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Neudesin, an extracellular heme-binding protein, suppresses adipogenesis in 3T3-L1 cells via the MAPK cascade

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ABSTRACT

Adult mice abundantly express neudesin, an extracellular heme-binding protein with neurotrophic activity, in white adipose tissues. At the early stage of adipocyte differentiation during adipogenesis, however, the expression of neudesin decreased transiently. Neudesin-hemin significantly suppressed adipogenesis in 3T3-L1 cells. The knockdown of neudesin by RNA interference markedly promoted adipogenesis in 3T3-L1 cells and decreased MAPK activation during adipocyte differentiation. The addition or knockdown of neudesin affected the expression of C/EBPα and PPARγ but not of C/EBPβ. These findings suggest that neudesin plays a critical role in the early stage of adipocyte differentiation in which C/EBPβ induces PPARγ and C/EBPα expressions, by controlling the MAPK pathway.

Introduction

White adipose tissue (WAT) plays a critical role in energy homeostasis [1,2]. Obesity, the excessive development of WAT, is a risk factor for several diseases, including type II diabetes, hypertension, cancer, and atherosclerosis; therefore, understanding the molecular and cellular mechanisms of the development of WAT is important. The development of WAT involves adipogenesis and an increased number of cells [3].

Adipogenesis is the process by which preadipocytes differentiate into mature adipocytes. Several transcription factors, which are essential for adipogenesis, have been identified through the study of cultured cells, including 3T3-L1 cells [4,5]. These factors include members of the CCAAT/enhancer binding protein (C/EBP) and peroxisome proliferator activated receptor (PPAR) families. C/EBPβ is expressed at a very early stage in the differentiation of preadipocytes, and PPARγ is expressed before the induction of C/EBPα, which is expressed at a relatively late stage of differentiation. A transcriptional cascade model is expected to accurately reproduce adipogenesis *in vivo* [4,5].

Neudesin is a secreted protein, previously identified as a neurotrophic factor, which is expressed abundantly in the developing brain and spinal cord of mouse embryos [6,7]. Mouse neudesin exhibits a high similarity (~90% identity) to human and rat neudesins. Neudesin is an extracellular heme-binding protein and requires the binding of heme to its cytochrome b5-like heme/steroid-binding domain for its neurotrophic activity [8].

Neudesin is also expressed in peripheral tissues, such as heart, kidney, and lung, but the biological function of neudesin in peripheral tissues remains unclear [6]. Since reverse transcriptase-polymerase chain reaction (RT-PCR) studies have revealed the abundant expression of neudesin mRNA in WAT, we examined the effect of neudesin on adipogenesis in an immortalized preadipocyte cell line, 3T3-L1, whose molecular mechanisms governing adipogenesis have been previously reported [9–12]. We found that neudesin suppresses adipogenesis in the early stage of adipocyte differentiation, suggesting the possibility that neudesin may play an important role in adipogenesis.

Materials and methods

Production of recombinant mouse neudesin protein. Recombinant baculoviruses containing mouse *neudesin* cDNA were obtained by

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cotransfection of Sf9 cells with a recombinant pBacPAK9 and a Bsu36I-digested expression vector, BacPAK6 (Clontech), as described elsewhere [6]. High Five cells infected with the recombinant baculovirus were cultured at 27 °C for 96 h in Grace's insect medium (Gibco) with 10% fetal bovine serum (FBS; Gibco). Recombinant mouse neudesin proteins were purified from the culture medium by affinity chromatography using Ni-NTA agarose (Qiagen) as described elsewhere [6].

Cell culture and differentiation. 3T3-L1 preadipocytes were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin–streptomycin solution (Gibco) with 10% FBS [13,14]. For the differentiation experiment, 3T3-L1 cells were plated in 24-well plates (3.0×10^5 cells per well) and cultured in a medium supplemented with 10% FBS for 2 days. Subsequently, the medium was replaced with DMEM containing 10% FBS and inducers (0.25 μM dexamethasone, 10 μg/ml insulin and 0.5 mM 3-isobutyl-1-methylxantine; MDI) for 2 days. The medium was then changed, and 10 μg/ml insulin added for 2 days and was further cultured in DMEM supplemented with 10% FBS.

Oil red O staining. Cultured cells were fixed with 4% paraformaldehyde for 30 min at 4 °C and washed with 60% isopropyl alcohol. Fixed cells were stained with oil red O staining solution (0.3% oil red O in 60% isopropyl alcohol) for 20 min at room temperature, washed with 60% isopropyl alcohol, and then washed several times with distilled water. Finally, the stained cells were destained with isopropanol and the optical density (OD) at 530 nm wavelength of the isopropanol was measured by spectrophotometry.

siRNA transfection. 3T3-L1 preadipocytes were seeded in 24-well plates (3.0×10^5 cells per well) and cultured in DMEM with 10% FBS. The next day, the cells were transfected with 10 nM of siRNA (Stealth™; Invitrogen) against neudesin (5′-AUCCAGUGACAUCU UGGCCACACCU-3′) and control (5′-AUCACCGUGAUACAGUUCACC GCCU-3′) using Lipofectamine RNAiMAX transfection reagent (Invitrogen). Six hours later, the medium was replaced with DMEM containing 10% FBS. Subsquently, the transfected cells were cultured in DMEM containing 10% FBS for 24 h and used for the following experiments. The knockdown of neudesin was examined by RT-PCR as previously described [8].

RNA extraction and real-time quantitative RT-PCR. Total RNA was extracted from adipose tissues and 3T3-L1 cells using an RNeasy Mini Kit (Qiagen). cDNAs were transcribed from the RNAs as templates with Molony murine leukemia virus reverse transcriptase (Gibco). The cDNAs were amplified by PCR with Taq DNA polymerase (NIPPON GENE). The primers used were 5′-GCCTGCTCCTCG CTGTCTAT-3′ and 5′-CCTAGAACCGGCTGCTTCTC-3′ for *neudesin*, 5′-ATGTGTGATGCCTTTGTGGGA-3′ and 5′-TCATGCCCTTTCATAAA CTCT-3′ for *aP2*, 5′-AAGAAGTCGGTGGACAAGAACAG-3′ and 5′-GT TGCGTTGTTTGGCTTTATCTC-3′ for *C/EBPα*, 5′-GACTACGCAACACAC GTGTAACT-3′ and 5′-CAAAACCAAAAACATCAACAACCC-3′ for *C/EBPβ*, 5′-TGGGTGAAACTCTGGGAGAT-3′ and 5′-CCATAGTGGAAG CCTGATGC-3′ for *PPARγ*, and 5′-ACGCTGAGCCAGTCAGTGTA-3′ and 5′-CTTAGAGGGACAAGTGGCG-3′ for *18S-ribosomal RNA* as a control [15]. Real-time quantitative RT-PCR analyses were performed using the DNA Engine Opticon-2 (MJ Research). Reactions were performed using 2× SYBR Green reaction buffer (TAKARA). After an initial incubation for 2 min at 50 °C, the cDNA was denatured at 94 °C for 10 min followed by 40 cycles of PCR (94 °C, 30 s, 58 °C, 42 s). For each condition, expression was quantified in duplicate.

Detection of endogenous neudesin proteins. Tissue samples were homogenized in 0.1 M sodium phosphate (pH 7.4), and a small sample was removed for protein determination. Samples were centrifuged at 10,000g for 20 min at 4 °C. The supernatants were analyzed by Western blotting (see below), and endogenous neudesin was detected using a rabbit anti-neudesin polyclonal antibody as described previously [8].

BrdU assay. 3T3-L1 preadipocytes were plated in 96-well plates (2×10^3 cells per well). After 24 h in culture, the cells were cultured in DMEM supplemented with 0.1% BSA for 24 h. The cells were then treated with neudesin-hemin, hemin, or FGF2 for 16 h and further cultured for 8 h in the presence of 5-bromo-2′-deoxyuridine (BrdU; 10 μM). Mitogenic activity was determined from five independent wells using the 5-Bromo-2′-deoxyuridine Labeling and Detection Kit III (Roche) as described elsewhere [16].

MTT assay. The survival of 3T3-L1 preadipocytes was evaluated by measuring MTT-reducing activity. siRNA-transfected 3T3-L1 preadipocytes were seeded at a density of 2×10^3 cells per well in 96-well plates. After 24 h in culture, the cells cultured in serum-free DMEM for 4 days. The cultured cells were then treated with 0.5 mg/ml MTT (MTT Cell Count Kit; Nacalai Tesque) at 37 °C for 4 h. The reduction product, MTT-formazan, was solubilized with isopropyl alcohol. Absorption at 570 nm of each sample solution was considered to represent the MTT-reducing activity of the cells.

Western blotting. 3T3-L1 preadipocytes were seeded at a cell density of $0.4 \times 10^5/cm^2$ in 35 mm dishes. After being cultured in serum-free DMEM containing 0.1% BSA for 24 h, the cells were used for experiments of addition of neudesin. siRNA-transfected 3T3-L1 preadipocytes were cultured in DMEM containing 10% FBS for 3 days. After the induction of differentiation, cells were further cultured for 1–8 days. The cells were lysed in TNE buffer. Proteins were detected by Western blotting using the following antibodies: rabbit antibodies against ERK1/2, phosphorylated ERK1/2, Akt, phosphorylated Akt (Ser473) (each 1:1000) (Cell Signaling), C/EBPα (14AA), C/EBPβ (C-19), PPARγ (H-100) (each 1:100) (Santa Cruz Biotechnology), and β-actin (1:1000) (Sigma). The secondary antibody was a HRP-conjugated goat anti-rabbit antibody (1:2000) (Vector). Immunoreactive bands were visualized

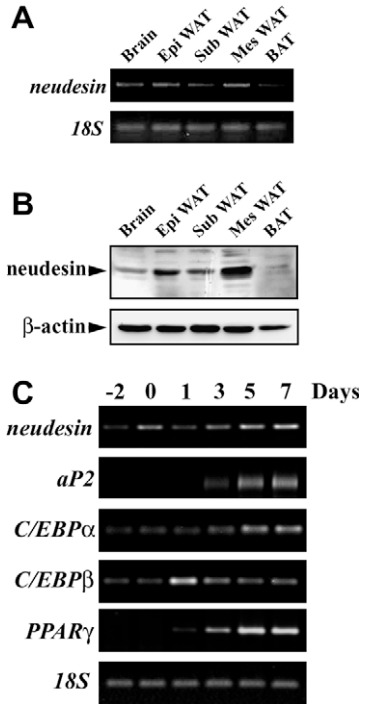

Fig. 1. Expression of neudesin in adipose tissues and during adipogenesis. (A) Expression of neudesin mRNA in mouse brain, WAT, and BAT (8 weeks postnatal). Epi, epididymal; Sub, subcutaneous; Mes, mesenteric. (B) Expression of endogenous neudesin protein (15.6 kDa) in adipose tissues. β-actin was used as a control. (C) Expression of neudesin mRNA during differentiation. 3T3-L1 preadipocytes were induced to differentiate in the presence of MDI. Total RNA was analyzed by RT-PCR. *18S* was used as a loading control.

using an enhanced chemiluminescence detection system as previously described [17,18].

Results

Expression of neudesin in adipose tissues and during adipogenesis

The expression of neudesin in adipose tissues was examined by RT-PCR and Western blotting. Both neudesin mRNA and protein were expressed in WAT but not in brown adipose tissue (BAT), and were expressed more in WAT than in the brain (Fig. 1A and B). Next, we compared the expression of neudesin with that of adipose differentiation marker genes (aP2, C/EBPα, C/EBPβ, and PPARγ) during adipogenesis in 3T3-L1 cells. Neudesin mRNA was detected in 3T3-L1 preadipocytes before the addition of MDI. The expression of neudesin decreased in the early stage of differentiation whereas that of C/EBPβ increased, and neudesin expression increased in the late stage of differentiation in parallel with that of aP2, C/EBPα, and PPARγ (Fig. 1C).

Effects of neudesin-hemin on adipogenesis in 3T3-L1 cells

To elucidate the role of neudesin in adipogenesis, the effect of neudesin-hemin on 3T3-L1 was examined. When neudesin-hemin was added with MDI to 3T3-L1 preadipocytes, MDI-induced adipocyte differentiation was suppressed. However, the effect of neudesin-hemin was not as marked as that of FGF2, a potent mitogen, which is reported to inhibit adipogenesis by activating the MAPK cascade in 3T3-L1 [19]. Hemin alone had no effects on adipocyte differentiation (Fig. 2A and B). Next, we examined the influence of neudesin-hemin on the expression of several adipogenic genes. The addition of neudesin-hemin to 3T3-L1 cells decreased the expression of C/EBPα and PPARγ at Day 4 in the middle and late stages of adipocyte differentiation, but had no effect on that of C/EBPβ at Day 2 in the very early stages (Fig. 2C and D).

Neudesin-hemin is reported to exhibit neurotrophic activity by promoting the phosphorylation of ERK1/2 and Akt in primary cultured neurons and neural precursor cells [6,7]. Here, in 3T3-L1 cells, neudesin-hemin promoted phosphorylation of ERK1/2, but showed no influence on that of Akt (Fig. 2E).

Effect of neudesin knockdown by RNAi on adipogenesis

To elucidate the role of endogenous neudesin in adipogenesis, we examined the effect of neudesin RNAi on 3T3-L1 cells. RT-PCR revealed that neudesin siRNA, but not the control siRNA, suppressed neudesin expression (Fig. 3A). Five days after stimulation with MDI, when adipogenesis was still in progress, 3T3-L1 cells treated with neudesin siRNA were more intensely stained with oil red O than those treated with control siRNA, indicating that the knockdown of neudesin promoted adipogenesis (Fig. 3B and C).

Next, we examined the effect of neudesin siRNA treatment on the expression of various adipogenic genes and on phosphorylation of ERK1/2 and Akt. There were no differences in the rapid and transient up-regulation of C/EBPβ during the early stages of adipogenesis between neudesin siRNA-treated and control siRNA-treated 3T3-L1 cells. However, neudesin siRNA treatment enhanced the expression of C/EBPα and PPARγ (Fig. 3D and E), known to be in-

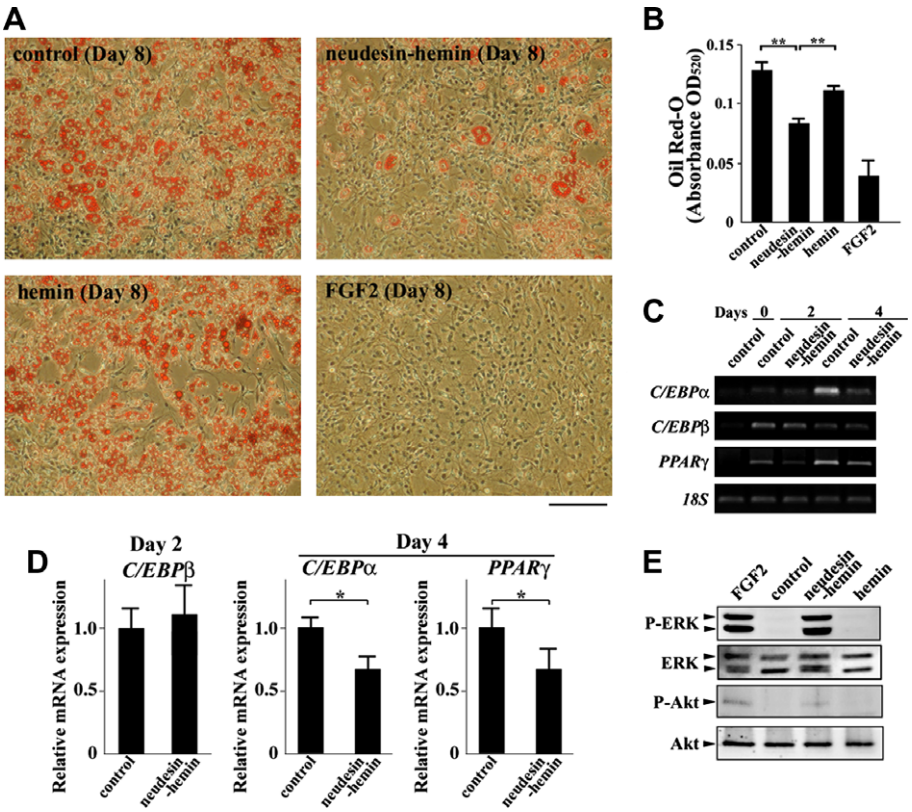

Fig. 2. Effects of neudesin-hemin on adipogenesis in 3T3-L1 cells. (A) Neudesin-hemin suppresses adipogenesis in 3T3-L1 cells. 3T3-L1 preadipocytes were stimulated with inducers (MDI) as well as neudesin-hemin (10 ng/ml), hemin (100 nM) or FGF2 (10 ng/ml). After 8 days, the cells were stained with oil red O to detect oil drops. Scale bar indicates 100 μm. (B) Oil red O staining. Results are means ± SD for four independent wells. $^{**}p < 0.005$. (C) Effect of neudesin-hemin on the expression of several adipogenic genes. Expression of C/EBPα, C/EBPβ and PPARγ in 3T3-L1 cells treated with and without neudesin-hemin (10 ng/ml) during differentiation. *18S* was used as a loading control. (D) The mRNA expressions of C/EBPβ, C/EBPα, and PPARγ were measured using quantitative real-time PCR. 18S mRNA expression was used as an internal control. $^{*}p < 0.05$. (E) Effect of neudesin-hemin, hemin, and FGF2 on the phosphorylation of ERK1/2 and Akt in 3T3-L1 cells. 3T3-L1 cells were cultured for 30 min in the absence or presence of neudesin-hemin (10 ng/ml), hemin (100 nM) or FGF2 (10 ng/ml).

duced by C/EBPβ and to play important roles in the middle and late stages of adipogenesis [4,5]. Similarly, neudesin siRNA treatment increased the protein levels of C/EBPα and PPARγ, but had no effect on that of C/EBPβ (Fig. 3F). There was no difference in phosphorylation of Akt between in neudesin siRNA-treated and control siRNA-treated cells. Interestingly, neudesin siRNA treatment significantly reduced the phosphorylation of ERK in 3T3-L1 cells on Days −1, 0 and 3–7, but not on Day 1 (Fig. 3G).

Effect of neudesin on proliferation of 3T3-L1 preadipocytes

Neudesin transiently promoted neural precursor cell proliferation early in the developmental process, which is reported to be mediated through the MAPK and PI3 kinase pathways [7]. Since we observed that neudesin activated the MAPK pathway but not the PI3 kinase pathway in 3T3-L1 cells, we tried to examine the effect of neudesin-hemin on 3T3-L1 cell proliferation. Neudesin-hemin exhibited mitogenic activity in a dose-dependent manner (0.1–10 ng/ml) as well as FGF2 (10 ng/ml). Hemin alone had no effect on proliferation of preadipocytes (Fig. 4A). PD98059, a specific inhibitor for MAP kinase kinase, significantly inhibited the mitogenic activity of neudesin-hemin (Fig. 4B). The MTT assay revealed that neudesin siRNA significantly decreased cell proliferation in 3T3-L1 preadipocytes (Fig. 4C).

Discussion

Neudesin is an extracellular heme-binding protein with intrinsic signal transducing neurotrophic activity [8]. Heme is reportedly

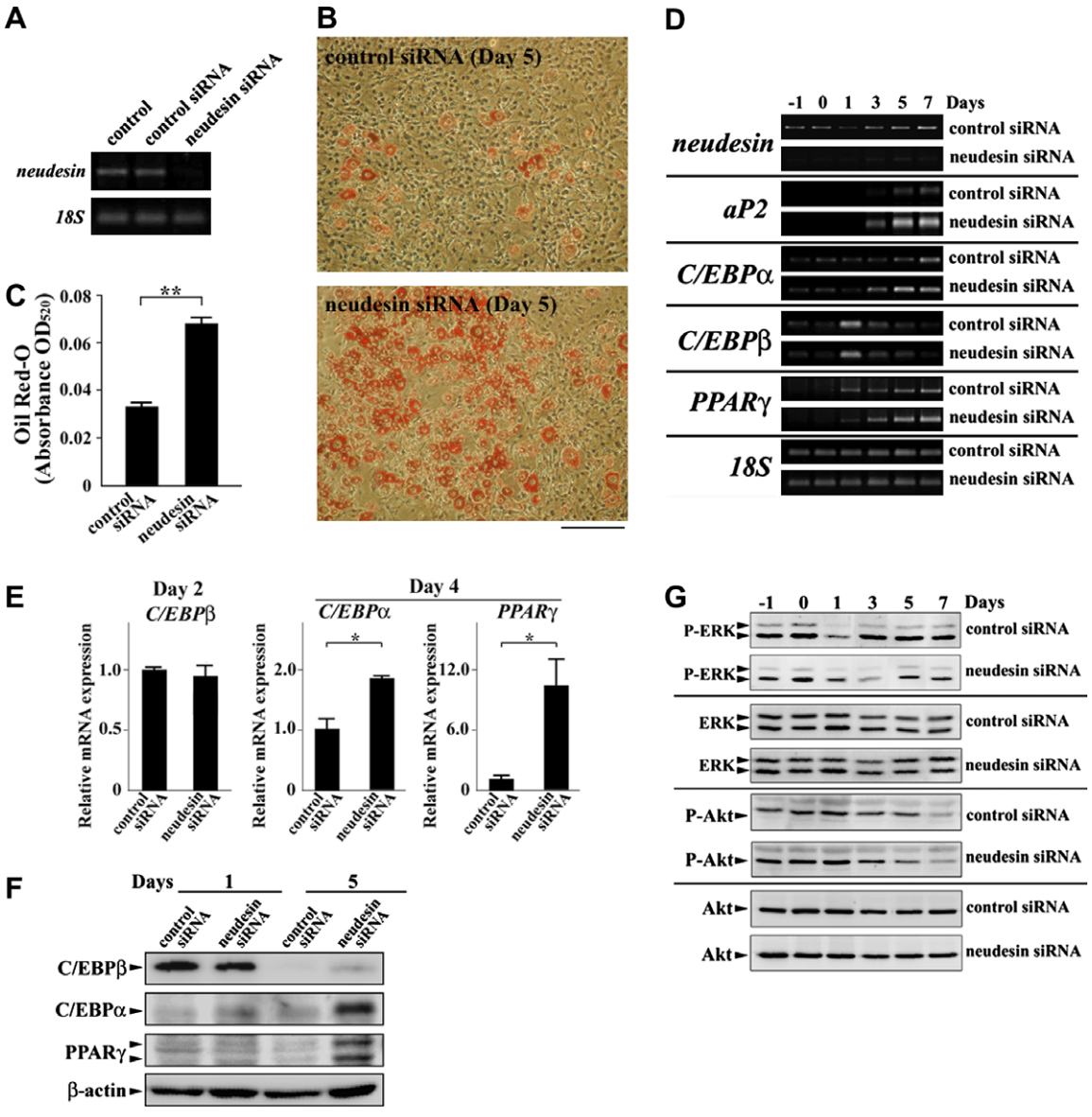

Fig. 3. Effect of knockdown of neudesin by RNAi on adipogenesis. (A) RT-PCR analysis of the knockdown of endogenous neudesin by neudesin siRNA in 3T3-L1 cells. After being treated with neudesin siRNA or control siRNA, 3T3-L1 cells were cultured for 1 day; *18S* was used as a loading control. (B) After treatment with neudesin siRNA or control siRNA, 3T3-L1 preadipocytes were stimulated with inducer (MDI). After 5 days, the cells were stained with oil red O to detect oil drops. Scale bar indicates 100 μm. (C) Oil red O staining. Results are means ± SD for four independent wells. $^{**}p < 0.005$. (D) Effect of neudesin siRNA treatment on the expression of various adipogenic genes. After treatment with neudesin siRNA or control siRNA, 3T3-L1 preadipocytes were induced to differentiate in the presence of MDI (Day 0). (E) The mRNA expressions of C/EBPβ, C/EBPα, and PPARγ were measured using quantitative real-time PCR. 18S mRNA expression was used as an internal control. $^{*}p < 0.05$. (F) Effect of neudesin RNAi on the expression of several adipogenic proteins. Expression of C/EBPα (42 kDa), C/EBPβ (45 kDa), and PPARγ (57 and 53 kDa) in 3T3-L1 cells treated with neudesin siRNA or control siRNA during differentiation. β-actin was used as a loading control. (G) Effect of neudesin siRNA treatment on phosphorylation of ERK1/2 and Akt. After being treated with neudesin siRNA or control siRNA, 3T3-L1 preadipocytes were induced to differentiate in the presence of MDI (Day 0).

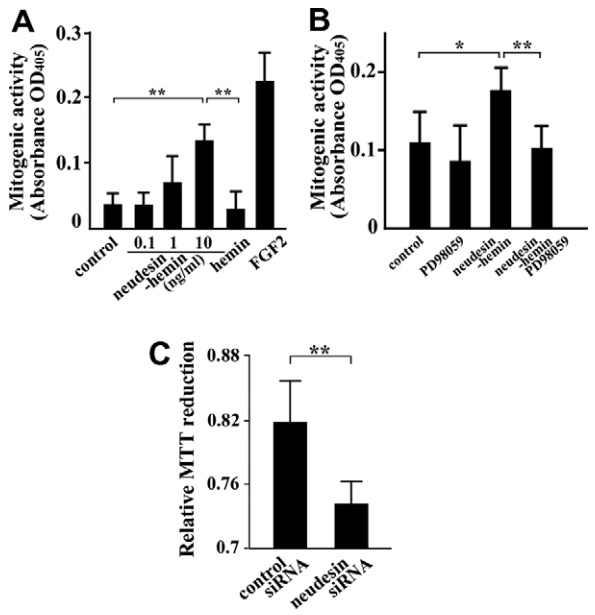

Fig. 4. Effect of neudesin on proliferation of 3T3-L1 preadipocytes. (A) Effect of neudesin-hemin (10 ng/ml), hemin (100 nM) and FGF2 (10 ng/ml) on mitogenic activity. Results are means ± SD for five independent wells. (B) The inhibitory effect of PD98059 (60 μM) on the proliferation of neudesin-hemin in 3T3-L1 preadipocytes via the MAPK cascade. Results are means ± SD for five independent wells. (C) MTT assay. After treatment with neudesin siRNA or control siRNA, 3T3-L1 preadipocytes were cultured for 4 days in serum-free DMEM. Results are means ± SD for five independent wells. $^{*}p < 0.05$; $^{**}p < 0.005$.

essential for the biological activity of neudesin because its activity decreases markedly without hemin [8]. Therefore, neudesin-hemin was used for testing neudesin activity in the present study. Although hemin alone is known to show biological activities [20,21], no activity was detected here (Figs. 2B and 4A). Although neudesin mRNA is expressed in various organs besides the brain, for example, the heart, kidney, and lung, the biological activity of neudesin in peripheral tissues is unclear. We found that both neudesin mRNA and protein were abundantly expressed in WAT but not in BAT (Fig. 1A), and their expressions transiently decreased in the early stage of adipose differentiation in 3T3-L1 cells (Fig. 1B); therefore, we used 3T3-L1 preadipocytes to elucidate the roles of neudesin in adipogenesis.

Our results indicated that neudesin plays a critical role as an inhibitory factor in adipogenesis. First, we determined that when 3T3-L1 preadipocytes were stimulated with MDI, the expression of neudesin mRNA decreased in the early stage of adipogenesis (Fig. 1B). Second, exogenous neudesin-hemin suppressed MDI-induced adipogenesis in 3T3-L1 cells (Fig. 2A and B). Third, our RNAi experiments revealed that suppression of endogenous neudesin expression promoted adipogenesis markedly (Fig. 3B and C). Finally, we observed neudesin-hemin promoted ERK phosphorylation (Fig. 2E). In contrast, neudesin siRNA suppressed ERK phosphorylation (Fig. 3G). It is known that activation of the MAPK cascade depresses adipogenesis in 3T3-L1 cells [19].

Next, we tried to identify the step at which neudesin acts. Neudesin mRNA decreased transiently 1 day after stimulation with MDI (Fig. 1B). It has been demonstrated that expression of C/EBPβ at a very early stage of adipogenesis is required for the induction of C/EBPα following PPARγ expression [5]. Treatment of 3T3-L1 cells with exogenous neudesin-hemin had no effect on the expression of C/EBPβ, but did reduce that of PPARγ and C/EBPα (Fig. 2C and D). Furthermore, although neudesin siRNA had no effect on the expression of C/EBPβ, it enhanced the expression of C/EBPα and PPARγ (Fig. 3D and E). These results suggest the possibility that the neudesin signal acts at the step at which C/EBPβ induces PPARγ and C/EBPα expressions.

It is known that activation of the MAPK cascade depresses and PI3 kinase promotes adipogenesis [22]. Furthermore, neudesin-hemin has enhanced phosphorylation of ERK1/2 and Akt in primary cultured neurons and neural precursor cells [6,7]. Here, in contrast, neudesin-hemin promoted phosphorylation of ERK1/2, but showed no influence on that of Akt in 3T3-L1 cells (Fig. 2E). The phosphorylation of ERK reduced transiently 1 day after the induction of adipogenesis, and recovered to the basal level on Days 3–7 (control siRNA in Fig. 3G). This suggests that a transient suppression of ERK may be concerned in the initiation of adipogenesis. Since ERK phosphorylation in cells treated with neudesin siRNA continued to decrease up to Day 7, the enhancement of adipogenesis by the knockdown of neudesin may be due to prolongation ERK suppression. This is supported by the observation that there was no difference in Akt phosphorylation between neudesin siRNA-treated and control siRNA-treated cells. Although PI3 kinase is reported to promote adipogenesis [22], it seems that the PI3 kinase cascade does not participate in the action of neudesin.

As we reported previously [7], neudesin promotes neural precursor cell proliferation early in the developmental process via the MAPK and PI3 kinase pathways. The fact that neudesin activated only the MAPK pathway in the process of adipogenesis of 3T3-L1 cells led us to examine the effect of neudesin on 3T3-L1 preadipocyte proliferation. Neudesin enhanced the proliferation of 3T3-L1 preadipocytes (Fig. 4A), whereas neudesin siRNA reduced it (Fig. 4C). In addition, the mitogenic activity of neudesin seemed to be mediated through the MAPK cascade because PD98059, a MAPKK inhibitor, cancelled the effect of neudesin (Fig. 4B). It has been reported that prolonged exposure of preadipocytes to FGF2, a potent mitogen, for more than 12–24 h inhibits adipogenesis via a mechanism that may be involved in activation of the MAPK pathway, whereas an initial 12 h exposure of preadipocytes to FGF2 induced a time-restricted burst of MEK activity that promoted a clonal expansion of preadipocytes [19]. However, it does not appear that neudesin participates in such a clonal expansion because the knockdown of neudesin had no effect on the initial differentiation of preadipocytes. In the present study, the exposure of 3T3-L1 cells to FGF2 for 2 days, under conditions in which cells showed accelerated proliferation, resulted in complete suppression of adipogenesis (Fig. 2A). The initiation of adipogenesis with MDI induced a decrease in ERK phosphorylation following reduced neudesin expression on Day 1, which appeared essential to continuation of adipogenesis. Neudesin siRNA enhanced MDI-induced adipogenesis, but produced lasting suppression of ERK phosphorylation on Days 3–7 (Fig. 3G). These results suggest that under ordinary conditions, the presence of neudesin may suppress adipogenesis of 3T3-L1 cells by sustaining the activation of the MAPK cascade. The initiation of adipogenesis may suppress neudesin expression, which may be accompanied by a transient reduction of neudesin protein, and thereby result in MAPK pathway suppression. Furthermore, the activation of ERK, which is profoundly involved in cell proliferation, may suppress a specific signal transducing step in adipogenesis, during which C/EBPβ leads to PPARγ and C/EBPα expressions.

The present results suggest that a neudesin level decrease may be a triggering factor for adipogenesis; this suggests that neudesin suppresses differentiation from preadipocytes to adipocytes. These findings provide further insight into the molecular mechanism of adipogenesis.

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