

Effects of Dedifferentiated Fat Cells on Neurogenic Differentiation and Cell Proliferation in Neuroblastoma Cells

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Effects of dedifferentiated fat cells on neurogenic differentiation and cell proliferation in neuroblastoma cells

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Abstract

Recent reports demonstrated that mesenchymal stem cells (MSCs) can induce differentiation of neuroblastoma (NB) cells. Dedifferentiated fat cells (DFAT) and MSCs have similar properties. The present study investigated whether DFAT can induce NB cell differentiation and suppress cell proliferation. DFAT was obtained from mature adipocytes isolated from adipose tissue from a ceiling culture. NB cells were cultured in a medium with/without DFAT, and subsequently in a DFAT-conditioned medium (CM) with/without phosphatidylinositol 3 kinase (PI3K) inhibitor. Length of neurites was measured, and the mRNA expression levels of the *neurofilament (NF)* and *tubulin beta III (TUB β 3)* were assessed using quantitative real-time reverse transcription polymerase chain reaction. Cell viability was assessed by the water-soluble tetrazolium salt-1 assay. NB cells cultured with DFAT elongated the neurites and upregulated the expression of *NF* and *Tub β 3* compared with the control. However, NB cells cultured in DFAT-CM demonstrated increased cell viability compared with the control. NB cells cultured with DFAT-CM and PI3K inhibitor suppressed cell viability and demonstrated increased neurite length and expression and upregulation of *Tub β 3*. Therefore, the combined use of DFAT-CM and PI3K inhibitors suppresses the proliferation of NB cells and induces their differentiation. DFAT may offer new insights into therapeutic approaches in NB.

Keywords

Neuroblastoma, Dedifferentiated fat cell, Differentiation, Pediatric, Phosphatidylinositol 3 kinase

Declarations

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1. INTRODUCTION

Neuroblastoma is an embryonal tumor derived from undifferentiated neural crest cells and is the most common extracranial solid tumor during childhood. Tumors may be derived from anywhere in the sympathetic nervous system. Primary tumors are mainly concentrated in the abdomen (65%), half of which occur in the adrenal medulla [1]. Most neuroblastomas that develop during infancy spontaneously dedifferentiate without treatment and have favorable prognosis. However, neuroblastomas that occurring 12 months after birth may have poor outcomes. Clinical features, including age at onset and molecular biological markers of tumor tissues, are closely related to the prognosis. *MYCN* amplification, found in 20%–30% of patients with neuroblastoma, is a strong indicator of poor prognosis. Risk stratification based on these indicators are used to make treatment decisions [1–3].

The standard treatment for high-risk neuroblastoma is a multimodal approach, comprising chemotherapy and radiation therapy, and autologous hematopoietic stem-cell transplantation. Over the last decade, high-dose chemotherapy combined with autologous hematopoietic stem cell transplantation has been shown to improve event-free survival compared with conventional chemotherapy [4–5]. However, despite clinical remission with such a treatment, the 3-year event-free survival rate remains low at 31%–47% because of recurrence from residual lesions [4–6].

Mesenchymal stem cells (MSCs) are fibroblast-like cells, isolated from the bone marrow, adipose tissue, and fetal appendage tissues (such as the umbilical cord, umbilical cord blood, and placenta). MSCs are multipotent cells that differentiate into various cell types, such as osteoblasts, adipocytes, and chondrocytes [7–8]. MSCs are not only multipotent but also promote regeneration and supply immunoregulatory signals and are expected to be therapeutic cells with immunotherapeutic potential.

MSCs induce differentiation of neuroblastoma cells by secreting neurotrophic factors and growth factors such as nerve growth factor (NGF), glial cell-derived neurotrophic factor, and brain-derived neurotrophic factor (BDNF) [9–10]. In a previous study, intertumoral injection of MSCs in a neuroblastoma mouse model inhibited tumor growth by inducing apoptosis. This resulted in longer survival in the MSC-treated mice [11]. Although MSCs have such high potential, clinical problems also have been reported. Harvest of bone marrow-derived MSCs is highly invasive. Additionally, geriatric patients with low bone density or those with osteoporosis do not have the required number of MSCs. Adipose tissue-derived stem cells (ASCs) could be an alternative to bone marrow-derived MSCs because they can be easily isolated without painful procedures or donor site injury [12, 13]. However, similar to bone marrow MSCs, ASCs also comprise a heterogeneous cell population in the stromal tissue. Additionally, ASCs at early passage contain endothelial cells, smooth muscle cells, and pericytes. Additionally, ASCs cultured with serial passages continue to express various

endothelial cell markers [12]. Therefore, other stem cell sources are required.

Harvesting of cells irrespective of age with minimal invasiveness is desirable. Additionally, the harvested cells must have high proliferative ability and homogeneity [14, 15]. As a cell source that solves these problems, Matsumoto et al. [16] demonstrated that dedifferentiated fat cells (DFAT), obtained from ceiling culture of mature adipocytes, isolated from the adipose tissue, can actively proliferate and have an ability to differentiate similar to MSCs. DFAT are recognized as cells that have acquired a trait similar to that of MSCs, because the cell surface antigen profile of DFAT is similar to that of bone marrow-derived MSCs and ASCs. Cytokines released from DFAT were also found similar to those released from bone marrow-derived MSCs and ASCs. Cytokines have been reported to contain neurotrophic factors, such as BDNF [17]. Therefore, DFAT may also induce differentiation of neuroblastoma cells like MSCs.

Various neurotrophic and growth factors secreted by MSCs may exert therapeutic effects by promoting the differentiation of neuroblastoma cells [9–10]. However, whether DFAT have a differentiation-inducing effect on neuroblastoma cells remains unclear. In the present study, we aimed to determine the effect of DFAT on neuronal differentiation and cell viability in a human neuroblastoma cell line.

2. METHODS

2.1. Human neuroblastoma cell lines

NB9 cells were acquired from the RIKEN Cell Bank (Tsukuba, Japan) and were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 15% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO) and 1% penicillin-streptomycin-glutamine (Thermo Fisher Scientific) at 37 °C in a 5% CO₂ incubator. Cells were subcultured to a fresh medium when the growth reached 90% confluence.

SK-N-SH cells were acquired from the American Type Culture Collection (ATCC Manassas, VA, USA). SK-N-SH cells were cultured as monolayers in Dulbecco's modified Eagle medium (Thermo Fisher Scientific) with 10% FBS, 1% Minimal Essential Medium non-essential amino acids (Thermo Fisher Scientific), 1% sodium pyruvate (Thermo Fisher Scientific), and 1% penicillin-streptomycin-glutamine, at 37 °C in a 5% CO₂ incubator. Cells were subcultured to a fresh medium when the growth reached 90% confluence.

2.2. Isolation and cell culture

Approximately 1–2 g of subcutaneous adipose tissue obtained from patients undergoing surgery was processed for preparing DFAT based on a previous report. The adipose tissue was cut into small

pieces and treated with 0.1% (w/v) collagenase solution (Sigma Aldrich) at 37 °C for 30 min under agitation. After agitation, the mixture was passed through a filter of pore size 250 µm to remove undigested tissue. The filtered liquid was centrifuged at 135 g for 3 min, and the floating top layer containing the mature adipocytes was collected. After washing with phosphate-buffered saline (PBS), the isolated mature adipocytes were placed in T-12.5 culture flasks that were filled with DMEM supplemented with 20% FBS and incubated at 37 °C in 5% CO₂. The mature adipocytes floated and attached to the upper surface of the culture flasks. After 7 days, the culture medium was removed, and the flasks were inverted such that the cells were situated at the bottom. The medium was replaced every 4 days until the cells attached to the ceiling surface reached confluence. After reaching confluence, the cells were subcultured on 100 mm dishes, and sixth-passage DFAT was used for the experiments. Samples of human subcutaneous fat were obtained from patients undergoing surgery in the Department of Pediatric Surgery of Nihon University Itabashi Hospital (Tokyo, Japan). The patients gave written informed consent. This study was approved by the Ethics Committee of Nihon University School of Medicine (Permission number: RK-160209-6).

2.3. DFAT conditioned medium

The sixth-passage DFAT were placed in 100 mm dishes and allowed to grow at 37 °C in 5% CO₂ until the cells reached 70% confluence. After washing with PBS, the medium was replaced with 5

mL of DMEM containing 5% FBS. The DFAT were incubated for 72 h, and the supernatant was collected and centrifuged at 1,500 g for 10 min. The supernatant was passed through a Millex-HV 0.45 μm filter (Merck Millipore, Burlington, MA). The collected DFAT-CM were frozen at $-80\text{ }^{\circ}\text{C}$ and thawed on the day of the experiments.

2.4. Co-culture of neuroblastoma cell lines and DFAT

NB9 cells (5×10^4) or SK-N-SH (1×10^5) were seeded in six-well plates. The neuroblastoma cells were incubated for 7 days in the absence (control group) or presence (co-culture group) of DFAT following 24 hours of seeding. In the co-culture group, DFAT (1×10^5) were seeded in the cell culture insert 0.4 μm PET membrane (BD Falcon). The cells were incubated in DMEM supplemented with 10% FBS. Seven days after incubation, the neurite length and the expression level of neural differentiation markers in NB cells were evaluated to assess the differentiation potential.

2.5. Cell proliferation

SK-N-SH or NB9 cells (5×10^3) were seeded in 96-well plates. Twenty-four hours after seeding, neuroblastoma cells were incubated for 3 days in the absence (control group) or presence of DFAT-CM (DFAT-CM group). In the DFAT-CM group, 100 μL of DFAT-CM was added to 100

μL of basal medium (DMEM containing 20% FBS). In the control group, 100 μL of DMEM was added to 100 μL of the basal medium. After culturing the neuroblastoma cells for 3 days under these conditions, the proliferation ability was evaluated using the WST-1 assay.

2.6. Combined effect of DFAT-CM and phosphatidylinositol 3 kinase (PI3K) inhibitor on neuroblastoma cells

The pharmacological inhibitor of PI3K, LY294002, was obtained from Tocris Bioscience (Bristol, England) and reconstituted according to the manufacturer's specifications. NB9 cells (5×10^4) were seeded into six-well plates. Twenty-four hours after seeding, neuroblastoma cells were incubated for 3 days in the absence (control group) or presence of DFAT-CM (DFAT-CM group), in combination with DFAT-CM and PI3K inhibitor (DFAT-CM + PI3K inhibition). In the LY294002 and DFAT-CM + LY294002 groups, the concentration of LY294002 was adjusted to 10 μM . After culturing the neuroblastoma cells for 3 days under these conditions, the differentiation potential of the neuroblastoma cells was evaluated. NB9 cells (5×10^3 cells/well) were seeded in 96-well plates. Twenty-four hours after seeding, the neuroblastoma cells were incubated under the above conditions for 3 days, and their proliferation ability was evaluated.

2.7. Neurite outgrowth assessment

For neurite outgrowth assessment in neuroblastoma cells, at least 15 random fields were photographed using a BZ-X710 phase-contrast light microscope (KEYENCE, Osaka, Japan). Neurite lengths were measured manually using the National Institutes of Health ImageJ software (Rasband, W. S., ImageJ, U. S. National Institutes of Health, Bethesda, ML, <https://imagej.nih.gov/ij/>, 1997–2018) [18–19]. Fifty cells were observed and counted to have neurites. A neurite was defined as a process outgrowth that was longer than twice the diameter of the cell body.

2.8. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted from the neuroblastoma cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Total RNA was reverse transcribed into complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). After obtaining cDNA, messenger RNA (mRNA) expression was analyzed with RT-PCR using the TaqMan probe. The mRNA expression levels of β III tubulin (*TUB β III*; Hs_00801390) and *neurofilament (NF)*; Hs_00193572) were analyzed as markers of neural differentiation. Real-time RT-PCR was performed with cDNA using TaqMan Fast Advanced Master Mix Product Insert (Thermo Fisher Scientific) in a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). *GAPDH* expression was measured as an internal standard. Each sample

was subjected to comparative CT analysis with respect to *GAPDH*, and the result was shown as relative to the expression level of the control.

2.9. Cell viability

SK-N-SH or NB9 cells (5×10^3) were seeded in 100 μ L of medium in 96-well plates and incubated for 3 days. The WST-1 assay was applied to the cell suspensions according to the manufacturer's protocol. Corresponding controls with analog concentrations of dimethyl sulfoxide were simultaneously performed. Absorbance readings of each well at 450 nm were recorded using a Microplate Manager6 microplate reader. The data is expressed as ratio of absorbance readings from the control wells.

2.10. Western blot analysis

NB9 cells in a 100-mm dish were pretreated with LY294002 for 1 h, followed by incubation with DFAT-CM for 24 h. For protein analysis, cell protein extracts were prepared by treating cells with cell lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and protease/phosphatase inhibitor cocktail (5872S, Cell Signaling Technology, MA, USA). Extracted cell lysates were boiled at 95 °C for 5 min. The lysates or Rainbow Molecular Weight Markers (RPN800E, GE Healthcare Life Sciences, England) were

loaded into separate lanes of an e-PAGEL polyacrylamide gel (E-T 10 L, ATTO, Tokyo, Japan) and separated using an electrophoresis system (AE-650, ATTO). Separated proteins were transferred to a polyvinylidene fluoride membrane (WSE-4051, ATTO) using a semi-dry blotting system (HorizeBLOT, ATTO) and treated with 3% bovine serum albumin (BSA) for 1 h at room temperature for blocking. For primary antibody response, the membranes were incubated with rabbit anti-AKT (pan) antibody (C67E7, Cell Signaling), rabbit anti-Phospho-Akt antibody (Ser473, Cell Signaling) at 1:1000 in 5% BSA at 4 °C overnight on a rocking platform. After washing, the membranes were incubated with anti-rabbit IgG-HRP antibody (NA9310V, GE Healthcare Life Sciences, England) as secondary antibodies at 1:2000 dilution for 1 h at room temperature. Immunoreactivity was detected using enhanced chemiluminescence western blotting detection reagents (RPN2209, GE Healthcare Life Science). Chemiluminescent signals were visualized using a Fusion Solo S system (Vilber Lourmat, France).

2.11. Statistical Analysis

The Mann–Whitney U test was used for statistical comparisons between the two groups. Statistical comparisons between multiple groups were performed using one-way analysis of variance followed by Tukey’s multiple comparison test for post hoc analysis. The analysis was performed using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [20]. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Effects of DFAT on neuroblastoma cells by co-culture

When the cell morphology of NB9 cells was observed with a phase-contrast microscope, the co-culture group had more cells with neurites than the control group (Fig. 1a). Based on the quantitative analysis of neurite lengths, the percentage of cells with neurite outgrowth was significantly higher in the co-culture group than in the control group (Fig. 1b). SK-N-SH cells had similar results in that neurite outgrowth was observed in the co-culture group (Figs. 1c and d). Subsequent to the neurite outgrowth assay to confirm NB9 cell differentiation, mRNA expression of neuronal specific markers (*NF* and *TUB β 3*) was assessed by real-time RT-PCR. The mRNA levels of *NF* and *TUB β 3* were significantly higher in the NB9 cells of the co-culture group than in those of the control group ($p < 0.05$) (Fig. 1e). SK-N-SH cells showed similar results; the mRNA levels of *NF* and *TUB β 3* were significantly increased in the co-culture group when compared to the control group ($p < 0.05$) (Fig. 1f).

3.2. Effects of DFAT-CM on neuroblastoma cells

To determine whether DFAT-CM can affect the viability of human neuroblastoma cells, the viability of NB9 and SK-N-SH cells was assessed by WST-1 assay. The cell viability of NB9 cells

cultured in DFAT-CM was significantly higher compared with that cultured in the control medium ($p < 0.05$) (Fig. 2a). Similar results were observed in SK-N-SH cells (Fig. 2b).

From the aforementioned results, it can be stated that DFAT-CM can induce neuronal differentiation in neuroblastoma cells. However, DFAT-CM was demonstrated to promote the proliferation of neuroblastoma cells.

3.3. Combined effect of DFAT-CM and PI3K inhibitor on neuroblastoma cells

According to literature, cell proliferation signaling pathways in neuroblastoma cells are activated by PI3K/AKT that exists an intermediate signaling pathway [21]. To determine whether inhibition of PI3K activity affects proliferative potential and the differentiation of neuroblastoma cells, we treated NB9 cells with LY294002 to inhibit PI3K and subsequently, incubated with DFAT-CM or control medium. First, the concentration of LY294002 required to suppress the proliferative potential of NB9 cells was determined. Twenty-four hours after seeding the NB9 cells, DFAT-CM and LY294002 were added and incubated. On the third day of culture, cell proliferation was evaluated using the WST-1 assay. LY294002, a selective inhibitor of PI3K, suppressed NB9 cell proliferation at 10 μ M (Fig. 3a). In addition, LY294002 significantly suppressed the cell proliferation ability promoted by DFAT-CM on NB9 cells at 10 μ M (Fig. 3a). These data indicate that the PI3K inhibitor has a suppressive effect on the cell growth-promoting action of DFAT-CM.

After NB9 cells were incubated for 3 days in combination with DFAT-CM and LY294002, the neurite outgrowth of neuroblastoma cells was analyzed. From the quantitative analysis of neurite length, neurite outgrowth cells were significantly higher in the DFAT-CM group than in the control group. Treatment of NB9 cells with LY294002 did not significantly increase the percentage of neurite outgrowth cells (Figs. 3b and c). The expression of *TUBβ3* was significantly increased in both the DFAT-CM and LY294002 groups compared with that in the control group (Fig. 3d). In the DFAT-CM+LY294002 group, the expression of *TUBβ3* was significantly increased not only in the control group but also in the DFAT-CM and LY294002 groups (Fig. 3d). These results suggest that PI3K inhibitors increase the mRNA level of *TUBβ3*, and this effect is enhanced by combination with DFAT-CM.

We investigated the changes in AKT phosphorylation in NB9 cells following the addition of PI3K inhibitors. We confirmed that the addition of DFAT-CM led to the phosphorylation of AKT in NB9 cells (Fig. 4). The phosphorylation of AKT was inhibited in the presence of the PI3K inhibitor LY294002 at a concentration of 10 μM (Fig. 4).

4. DISCUSSION

In this study, we demonstrated that DFAT can induce differentiation in neuroblastoma cells. Our data demonstrated that co-culturing neuroblastoma cells with DFAT promoted neurite formation.

Neurite outgrowth suggests differentiation of neuroblastoma into nerve cells and is a phenomenon observed when differentiation is induced in the neural progenitor cells by factors such as retinoic acid and NGF [22]. The neurite outgrowth of neuroblastoma cells indirectly co-cultured with DFAT via cell culture inserts suggests that the factors secreted from DFAT induced the differentiation of neuroblastoma cells. We also found that the expression of neuronal specific markers, including *NF* and *TUB β 3*, from neuroblastoma cells was enhanced by indirect co-culture with DFAT cells. This suggests that DFAT may induce the differentiation of neuroblastoma cells, even at the mRNA level. The differentiation-inducing effect of DFAT on neuroblastoma cells was observed not only in the *MYCN* non-amplified cell line but also in the *MYCN*-amplified cell line. Therefore, DFAT may be a promising treatment for highly malignant neuroblastomas.

Further, we investigated the effect of factors secreted from DFAT on the proliferative ability of neuroblastoma cells. DFAT induced differentiation of NB cells. As a result, it was expected that DFAT would suppress the proliferation of NB cells. However, it was shown that the proliferation of neuroblastoma cells was enhanced in both *MYCN*-amplified and non-amplified cells by DFAT-CM. Therefore, DFAT may release factors supportive of differentiation and growth in NB cells such as BDNF, TGF β , and NGF.

The PI3K/AKT pathway is an intermediate signaling pathway in the proliferation of NB cells [23–26]. Therefore, we also evaluated the effect of DFAT-CM on neuroblastoma cells treated with a

PI3K inhibitor.

Culturing neuroblastoma cells with DFAT-CM in the presence of LY294002 increased *TUB β 3* expression compared with cells cultured with DFAT-CM alone. Additionally, LY294002 suppressed the cell proliferation effect of DFAT-CM. Therefore, when PI3K is inhibited, DFAT-CM suppress the proliferation of neuroblastoma cells and may, thus, efficiently induce differentiation of NB cells.

In this study, the combined use of DFAT-CM and PI3K inhibitors induced differentiation and the suppression of proliferation of NB cells. Therefore, the combined administration of DFAT-CM and PI3K inhibitor, which are already used in the treatment of malignant tumors, may improve the prognosis of patients with neuroblastoma.

Our study had several limitations. The effect of DFAT on neuroblastoma mouse models is yet to be known. Additionally, we did not compare DFAT with the other types of cells, such as MSCs. Further studies are required to evaluate the effect of the combined administration of DFAT-CM with a PI3K inhibitor in improving neuroblastoma.

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FIGURE LEGENDS

Fig 1 Figure showing the effect of human dedifferentiated fat cells (DFAT) on neuroblastoma cell differentiation. NB9 or SK-N-SH cells were cultured in the presence or absence of DFAT. (a) Photograph showing NB9 cells using phase-contrast light microscopy seven days after culture (scale bar, 100 μm). (b) Neurite outgrowth was measured using National Institutes of Health ImageJ software. The neurite outgrowth of NB9 cells is enhanced by DFAT. (c) Photograph showing SK-N-SH cells using phase-contrast light microscope seven days after culture (scale bar, 100 μm). (d) The neurite outgrowth of SK-N-SH cells is enhanced by DFAT. Bar: mean \pm SE. Data are shown for triplicate wells, * $p < 0.05$.

(e) Figure showing the expression analysis of neural differentiation markers in NB cells that were cultured in the presence or absence of DFAT for seven days. Total RNA was then extracted and *NF* and *TUB β 3* were quantitated using real-time RT-PCR. Relative expression was analyzed using the comparative CT method with *GAPDH* as the internal control. The expression of *NF* and *TUB β 3* in NB9 cells was increased by co-culturing with DFAT cells. (f) The expression of *NF* and *TUB β 3* in SK-N-SH cells was increased by co-culturing with DFAT. Bar: mean \pm SE. Data are shown for triplicate wells, * $p < 0.05$.

Fig 2 Figure showing the effect of human dedifferentiated fat cells (DFAT) on neuroblastoma (NB) cell proliferation. NB cells were cultured in the presence or absence of DFAT-conditioned medium

(DFAT-CM). Neuroblastoma cell proliferation was evaluated using WST-1 three days after culture. (a)

The proliferation of NB9 cells was enhanced by DFAT-CM. (b) The proliferation of SK-N-SH cells was enhanced by DFAT-CM. Bar: mean \pm SE. Data shown for triplicate wells, * $p < 0.05$.

Fig 3 Figure showing the effect of human dedifferentiated fat cells (DFAT) in combination with a phosphatidylinositol 3-kinase (PI3K) inhibitor on neuroblastoma cell proliferation. (a) NB9 cells were cultured in the presence or absence of DFAT-conditioned medium (DFAT-CM), in combination with a PI3K inhibitor. Neuroblastoma cell proliferation was evaluated using WST-1 three days after culture. The proliferation of NB9 cells promoted by DFAT-CM was suppressed by the PI3K inhibitor at 10 μ M. Bar: mean \pm SE. Data shown for triplicate wells, * $p < 0.05$. (b) Figure showing the effect of human DFAT-CM in combination with a PI3K inhibitor on NB cell differentiation. NB9 cells were cultured in the presence or absence of DFAT-CM, in combination with a PI3K inhibitor for three days. NB9 cells were photographed using a phase-contrast light microscope after culture (scale bar, 100 μ m). (c) Neurite outgrowth of NB9 cells was enhanced by DFAT-CM, but the PI3K inhibitor did not affect neurite outgrowth. (d) Total RNA was extracted, and *TUB β 3* was quantified using real-time reverse transcription polymerase chain reaction. Relative expression was analyzed using the comparative CT method with *GAPDH* as the internal control. Expression of *TUB β 3* was increased by DFAT-CM and was further increased by the combined use of a PI3K inhibitor. Bar: mean \pm SE. Data shown for triplicate wells,

*p<0.05.

Fig 4 Image showing the results of Western blotting analysis for phospho-AKT expression in NB9 cells.

NB9 cells were cultured in the presence or absence of dedifferentiated fat cells-conditioned medium (DFAT-CM), in combination with a phosphatidylinositol 3-kinase (PI3K) inhibitor for 24 h. The cell protein extracts were then extracted and Western blotting analysis for phospho-AKT expression in NB9 cells was performed. DFAT-CM led phosphorylation of AKT in NB9 cells. The phosphorylation of AKT was inhibited in the presence of LY294002.

Figures

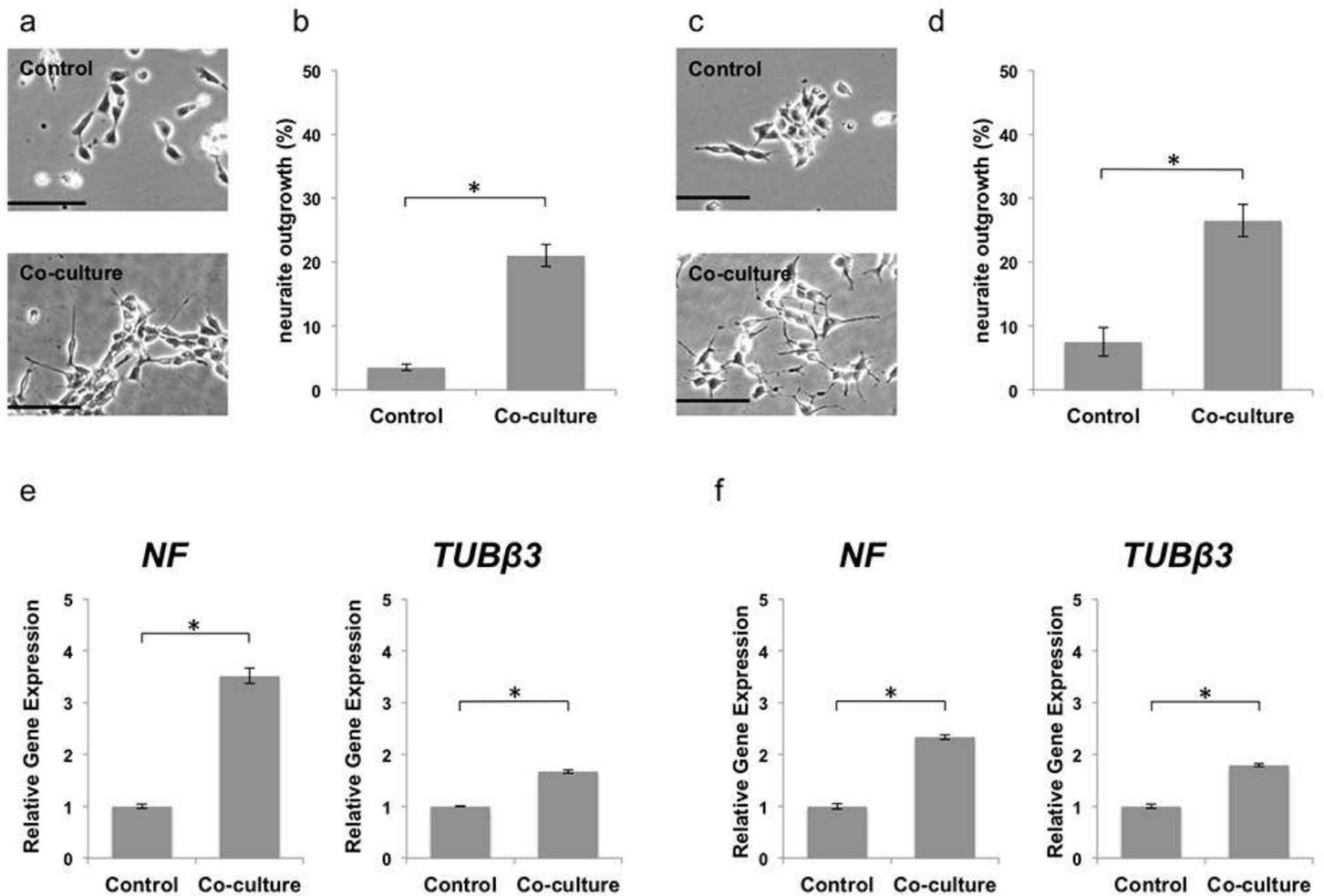


Figure 1

Figure showing the effect of human dedifferentiated fat cells (DFAT) on neuroblastoma cell differentiation. NB9 or SK-N-SH cells were cultured in the presence or absence of DFAT. (a) Photograph showing NB9 cells using phase-contrast light microscopy seven days after culture (scale bar, 100 μ m). (b) Neurite outgrowth was measured using National Institutes of Health ImageJ software. The neurite outgrowth of NB9 cells is enhanced by DFAT. (c) Photograph showing SK-N-SH cells using phase-contrast light microscope seven days after culture (scale bar, 100 μ m). (d) The neurite outgrowth of SK-N-SH cells is enhanced by DFAT. Bar: mean \pm SE. Data are shown for triplicate wells, * p <0.05. (e) Figure showing the expression analysis of neural differentiation markers in NB cells that were cultured in the presence or absence of DFAT for seven days. Total RNA was then extracted and NF and TUB β 3 were quantitated using real-time RT-PCR. Relative expression was analyzed using the comparative CT method with GAPDH as the internal control. The expression of NF and TUB β 3 in NB9 cells was increased by co-culturing with DFAT cells. (f) The expression of NF and TUB β 3 in SK-N-SH cells was increased by co-culturing with DFAT. Bar: mean \pm SE. Data are shown for triplicate wells, * p <0.05.

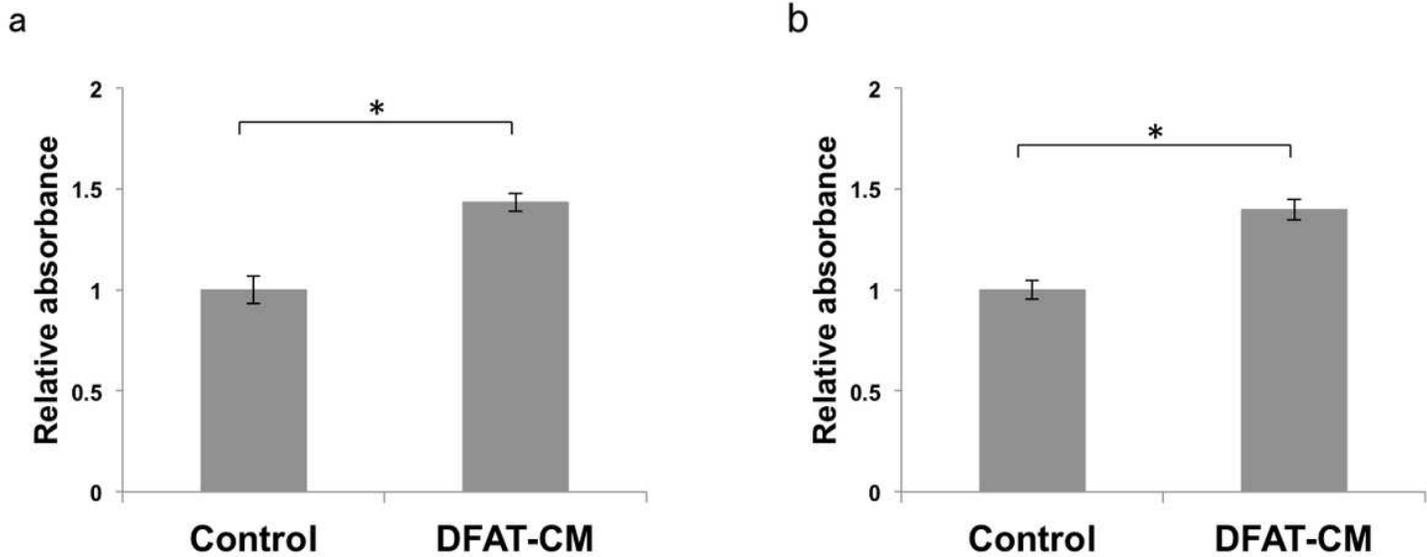


Figure 2

Figure showing the effect of human dedifferentiated fat cells (DFAT) on neuroblastoma (NB) cell proliferation. NB cells were cultured in the presence or absence of DFAT-conditioned medium (DFAT-CM). Neuroblastoma cell proliferation was evaluated using WST-1 three days after culture. (a) The proliferation of NB9 cells was enhanced by DFAT-CM. (b) The proliferation of SK-N-SH cells was enhanced by DFAT-CM. Bar: mean \pm SE. Data shown for triplicate wells, * $p < 0.05$.

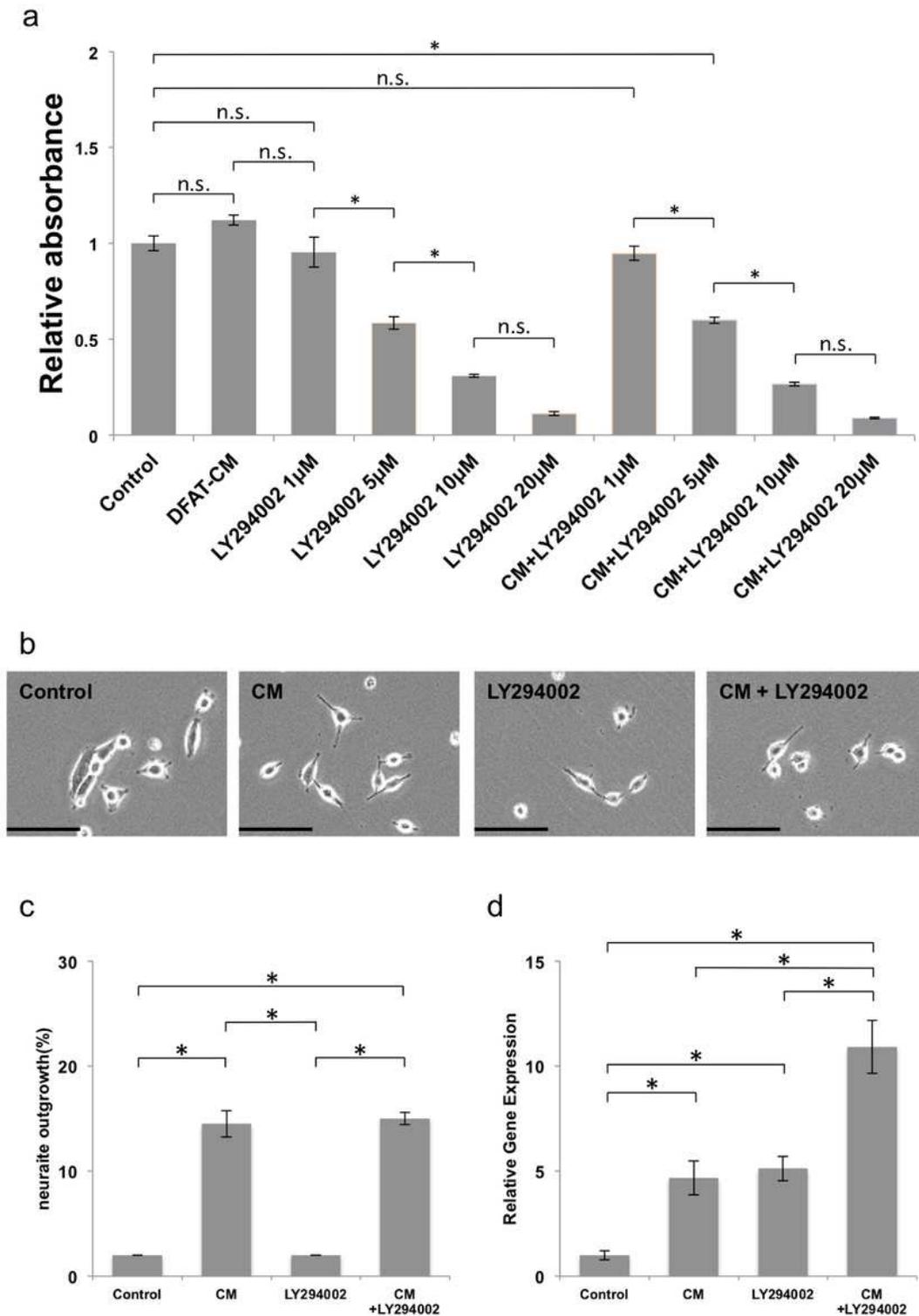


Figure 3

Figure showing the effect of human dedifferentiated fat cells (DFAT) in combination with a phosphatidylinositol 3-kinase (PI3K) inhibitor on neuroblastoma cell proliferation. (a) NB9 cells were cultured in the presence or absence of DFAT-conditioned medium (DFAT-CM), in combination with a PI3K inhibitor. Neuroblastoma cell proliferation was evaluated using WST-1 three days after culture. The proliferation of NB9 cells promoted by DFAT-CM was suppressed by the PI3K inhibitor at 10µM. Bar:

mean \pm SE. Data shown for triplicate wells, * $p < 0.05$. (b) Figure showing the effect of human DFAT-CM in combination with a PI3K inhibitor on NB cell differentiation. NB9 cells were cultured in the presence or absence of DFAT-CM, in combination with a PI3K inhibitor for three days. NB9 cells were photographed using a phase-contrast light microscope after culture (scale bar, 100 μm). (c) Neurite outgrowth of NB9 cells was enhanced by DFAT-CM, but the PI3K inhibitor did not affect neurite outgrowth. (d) Total RNA was extracted, and TUB β 3 was quantified using real-time reverse transcription polymerase chain reaction. Relative expression was analyzed using the comparative CT method with GAPDH as the internal control. Expression of TUB β 3 was increased by DFAT-CM and was further increased by the combined use of a PI3K inhibitor. Bar: mean \pm SE. Data shown for triplicate wells, * $p < 0.05$.

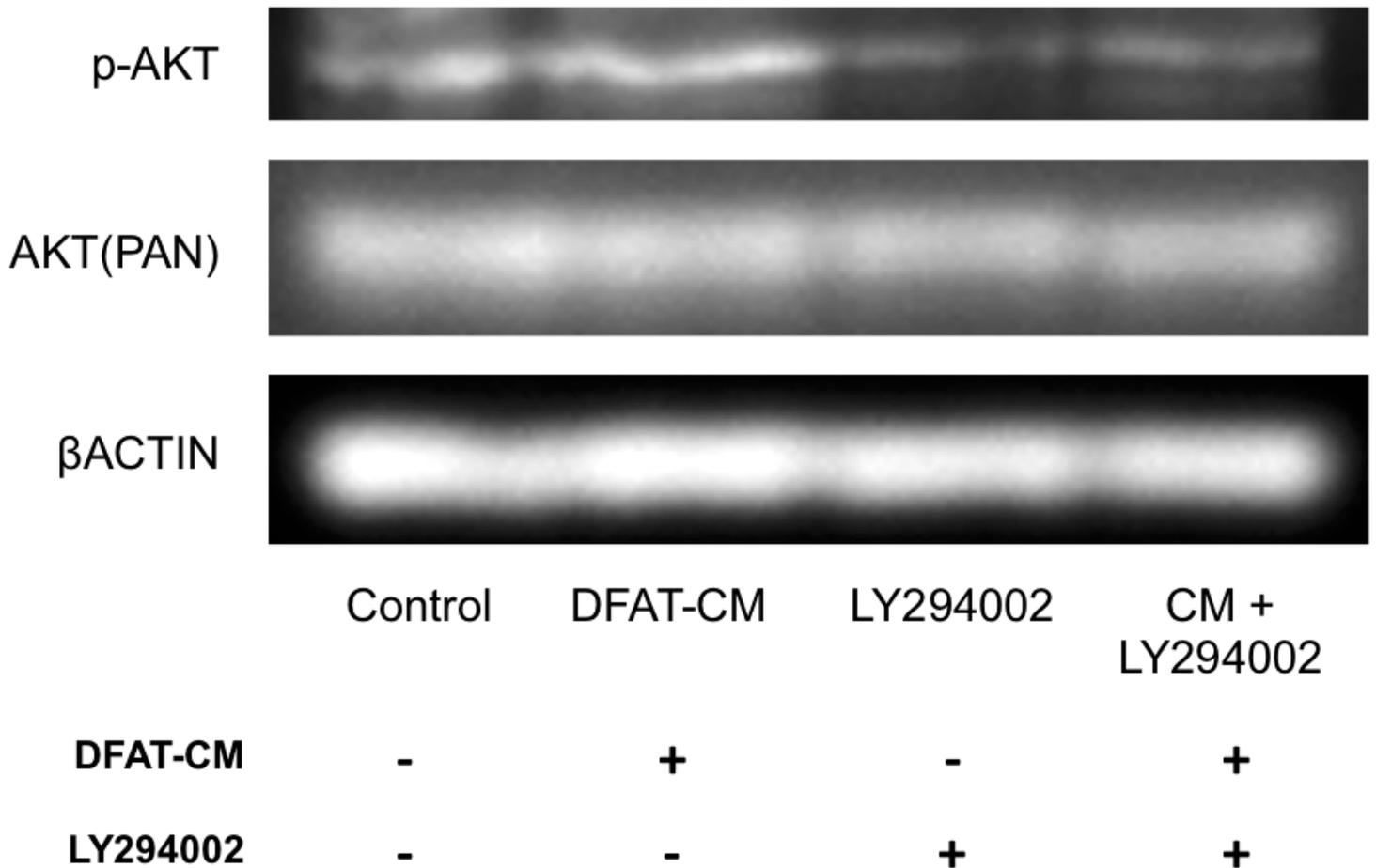


Figure 4

Image showing the results of Western blotting analysis for phospho-AKT expression in NB9 cells. NB9 cells were cultured in the presence or absence of dedifferentiated fat cells-conditioned medium (DFAT-CM), in combination with a phosphatidylinositol 3-kinase (PI3K) inhibitor for 24 h. The cell protein extracts were then extracted and Western blotting analysis for phospho-AKT expression in NB9 cells was performed. DFAT-CM led phosphorylation of AKT in NB9 cells. The phosphorylation of AKT was inhibited in the presence of LY294002.