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Nampt expression increases during osteogenic differentiation of multi- and omnipotent progenitors

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ABSTRACT

Despite emerging data showing that metabolic changes occur with stem cell differentiation, the cross-talk between factors governing energy metabolism and epigenetic modification is not understood. Nicotinamide adenine dinucleotide (NAD) participates in both energy metabolism and protein modification processes. Changes of the intracellular NAD concentration have been shown to correlate with differentiation of adult and embryonic stem cells. In the present study, we investigated the expression pattern of Nampt, the rate-limiting enzyme in NAD salvaging pathway, during osteogenic differentiation of the multipotent mouse fibroblast C3H10T1/2 and the omnipotent preosteoblast MC3T3-E1 cells. We found that Nampt was increasingly expressed during differentiation in both cell models. The increase of Nampt was associated with higher NAD concentration and Sirt1 activity. Knockdown of Nampt or addition of its specific inhibitor FK866 leads to lower intracellular NAD concentration and decline in osteogenesis. These findings indicate that osteogenic differentiation correlates with intracellular NAD metabolism in which Nampt plays a regulatory role.

1. Introduction

Stem cells (MSCs) reside in embryos as well as in a variety of adult tissues and are defined by two fundamental properties: multi-lineage differentiation and self-renewal [1]. During development, the potency of stem cells is reduced over time from totipotent (morula), to pluripotent (embryonic stem cells), to multipotent (fetal and adult stem cells), and to omnipotent (precursor cells), due to progressive gene silencing [2]. This progress is controlled by epigenetic modifications, which involves chromatin remodelling and modifications, resulting in selective expression of the lineage specific transcription factors [3].

Recently, the metabolic changes occurring during differentiation have attracted attention among stem cell researchers. The initial work of Cho et al. [4] and Facucho-Oliveira et al. [5] showed that the energy metabolism in embryonic stem cells switches from anaerobic glycolysis to oxidative phosphorylization as the mitochondria become mature and functionally active. Later studies of Yanes et al. [6] confirmed that embryonic stem cells are characterized by abundant metabolites with highly unsaturated structures whose levels decrease upon differentiation. A recently published paper by Chen et al. [7] demonstrated that similar metabolic changes also occurred in adult human mesenchymal stem cells: upon osteogenic induction the copy number of mitochrondrial DNA, protein subunits of the respiratory enzymes, oxygen consumption rate, and intracellular ATP content were increased. This indicates that there is an upregulation of aerobic mitochondrial metabolism. Despite the emerging evidence correlating metabolic change with cell differentiation, the cross-talk between factors governing energy metabolism and the epigenetic modifications/gene regulation is not understood.

Nicotinamide adenine dinucleotide (NAD) is found in all living organisms. It consists of two nucleotides joined through their phosphate groups. One nucleotide contains an adenine base and the other nicotinamide. In energy metabolism, NAD functions as a redox co-enzyme. In each reaction, the oxidizing agent, NAD^+, accepts electrons from other molecule(s) and forms NADH, which then is used as a reducing agent donating electrons in the next reaction. This way, NAD transports electrons from one reaction to another, facilitating energy release from nutrients [8]. In eukaryotic cells, NAD also functions as substrate in protein modification processes, such as protein deacetylations mediated by sirtuins [9]. In such reactions, NAD accepts the acetyl group from the target protein and bonds it to its ADP-ribose moiety [10]; the product is then cleaved into nicotinamide and O-acetyl-ADP-ribose [11]. To replenish the NAD pool which would otherwise be depleted by the NAD-consuming processes, an NAD salvaging pathway has

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evolved in mammalian cells. The rate limiting step of this pathway is catalyzed by nicotinamide phosphoribosyltransferase (Nampt), by which nicotinamide and phosphoribosyl pyrophosphate is converted into nicotinamide mononucleotide (NMN). NAD is then formed from NMN and ATP by the second enzyme, nicotinamide mononucleotide adenylyltransferase (Nmnat) [12].

Accumulating data indicate that the NAD-dependent protein modifications influence gene expression. One typical example is Sirt1-mediated deacetylations, which preferentially target the acetylated lysines in the N termini of histones [13], leading to the formation of facultative heterochromatin with resultant gene silencing [14,15]. Although controversy exists, changes of the intracellular NAD concentrations have been demonstrated in both adult and embryonic stem cells during differentiation [16–20]. We previously studied the roles of Sirt1 and Nampt in lineage fate determination of mesenchymal stem cells [21,22]. In the present study, we investigated the expression of Nampt during osteogenic differentiation of multipotent mouse fibroblast C3H10T1/2 cells and the omnipotent preosteoblast MC3T3-E1 cells. We found that Nampt was increasingly expressed during osteogenic differentiation in both cell models. The increase in Nampt was associated with higher NAD concentration and higher Sirt1 activity. These findings indicate that osteogenic differentiation correlates with cellular NAD metabolism in which Nampt plays a regulatory role.

2. Materials and methods

2.1. Cell culture

The murine fibroblast C3H10T1/2 cells and preosteoblastic MC3T3-E1 cells were obtained from the American Type Culture Collection (ATCC). The cells were cultured in Modified Eagle's medium alpha (α-MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-Glutamine and 50 μg/ml Gentamicin at 37 °C in a humidified 5% CO_2 atmosphere. For osteoblast differentiation, cells were cultured in α-MEM medium supplemented with 10% FBS, 10 mM β-glycerophosphate, 50 μg/ml α-ascorbic acid and 0.1 μM dexamethasone (osteogenic medium) in 2 weeks with medium changes twice a week.

2.2. Cell growth assay

Cell growth was measured using the WST-1 assay (Roche Molecular Biochemicals) in 96-well plates according to the recommendation of the manufacturer. Briefly, 2×10^3 cells were seeded per well on the 96-well plates and incubated for 24 h. Then the cells were treated with different concentrations of FK866 (0–100 nM, Sigma) or vehicle. Cell growth was analyzed on day 3 of culture. The absorbance was measured at 450/650 nm. At least 4 wells were used for each concentration of tested reagent and values are expressed as mean ± S.E.M.

2.3. Alkaline phosphatase (ALP) staining and quantification of ALP activity

TRACP and ALP double-stain kit (Karara Bio. Inc., Otsu, Japan) was used for staining of alkaline phosphatase in the cell cultures. The ALP activity was quantified by Phosphatase Substrate Kit (Pierce, IL, USA) containing PNPP (*p*-nitrophenyl phosphate disodium salt). The detailed procedures were described previously [22]. ALP activity was normalized with protein concentration of the cell lysates.

2.4. Staining for mineralization

MC3T3-E1 cells were cultured in 24-well plate for 3 weeks, and alizarin red staining was used. Briefly, the cells were washed with PBS and fixed in ice-cold 70% ethanol for 60 min. Then the cells were incubated with 2% alizarin red with pH 4.2 for 10 min at RT and subsequently washed with distilled water.

2.5. Gene transfection of murine preosteoblastic cell line MC3T3-E1 cells

Cells were plated at 5×10^4/well in 24-well plate and incubated at 37 °C for 18 h. Then cells were exposed to MISSION Nampt shRNA or Non-target scramble shRNA Lentiviral transduction particles (Sigma), in the presence of 8 μg/ml hexadimethrine bromide (Sigma) for 20 h. Following transduction, cells were selected with 1.8 μg/ml puromycin.

2.6. Western blot analysis

Cells were lysed in M-PER mammalian protein extraction reagent supplemented with Halt™ protease inhibitor cocktail (Thermoscientific). Protein concentrations were measured with Bio-Rad protein assay (Bio-Rad) using bovine serum albumin as a standard. Thirty micrograms of protein was mixed with Laemmli buffer and boiled at 97 °C for 10 min before loading onto SDS–PAGE Gels. Proteins were transferred to PVDF membranes by electroblotting after the membranes were blocked with 5% milk. The membranes were probed with rabbit anti-Nampt (BETHYL), rabbit anti-Sirt1 (Upstate) and rabbit anti-GAPDH (abcam) antibodies diluted in blocking solution followed by horseradish peroxidase-conjugated with anti-rabbit IgG secondary antibody. The blots were visualized using Immun-Star™ HRP Chemiluminescence Kit (Bio-Rad) and exposed to X-ray films.

2.7. RNA preparation and quantitative real-time PCR analysis

Total RNA was extracted with RNeasy Mini Kit (Qiagen, Germany) according to manufacturer's instruction. cDNA synthesis was performed with 500 ng RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitativereal-time PCR was performed with Maxima SYBR/ROX qPCR Master Mix (Fermentas) and monitored with a LightCycler 480 (Roche). The gene primer was used for mouse Nampt (Mm_Nampt_1_SG QuantiTect Primer, Qiagen). The specific primer sequences used for mouse Sirt1, OCN, Runx2, OPN, OPG and β-actin were listed as follows: Sirt1: forward 5′-CAC ATG CCA GAG TCC AAG TT-3′, reverse 5′-AAA TCC AGA TCC TCC AGC AC -3′; OCN: forward 5′-GCA ATA AGG TAG TGA ACA GAC TCC-3′, reverse 5′-GTT TGT AGG CGG TCT TCA AGC-3′; Runx2: forward 5′-AAG GAG GGA CTA TGG CGT CAA A-3′, reverse 5′-GGC TCA CGT CGC TCA TCT TG-3′; OPN: forward 5′-GAT TTG CTT TTG CCT GTT TGG-3′, reverse 5′-TGA GCT GCC AGA ATC AGT CAC T-3′; OPG: forward 5′-CAT CCA AGA CAT TGA CCT CT-3′, reverse 5′-TCT TCT GGG CTG ATC TTC TTC C-3′; β-actin: forward 5′-AAG ACC TCT ATG CCA ACA CAG TG-3′, reverse 5′-CAG GAG GAG CAA TGA TCT TGA TCT-3′. The reaction protocol included preincubation at 95 °C for 10 min, amplification of 40 cycles that was set for 15 s at 95 °C, annealing for 30 s at 60 °C and extending at 72 °C for 30 s. Levels of gene expression were shown relative to the internal standard (mouse β-actin).

2.8. Measurement of Sirt1 deacetylase activity

Sirt1 deacetylase activity was measured by fluorometric SIRT1 assay kit (Sigma) following the manufacturer's instructions. The assays were performed by incubating 20 μl protein extract with

SIRT1 substrate solution at 37 °C for 30 min. After addition of the developing solution and incubation at 37 °C for 10 min, fluorescent intensity was measured at 460 nm (excitation 355 nm) by using a fluorescence plate reader and normalized by protein content.

2.9. Measurement of NAD amount

Intracellular NAD was measured with a NAD^+/NADH quantification kit (Bio Vision) following the manufacturer's instructions. The optical density was read at OD 450 nm by a kinetic ELISA reader (Spectra MAX 250, Molecular Devices, CA, USA). The NAD amount from each sample was calculated as total NAD divided by the protein concentration.

3. Results

3.1. NAD concentration and Nampt expression increased during osteogenic differentiation of C3H10T1/2 and MC3T3-E1 cells

To investigate the NAD fluctuations during osteogenic differentiation, we firstly compared the total NAD concentration between undifferentiated cells (day 0) and cells that have been cultured in osteogenic media for 9 days. As shown in Fig. 1A, in both C3H10T1/2 and MC3T3-E1 cell models, NAD concentration was significantly higher at the later differentiation stages (day 9). A time-dependent increase of Nampt expression was shown with both cell models (Fig. 1B). However, although relative higher Sirt1 activity was detected in the differentiated cells (Fig. 1C) the expression of Sirt1 protein remains changed (Fig. 1B).

3.2. Osteogenic differentiation of MC3T3-E1 cells was inhibited by knock-down of Nampt and addition of Nampt inhibitor, FK866

To investigate the role of Nampt on osteogenic differentiation, we generated Nampt deficient MC3T3-E1 cells by transfecting the cells with Nampt shRNA lentiviral transduction particles. As indicated in Fig. 2A, Nampt expression at both protein and mRNA levels were successfully diminished as compared with the cells tranfected with the non-target scramble shRNA lentiviral particles. After 9 days of ostegenic differentiation, the activity of the osteoblast marker, alkaline phosphatase (ALP), was lower in Nampt knockdown cells as demonstrated by ALP staining and PNPP semi-quantification (Fig. 2B). Such effects were additionally supported by Q-PCR analysis, which showed that the expression of the osteoblast specific marker genes, OCN, OPN and OPG were markedly decreased (Fig. 3C).

We next tested the effects of a potent and specific Nampt inhibitor, FK866 [23], on osteogenic differentiation. We found that at concentrations higher than 10 nM FK866 exerted apparent cytotoxic effect on MC3T3-E1 cells (data not shown), while at the non-toxic concentration 1 nM, FK866 significantly decreased ALP activity (Fig. 3D). Consequently, the mineral nodule formation, as

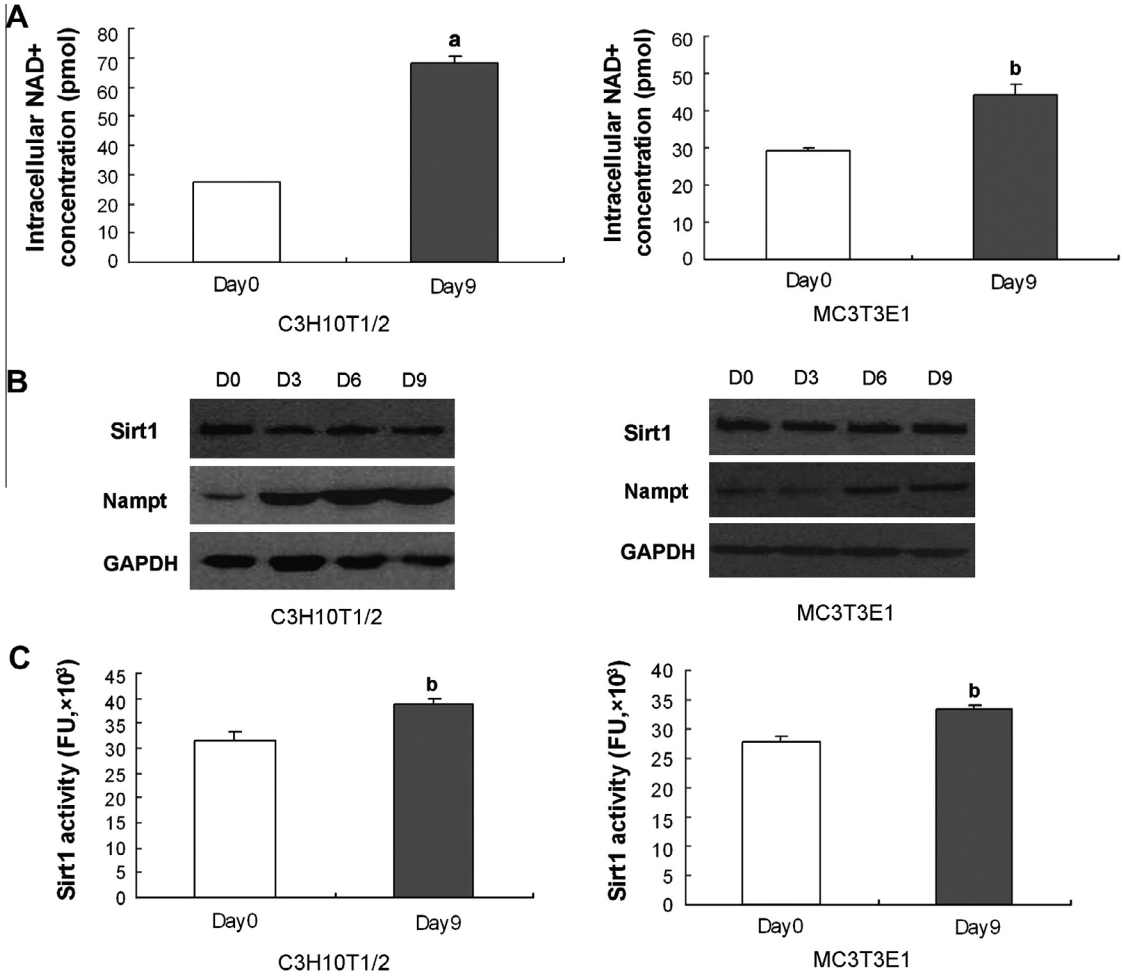

Fig. 1. C3H10T1/2 and MC3T3-E1 cells were cultured in osteogenic media for 9 days. NAD concentration was significantly higher at the later differentiation stages (A) time-dependent increase of Nampt expression was shown with both cell models (B). However, although relative higher Sirt1 activity was detected in the differentiated cells (C). The expression of Sirt1 protein remains changed (B). (a, $p < 0.01$; b, $p < 0.05$ with student t-test).

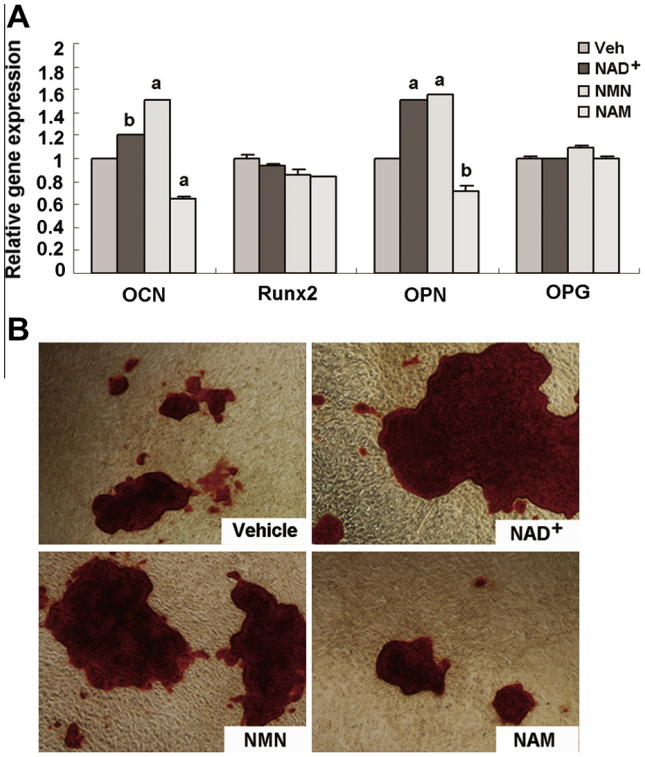

Fig. 2. Nampt deficient MC3T3-E1 cells were generated by transfecting the cells with shRNA lentiviral transduction particles. Nampt expression at both protein and mRNA level were significantly decreased in cells tranfected with Nampt shRNA as compared with the cells tranfected with the non-target scramble shRNA lentiviral particles (A). Osteoblast differentiation was shown by ALP staining and quantified by PNPP analysis (B). The gene expression of OCN, Runx2, OPN and OPG were analyzed by quantitative real-time PCR (C). Additionally, effects of the specific Nampt inhibitor FK866, were investigated on the osteoblast differentiation of MC3T3-E1 cells. ALP activity was quantified by PNPP analysis (D). In vitro mineralization was shown by alizarin red staining (E). The gene expression of the osteoblast key transcription factor Runx2, as well as the osteoblast marker genes, OCN, OPN and OPG were analyzed with quantitative real-time PCR (F) (a, $p < 0.05$; b, $p < 0.01$ with student t-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this book.)

demonstrated by alizarin red staining, was reduced with the treatment of 1 nM FK866 (Fig. 3E). The inhibitory effects on osteogenic differentiation were further confirmed by Q-PCR analysis, which showed that the expression of the osteoblastic key transcription factor Runx2, as well as the osteoblast specific marker genes, osteocalcin (OCN) and OPG were markedly down-regulated (Fig. 3F).

3.3. Nampt deficiency attenuated Sirt1 activity in MC3T3-E1 cells by reducing intracellular NAD$^+$ concentration

To find out whether the decreased osteoblast differentiation with Nampt deficiency in MC3T3-E1 cells was mediated by Sirt1 we examined the expression of Sirt1 protein. As shown in Fig. 3A, no significant change in Sirt1 protein expression was found between cells transfected with Nampt shRNA and scramble shRNA lentiviral particles. However, when Sirt1 activity was investigated, there was a significant reduction in Nampt deficient cells (Fig. 4B). Furthermore, we analyzed the intracellular concentration of NAD. As demonstrated in Fig. 4C, the NAD concentration in Nampt deficient cells was markedly lower.

3.4. Osteogenic differentiation of MC3T3-E1 cells was enhanced by the addition of NAD and NMN, while it was inhibited by nicotinamide

To understand the role of NAD metabolism in osteogenic differentiation, we next examined the effects of different intermediates in the NAD salvaging pathway on MC3T3-E1 cell differentiation. As shown in Fig. 4A, two of the osteoblastic marker genes, OCN and OPN, were up-regulated with the treatment of 100 μM NAD or 100 μM NMN in 9 days. The expressions of the same genes were down-regulated with the treatment of 100 μM NAM. In consistence with the above findings, matrix mineralization analysis by alizarin red staining showed that bone nodule formation was more were pronounced with the treatment of NAD and NMN, while it was attenuated with the treatment of NAM (Fig. 4B).

4. Discussion

Accumulating data show that the progressive transition of pluripotent stem cells to the lineage-specific differentiated stages involves dynamic changes in energy demand and in the relative contributions of oxidative and glycolytic metabolic pathways [4–7]. However, little attention has been paid to the molecular link between energy metabolism and cell differentiation. In the present study, we investigated the roles of the NAD salvaging pathway in the osteogenic differentiation of the well-established murine models C3H10T1/2 and MC3T3-E1 cells. The C3H10T1/2 cell line was derived from mouse embryonic tissue [24]. Under proper stimulation, these cells can differentiate towards myocytes [25], osteoblasts [26], adipocytes and chondrocytes [27], representing a suitable model to study the lineage fate determination of multipotent stem cells. The MC3T3-E1 cell line was derived from calvaria of newborn mice. When cultured in osteogenic medium the cells differentiate along the osteoblastic lineage and produce mineralized matrix within 2 weeks [28], therefore representing a model for the omnipotent preosteoblasts. With both osteogenic models, we show for the first time that the intracellular NAD concentration and Nampt expression increase during osteogenic differentiation. We then focused the investigation to the role of the Nampt-NAD-Sirt1 pathway in the differentiation of MC3T3-E1 cells. The reason that we focused on MC3T3-E1 cells is because 10–20% of the C3H10T1/2 cells showed adipocytic phenotype upon osteogenic induction [21]. This heterogeneity prevents their usage for the lineage-specific differentiation study. In addition, Nampt is known to be increasingly expressed during adipocyte differentiation [29].

NAD plays dual roles in the biochemical process in mammalian cells: it function as a co-enzyme in redox reactions, and is consumed as a substrate in protein modifications [8]. Since the reducing agent NADH can emit intrinsic fluorescence the fluctuations of intracellular NADH can be monitered with optical techniques in the living cells [30]. Interestingly, although several studies showed the differentiation-induced changes in fluorescence of NADH no consensus has been reached on whether the absolute NADH concentration increased or decreased with differentiation [16–20]. In the present study, we used the non-fluorescence technique to measure intracellular NAD concentrations. The advantage with this method is that it can include the oxidized agent NAD$^+$ which has no functional fluorophore and cannot be detected by the optical techniques [30]. We found that in both osteogenic models the NAD concentrations were significantly higher in the differentiated cell than cells before osteogenic induction (Fig. 1). Since upregulation of aeroboic mitochondrial metabolism has been shown to be associated with osteogenic differentiation of mesenchymal stem cells [7] we hypothesize that the increased intracellular NAD pool, like the increased total ATP content reported by Chen et al. [7], reflects a metabolic adaptation of the differentiated cells to meet the higher demand of mitochondrial respiratory enzymes.

When NAD serves as a substrate, it is known to be consumed in the adenosine 5′-dephosphate (ADP)-ribosylation reactions catalyzed by poly (ADP-ribose) polymerases (PARPs) or the protein deacetylations catalyzed by Sirtuins; the former is involved in

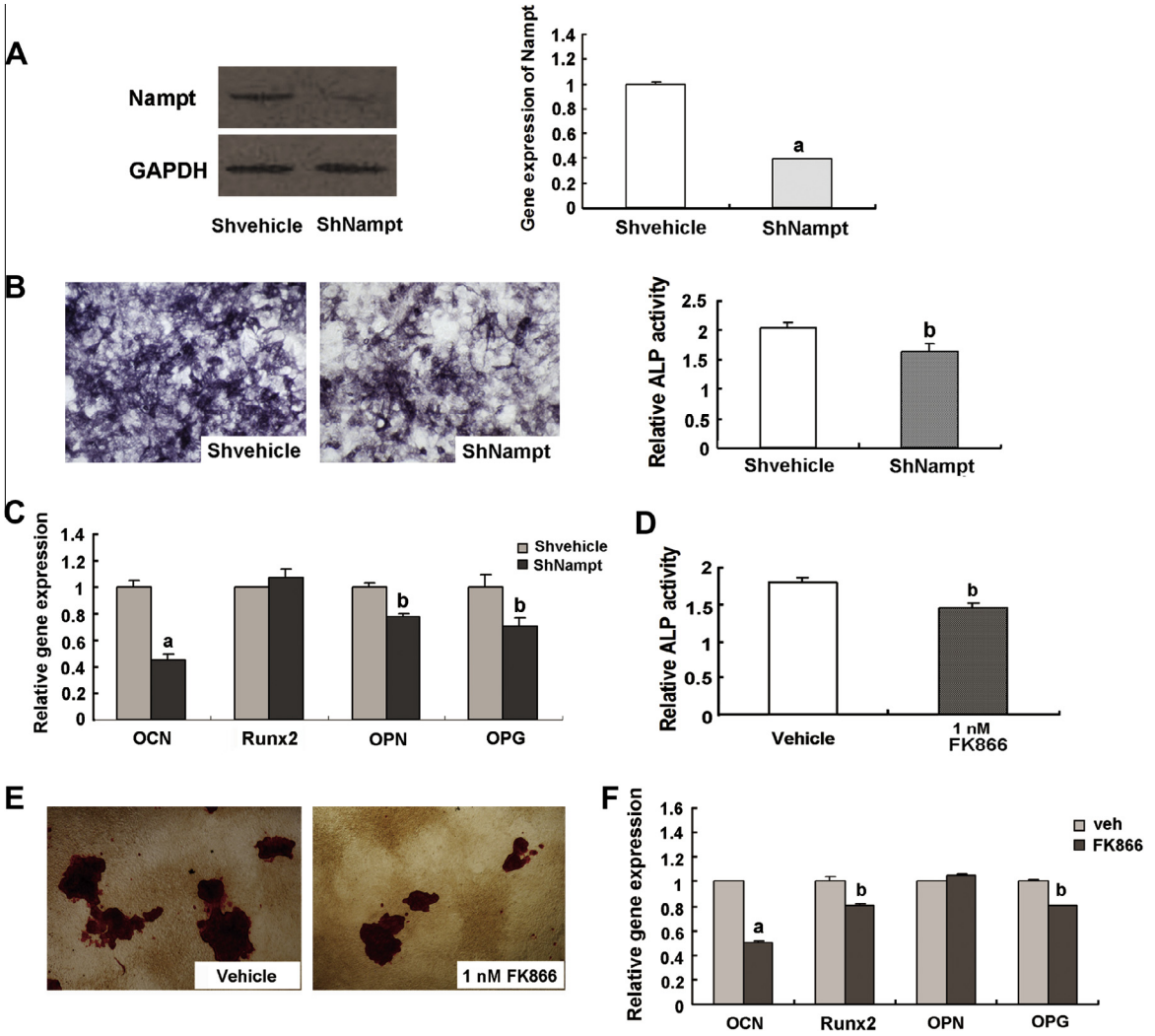

Fig. 3. Sirt1 activity was downgraded in Nampt deficient MC3T3-E1 cells by reducing intracellular NAD concentration. Sirt1 protein expression was shown by Western blot analysis (A). Sirt1 deacetylase activity was measured by fluorometric SIRT1 assay kit (B). The total intracellular NAD level was examined with NAD^+/NADH quantification kit (C).

cell-death programmes [31] and the latter is considered as key regulators in stress/senescence related gene regulation [32]. In the present study, we evaluated the influence of NAD concentration on the activity of Sirt1. At the level of chromatin, Sirt1-mediated deacetylation targets histone H1 at Lys26, H3 at Lys9 and Lys4, and histone H4 at Lys16. These modifications are supposed to promote the formation of facultative heterochromatin with resultant gene silencing [14,15]. Sirt1 can also target the lysine residues in transcriptional factors, many of which are involved in differentiation, such as p53 and Foxos [33–35]. The combined epigenetic and transcriptional effects enable Sirt1 to affect the differentiation of several cell lineages, including neurons [36], myocytes [37] and adipocytes [38]. Regarding osteoblasts, previous studies showed that overexpression of Sirt1 gene or addition of its activator resveratrol enhanced in vitro differentiation of osteoprogenitors [21,39,40]. We showed here that although Sirt1 protein expression remains constant, its enzymatic activity was significantly higher in the differentiated cells (Fig. 1). Furthermore, reduction of NAD concentration by knockdown of Nampt or addition of FK866 result lower Sirt1 activity (Figs. 2 and 3). Therefore, the NAD accumulation might enhance osteogenic differentiation through a Sirt1-mediated pathway. On the other hand, a recent report of Liu et al. showed that NAD-dependent Sirt1 and 6 proteins coordinate a switch from glucose to fatty acid oxidation during the acute inflammatory response in human leukocytes and murine splenocytes [41]. Since similar changes in energy metabolism also occurs in differentiation we hypothesize that the increased Sirt1 activity might also facilitate the transition from glycolytic to oxidative metabolic pathways. Therefore, the Sirtuins might be involved in the interaction between energy metabolism and differentiation in stem cells.

Although most organisms can synthesize NAD from simple components, tryptophan or aspartic acid (*de novo* pathway) [8], the Nampt-mediated NAD salvage reactions present the major NAD source in mammalian cells [42]. The reduced intracellular NAD concentration in Nampt-defiant cells (Fig. 3C) indicate that the intracellular NAD accumulation during differentiation is caused by the enhanced Nampt activity. This is confirmed by the facts that Nampt dysfunction resulted in reduction of Sirt1 activity and inhibition of osteogenic differentiation (Fig. 3B). In vivo, Nampt catalyzes the conversion of NAM–NMN, which has been supposed to be the rate limiting step for NAD biosynthesis. NMN can then be used in the synthesis of the final product, NAD. We found that the addition of NAD and its immediate precursor NMN promoted the expression of osteoblastic markers (Fig. 4), indicating that, besides inducing the enzyme, osteogenic

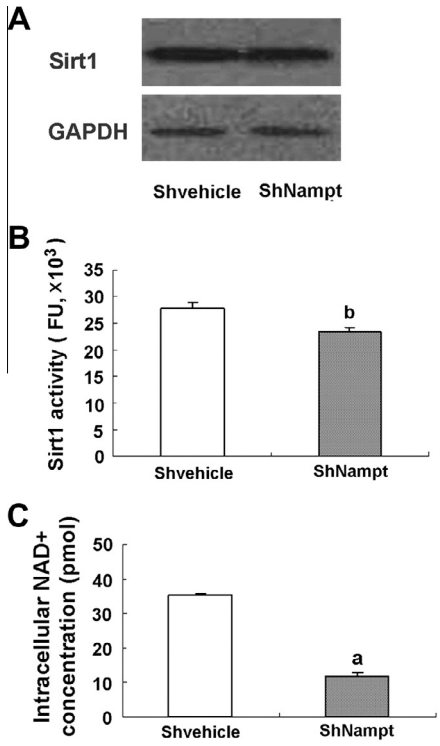

Fig. 4. With the treatment of different exogenous intermediates in the NAD$^+$ salvaging pathway, the osteoblast differentiation of MC3T3-E1 cells was analyzed by quantitative real-time PCR (A). In vitro matrix mineralization was shown by alizarin red staining (B). (a, $p < 0.05$; b, $p < 0.01$ with student t-test).

differentiation can also be enhanced through supplying molecules in the NAD salvaging pathway. Although NAM is a precursor for NMN it exerts totally contradictory effects in osteogenic differentiation as compared with NAD and NMN (Fig. 4). We suppose that this is because NAM can non-competitively inhibit the sirt1 catalyzed deacetylation [43,44]. The paradox further confirms that the effects of NAD on cell differentiation is Sirt1-mediated. These results indicate that the stem cell differentiation might be modulated through the application of small molecules that influence the NAD-Sirt1 pathway, which might be a future target for the treatment of degenerative diseases.

In summary, the present study indicates that during osteogenic differentiation, the stem cells enhance the activity of the NAD salvaging pathway by increasing Nampt expression. This leads to accumulation of intracellular NAD and to increased activities of the NAD-dependent enzymes, such as Sirt1. The enhanced Sirt1 activities, in turn, promote osteogenic differentiation and this positive feedback might drive the cells towards the stage of terminal differentiation.

Acknowledgments

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