells. Cells were observed and imaged using Leica DM 760 microscope (Leica Microsystems, Heerbrugg, Switzerland).

Visfatin accelerated the mineralization of MDPC-23 dentin cells with associated increases, a well-known indicator of dentin differentiation (Figure 3 (A) and (B)). In addition, to determine whether Visfatin induced dentin differentiation in MDPC-23 cells, we measured expression levels of DMP-1 and DSPP, a well-known representative marker of dentin differentiation [25, 26]. DSPP, DMP-1, and NAMPT expression increased in the Visfatin treatment group, respectively. (Fig. 2(c)). These results indicate that Visfatin promoted MDPC-23 cell differentiation.

However, the cell viability of MDPC-23 cells gradually decreased by FK-866 in a dose-dependent manner. Treatment with 5 and 10 nM FK-866 decreased MDPC-23 cell viability by 73% and 47%, respectively, compared to the non-treated control (Fig.4(A)). We performed a cell survival assay to confirm that Visfatin had MDCP-23 cell-specific cytotoxic effects (Fig. 4(B)). In contrast to Visfatin, 5 and 10 nM FK-866 decreased the total number of FaDu pharyngeal carcinoma cells and increased the number of cells stained red by ethidium homodimer-1, a marker of membrane-impermeable cell death. Along with this, to elucidate the mechanism by which FK-866 induces MDPC-23 cell death, DAPI staining was performed to

investigate alterations in chromatin condensation (Fig. 4 (B)). The number of MDPC-23 cells with condensed chromatin gradually increased in response to FK-866 in a dose-dependent manner. Chromatin condensation is a hallmark of apoptosis [27].

Apoptosis is mainly regulated by two general pathways: intrinsic (mitochondrial) or extrinsic (death receptor) pathways [28]. Fas, an important regulator of apoptosis, binds to receptor FasR across the surface of the target cell and then initiates a death receptor-mediated exogenous apoptosis pathway through activation of caspase-8, -3 and PARP [29]. In the registered pathway, mitochondrial outer membrane penetration is initiated by an internally originated signal [30]. Anti-apoptotic mitochondrial proteins, such as Bcl-2 and Bcl-xL and pro-apoptotic proteins, such as Bax and Bad, are important regulators of cytochrome c release in mitochondria [31]. In our work, FK-866 treatment increased the level of Bax and Bad protein expressions but decreased the level of Bcl-2 and Bcl-xl protein expressions in MDPC-23 cells (Fig. 5B). Level changes in these anti-apoptotic factors associated with mitochondrial-dependent eigenpaths subsequently induced active cascades of caspase-9, caspase-3 and PARP in FaDu cells treated with FK-866 (Fig. 5(C)). These results indicate that FK-866 induces apoptosis in MDPC-23 cells containing death receptors and mitochondrial-signaling pathways.

These results, therefore, suggested that FK-866 induced apoptosis of MDPC-23 cells. Activation of caspase is a key function of the apoptotic signaling pathway [32]. Therefore, to determine whether FK-866-induced apoptosis is mediated by caspase activation, we investigated caspase-3/7 activation analysis in FK-866 treated MDPC-23 cells using PhiPhiLux, a fluorinated caspase substrate (Fig. 6(A)). Caspase-3 (CPP-32, Apopain, Yama, SCA-1) is a critical executioner of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) [33]. In our data, FK-866 was treated on MDPC-23 cells, and Caspase-3 was increased in a concentration-dependent manner (Fig 6 (B)). These results suggest that FK-866 induces cell death through Caspase-3 in MDPC-23 cells.

5. Conclusion

In conclusion, Visfatin activation induced proliferation and differentiation of MDPC-23 cells. In addition, when treated with the NAMPT inhibitor FK-866, it activated the death of MDPC-23 cells. Taken together, our data show that NAMPT is an essential regulatory molecule for cell survival and differentiation, and cells die if the expression of NAMPT is inhibited. Therefore, this study suggests that NAMPT may be important in the survival and differentiation procedures of odontoblast.

Conflict of interests

The authors have no conflicts with any step of the article preparation.

Consent for publications

The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed consent

The authors declare that no patients were used in this study.

Availability of data and material

The data in this article can be obtained from the corresponding author under reasonable circumstances**.**

Author's contributions

K.R.K., J.Y.S., H.I.L., J.H.P., and D.K.K. contributed to the cell based experimental design and collected the data. J.S.K., H.S.C., H.J.K., S.K.Y., and C.S.K. contributed to the data analysis and interpretation. K.R.K. and D.K.K. did the writing and revisions of manuscript.

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