Original Article

Increase of gremlin 2 with age in human adipose-derived stromal/stem cells and its inhibitory effect on adipogenesis

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ABSTRACT

Introduction: Adipose-derived stromal/stem cells (ASCs) have attracted attention as a promising material for regenerative medicine. Previously, we reported an age-related decrease in the adipogenic potential of ASCs from human subjects and found that the individual difference in this potential increased with age, although the mechanisms remain unclear. Recently, other groups demonstrated that a secreted antagonist of bone morphogenetic protein (BMP) signaling, Gremlin 2 (GREM2), inhibits the differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) into osteoblasts and the adipogenesis of 3T3-L1 cell. Here, we examined the effects of GREM2 on the differentiation of ASCs into adipocytes.
Methods: To examine changes in GREM2 expression levels with age, immunohistochemistry was performed on subcutaneous adipose tissues from subjects 12—97 years of age. Next, GREM2 gene expression levels in ASCs collected from subjects 5—90 years of age were examined by RT-PCR, and the change with age and correlation between the expression level and the adipogenic potential of ASCs were analyzed. In addition, to assess whether GREM2 affects adipogenesis, ASCs (purchased from a vendor) were cultured to induce adipogenesis with recombinant GREM2 protein, and siRNA-induced GREM2 knockdown experiment was also performed using aged ASCs.
Results: In adipose tissues, GREM2 expression was observed in cells, including ASCs, but not in mature adipocytes, and the expression level per cell increased with age. GREM2 expression levels in ASCs cultured in vitro also increased with age, and the individual differences in the level increased with age. Of note, partial correlation analysis controlled for age revealed that the adipogenic potential of ASCs and the GREM2 gene expression level were negatively correlated. Furthermore, based on a GREM2 addition experiment, GREM2 has inhibitory effects on the adipogenesis of ASCs through activation of Wnt/β-catenin signaling. On the other hand, GREM2 knockdown in aged ASCs promoted adipogenesis.
Conclusions: The GREM2 expression level was confirmed to play a role in the age-related decrease in adipogenic potential observed in ASCs isolated from adipose tissues as well as in the enhancement of the individual difference, which increased with age. GREM2 in adipose tissues increased with age, which suggested that GREM2 functions as an inhibitory factor of adipogenesis in ASCs.

1. Introduction

Gremlin 2 (GREM2), also called PRDC, is a secreted protein and a DAN family antagonist of bone morphogenetic protein (BMP) of the TGF-β family [1–6]. GREM2 was identified as a gene involved in development by gene trapping in mouse embryonic stem (ES) cells [7]. GREM2 expression is induced in cardiac progenitor cells during cardiogenesis in model organisms and was reported to play an important role in differentiation into cardiomyocytes [8–11]. The expression of GREM2 was also found in adult tissues, such as the ovaries, spleen, and brain, and the activity of BMP-responsive promoters was blocked by the binding of GREM2 to BMP-2 or BMP-4 [12,13]. Recently, GREM2 was reported to inhibit the differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) into osteoblasts as well as the adipogenesis of 3T3-L1 cells [9,10,14–17]. This suggests that GREM2 plays an important role in tissue homeostasis by promoting or inhibiting the differentiation of stem cells.

We have been investigating the differentiation potential of mesenchymal stem cells (MSCs) in body tissues. MSCs are stem cells known to have the potential to differentiate into mesenchymal cells, including osteoblasts, adipocytes, chondrocytes, and myocytes, and reside in tissues such as bone marrow, fat, muscle, skin, and umbilical blood. MSCs in such tissues have similar morphological characteristics and immunophenotypes [18–20]. Adipose-derived stromal/stem cells (ASCs), a type of MSC, can be isolated from adipose tissues by treating with collagenase followed by centrifugation, and they include a high percentage of stem cells. Therefore, ASCs have attracted attention as a promising material for regenerative medicine [20–22]. We isolated ASCs from subcutaneous adipose tissues from 260 subjects (5—97 years of age), and analyzed their proliferation potential and differentiation potential into adipocytes, osteoblasts, and chondrocytes, revealing that the differentiation potential into any type of cell varied from individual to individual, and that the difference among individuals increased with age. In particular, the adipogenic potential decreased with age [23]. The decrease in the adipogenic potential of ASCs with age has been also reported by other groups [24–27], but the mechanism is not understood. Here, we hypothesized that the age-related decrease in adipogenic potential and the increase in individual differences in this potential are caused by GREM2, and thus examined the age-related change in GREM2 expression in adipose tissues. We also investigated the effects of GREM2 on the differentiation of ASCs into adipocytes.

2. Materials and methods

2.1. Collection of adipose tissues and ASCs

Human adipose tissues were obtained from patients who had skin surgery. Normal subcutaneous adipose tissues were trimmed out from surgically excised tissues. Prior to surgery, informed consent was obtained from each patient. A total of 100 specimens of subcutaneous adipose tissue were collected from subjects (5—97 years of age). Among them, immunohistochemistry was performed on 36 specimens (12—97 years of age; median age, 66 years; 17 men) which were derived from body parts including abdomen (16/36), crus (7/36), back (5/36), buttock (3/36), chest (3/36), and upper arm (2/36). The rest 64 specimens (5—90 years of age; median age, 63 years; 24 men) were used to isolate ASCs, and the adipogenic potential of ASCs was tested at passage 5 [23]. The adipogenic potential of ASCs, donor age, sex, and body part from which adipose tissue was collected are listed in Supplementary Table S1. This study was conducted after approved by the Research and Ethics Committee of Fujita Health University School of Medicine (approval No. HM17-223).

2.2. Histological analysis

Subcutaneous adipose tissues were fixed in 4% PFA and embedded in paraffin, and then 4-μm thick sections were prepared. HE staining (Dako North America, CA, USA) was performed in a conventional way, and the samples were observed using a phase contrast microscope. For immunohistochemistry, Proteinase K treatment (S3004, Dako) was performed in a 1:50 dilution to activate antigens. Then, sections were incubated with GREM2 antibodies (GeneTex, CA, USA; in a 1:150 dilution) followed by secondary staining with Alexa Fluor™ 594 donkey anti-rabbit IgG (H+L) (Invitrogen, NY, USA; in a 1:500 dilution) and DAPI. All slides were observed for GREM2 expression with a fluorescence microscope (DMI 6000B-AFC, LAS X ver.1. x; Leica Microsystems, Wetzlar, DEU). For analysis of immunohistochemically images, integrated fluorescent intensity of GREM2 expression signal per unit area (200 μm × 200 μm) was calculated with MetaMorph (Molecular Devices, CA, USA), and the DAPI-stained nuclei observed among mature adipocytes in each unit area were counted for the number of cells, which include ASCs. For each specimen, 9 areas were analyzed, and the mean was plotted and used for the following statistical analysis.

2.3. Cell culture

ASCs collected from adipose tissues [23] and human adipose derived stem cells (ADSCs; from a Hispanic female 34 years of age; cell strain No. 01171; KURABO, Osaka, JPN) were cultured in complete medium (D/α medium), which consists of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and α minimum essential medium (α MEM, Invitrogen) with a 1:1 ratio, supplemented with 1% fetal bovine serum (Sigma—Aldrich, MO, USA), 1 × ITS-X (Invitrogen), 10 ng/mL basic FGF (PeproTech, NJ, USA), 0.4 μg/mL hydrocortisone, and 1% Antibiotic-Antimycotic (Gibco BRL, MD, USA) at 37 °C in a humidified atmosphere with 5% CO_2. To induce adipogenesis, confluent ADSCs were cultured for 8 days with medium changed every 2—3 days in the following differentiation medium: DMEM with 10% fetal bovine serum, 33 μM Biotin, 10 μg/mL Insulin, 1 μM dexamethasone, 0.5 mM 3-isobuthl-1-methylxanthine, 0.2 mM indomethacin, and 1% Antibiotic-Antimycotic (Gibco). DMEM and all other ingredients unless otherwise indicated were purchased from Sigma—Aldrich. To examine the effect of GREM2 on differentiation, human recombinant PRDC/GREM2 Protein (NOVUS Biologicals, CO, USA) was added to differentiation medium. Evaluation of adipogenesis by staining with Oil Red O was conducted as previously described [23].

2.4. siRNA transfection

Stealth siRNAs against GREM2, siRNA1(HSS127558) and siRNA2(HSS184951), and a non-silencing negative control (NC) siRNA were purchased from Invitrogen. ASCs from aged subjects (#48 and #54 in Table S1), in which GREM2 expression level was high were cultured until subconfluent and transfected with siRNA. Solutions of 100 nM siRNAs in OPTIMEM (Gibco) were prepared and used for transfection of ASCs with Lipofectamine RNAiMAX (Invitrogen) at 37 °C for 4 h in a humidified atmosphere with 5% CO2. Cells were cultured in adipocyte differentiation medium for four days and total RNA was collected (n = 3 independent experiments for each condition).

Table 1
Primers used for real-time quantitative RT-PCR.

| Gene	Forward sequence	Reverse sequence
GREM2	AAGGCAGAGGGAGAGGGAGA	CACCAGGAACAAGGACAGGGA
PPARG	GAACGACCAAGTAACTCTCCTCAAAT	TCTTTATTCATCAAGGAGGCCAGCATT
ADIPOQ	AGGCCGTGATGGCAGAGAT	TCACCGATGTCTCCCTTAGGA
CEBPA	GGGTCTGAGACTCCCTTTCCTT	CTCATTGGTCCCCCAGGAT
LEF1	CAGGAGCCCTACCACGACAA	GCCTCCATCTGGATGCTTTC
TCF7L2	ATGAAATGGCCACTGCTTGAT	GCATCCTTGAGGGCTTGTCTA
DKK2	AAGGAGACCCCTGCCTACGAT	CAGAAATGACGAGCACAGCAA
GAPDH	TGCACCACCAACTGCTTAGC	TCTTCTGGGTGGCAGTGATG

2.5. Gene expression analysis

Total RNA was purified from confluent cells with TRI REAGENT®(Molecular Research Center, OH, USA) in a conventional way. One μg of RNA was reverse transcribed into cDNA with PrimeScript TM RT Master Mix (Takara Bio, Shiga, JPN). Quantitative real-time PCR was performed using THUNDERBIRD® SYBR®qPCR Mix (TOYOBO, Osaka, JPN) with a StepOnePlus (Life Technologies Japan, Tokyo, JPN). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for normalization, and the
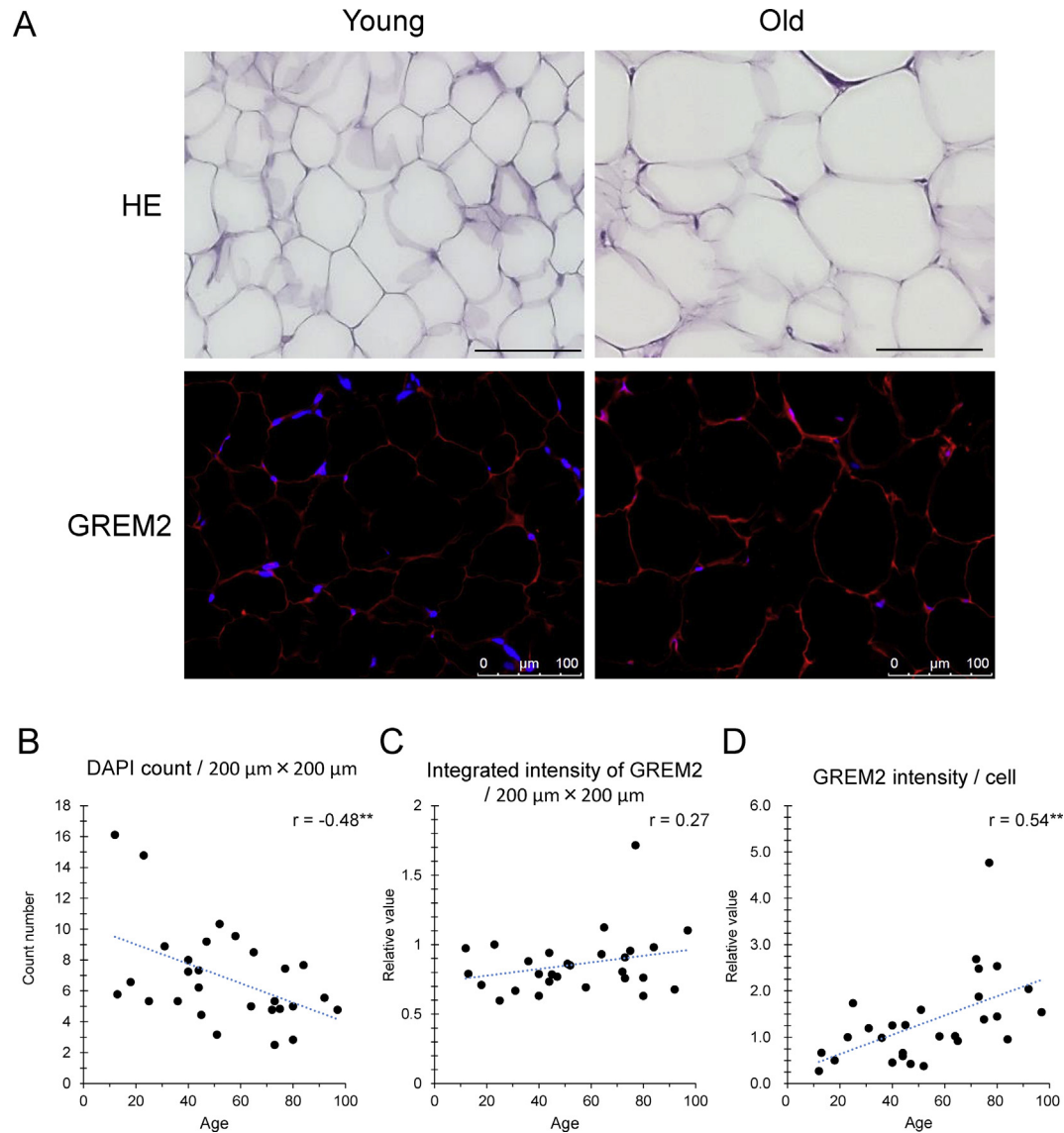
Fig. 1. GREM2 expression in young and old adipose tissues. Immunohistochemistry was performed on subcutaneous adipose tissues obtained from 36 subjects 12—97 years of age (A) Representative HE staining images of adipose tissues (upper panel) and immunofluorescence images against GREM2 (lower). Young, adipose tissue from the back of a 23-year-old women; old, from the lumbar region of a 69-year-old men. Bars = 100 μm (B) The average numbers of cells stained with DAPI except mature adipocytes in immunofluorescence images of subcutaneous adipose tissue areas (200 μm × 200 μm) were plotted (C) Integrated fluorescent intensities of GREM2 were calculated for each area. The value for the young sample shown in (A) derived from a 23-year-old subject was set as 1, and the relative values of GREM2 integrated fluorescent intensities were plotted (D) GREM2 integrated fluorescent intensities adjusted by cell number were plotted. For each, Pearson's product—moment correlation analysis (a parametric method) was performed to assess the degree of relationship. **$p < 0.01$.

A

GREM2 mRNA expression

ρ = 0.62***

B

Change in standard deviation

r = 0.89***
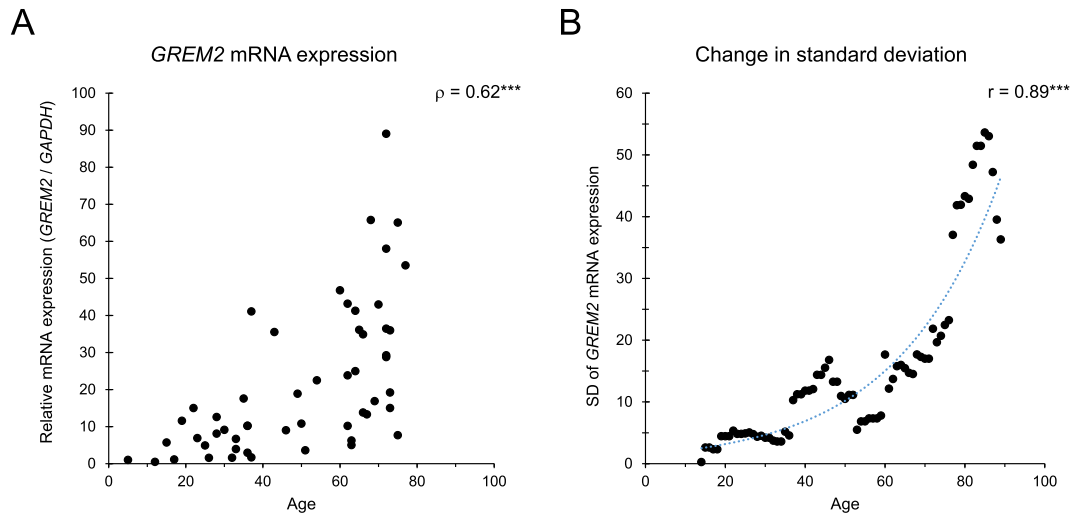
Fig. 2. Effect of donor age on *GREM2* mRNA expression in ASCs. (A) *GREM2* gene expression level was analyzed in ASCs from 64 donors (shown in Supplementary Table S1) 5–90 years of age. The expression levels were adjusted using *GAPDH* as an internal control, and the relative values when the value of strain ID #1 (from a 5-year-old subject) was set as 1, were plotted against donor age (B) At each donor age ± 5 years (i.e., in the range of 10 years). standard deviations of *GREM2* expression level were calculated and plotted. The individual difference in *GREM2* gene expression level exponentially increased as the age increased. ***$p < 0.001$.

relative differences among samples were analyzed with a ΔΔ*Ct* method. The primers used for GREM2, PPARG, ADIPOQ, CEBPA, LEF1, TCF7L2, DKK2 and GAPDH are listed in Table 1.

2.6. Statistical analysis

All statistical analyses were performed by R program (version 3.1.1, R Development Core Team 2012). The mean values were used as results, and error bars represent standard deviation. Unless otherwise stated, correlation analysis was performed by Spearman's rank correlation analysis for non-normally distributed data and by

r = -0.43***

Fig. 3. Correlation between *GREM2* gene expression level in ASCs and adipogenic potential. Regression residuals against donor age of log-transformed adipogenesis value and those of log-transformed *GREM2* expression level in ASCs were calculated and plotted on the x and y axis, respectively. Pearson's product−moment correlation coefficient r was -0.43 (***$p < 0.001$).

Pearson's product moment correlation analysis for normally distributed data. Statistical significances were tested employing t-test. A p value of <0.05 was considered statistically significant.
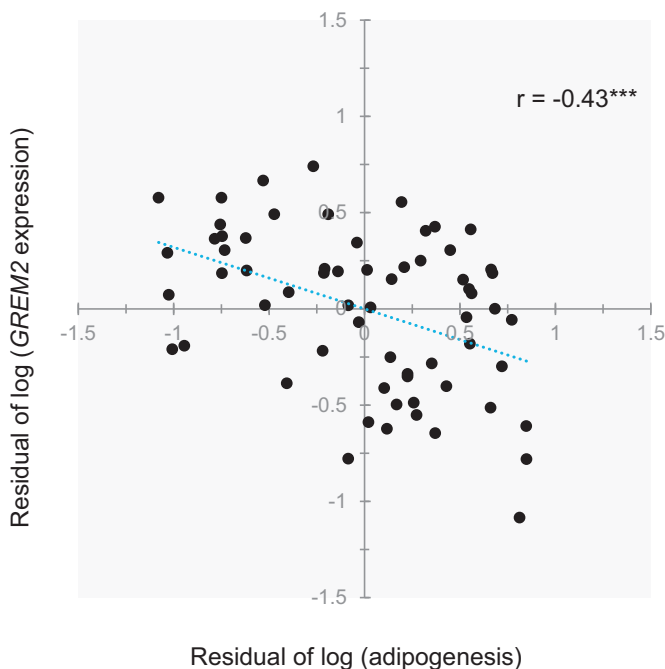
3. Results

3.1. GREM2 increased with age in human subcutaneous adipose tissues

To assess the difference in human subcutaneous adipose tissues between young and old subjects, sections were prepared from tissues of 36 subjects 12−97 years of age for histological observation. The adipose tissues from elderly subjects contained many enlarged mature adipocytes (hypertrophic adipocytes) across the tissues, compared with those from young subjects (HE in Fig. 1A). Immunohistochemistry against GREM2 revealed GREM2 expression in cells, including ASCs, located among mature adipocytes in both young and old tissues (GREM2 in Fig. 1A). The number of DAPI-stained cells per unit area (200 μm × 200 μm) in each sample was counted and the number of cells significantly decreased with age (r = -0.48, p < 0.01; Fig. 1B). On the other hand, no notable changes with age were found on comparison of the integrated fluorescence intensity of GREM2 signals in the same area (r = 0.27; Fig. 1C). Therefore, the GREM2 expression level per cell was considered to significantly increase with age (r = 0.54, p < 0.01; Fig. 1D) in human adipose tissues.

3.2. GREM2 expression level increased with age in ASCs

To examine if ASCs express GREM2 and its expression level changes with age, total mRNA was collected from ASCs (at passage 5) obtained from 64 donors 5−90 years of age. The expression level of GREM2 gene in ASCs from the youngest donor (#1 in Table S1) was used as a standard, and relative GREM2 expression levels in ASCs from each donor were plotted (Fig. 2A). At younger ages, GREM2 expression levels were low, and as the age increased, there were more ASCs in which GREM2 was highly expressed and the individual differences in GREM2 expression level gradually increased. As the individual differences were not equally distributed at different ages, the correlation between GREM2 gene expression and age was examined using Spearman's

rank correlation coefficient (non-parametric method). The value of the coefficient rho (ρ) was 0.62 (p < 0.001), indicating a significant correlation. To examine the change in the individual difference of the GREM2 gene expression level with age, standard deviations of relative GREM2 expression values (Fig. 2A) at each age ±5 years were calculated and plotted (Fig. 2B). This confirmed that the individual difference in GREM2 expression exponentially increased with age. Therefore, ASCs express GREM2, and its expression level and the individual difference in expression level increase with age.

3.3. The GREM2 expression level was correlated with the adipogenic potential of ASCs

To statistically analyze the correlation between the *GREM2* expression and the adipogenic potential in ASCs at passage 5, a partial correlation was calculated between the log-transformed GREM2 gene expression level and log-transformed adipogenesis value controlling for age (Fig. 3). As a result, the GREM2 gene expression level and adipogenic potential of ASCs were significantly negatively correlated (r = -0.43, p < 0.001).

3.4. GREM2 inhibited adipogenesis of human ADSCs

To examine the effects of GREM2 on ADSCs, which are MSCs originating from adipose tissues, commercially available ADSCs were used to induce differentiation into adipocytes, and GREM2 was added to the culture to assess its effects on differentiation. In the presence of GREM2, neutral fat stained with Oil Red O was observed less than in the absence of GREM2 (Fig. 4A). In addition, the amount of neutral fat per cell number (adjusted using CCK-8; DOJINDO Laboratories, Kumamoto, JPN) significantly decreased in a GREM2 dose-dependent manner (Fig. 4B). In addition, the gene expression of two adipogenic markers, PPARG and ADIPOQ, was significantly suppressed when GREM2 was added to the culture (Fig. 4C and D). These findings suggested that GREM2 inhibited the adipogenesis of ADSCs.

Next, since GREM2 was known to function via Wnt/β-catenin signaling [16], we examined if GREM2 affects the expressions of the downstream genes of the signaling pathway. The expressions of *CEBPA* [28,29] and *LEF1* [30,31] genes, both of which are known to be down-regulated by the activation of Wnt/β-catenin signaling, were decreased in the presence of GREM2 (Fig. 4E and F), while *TCFL2*
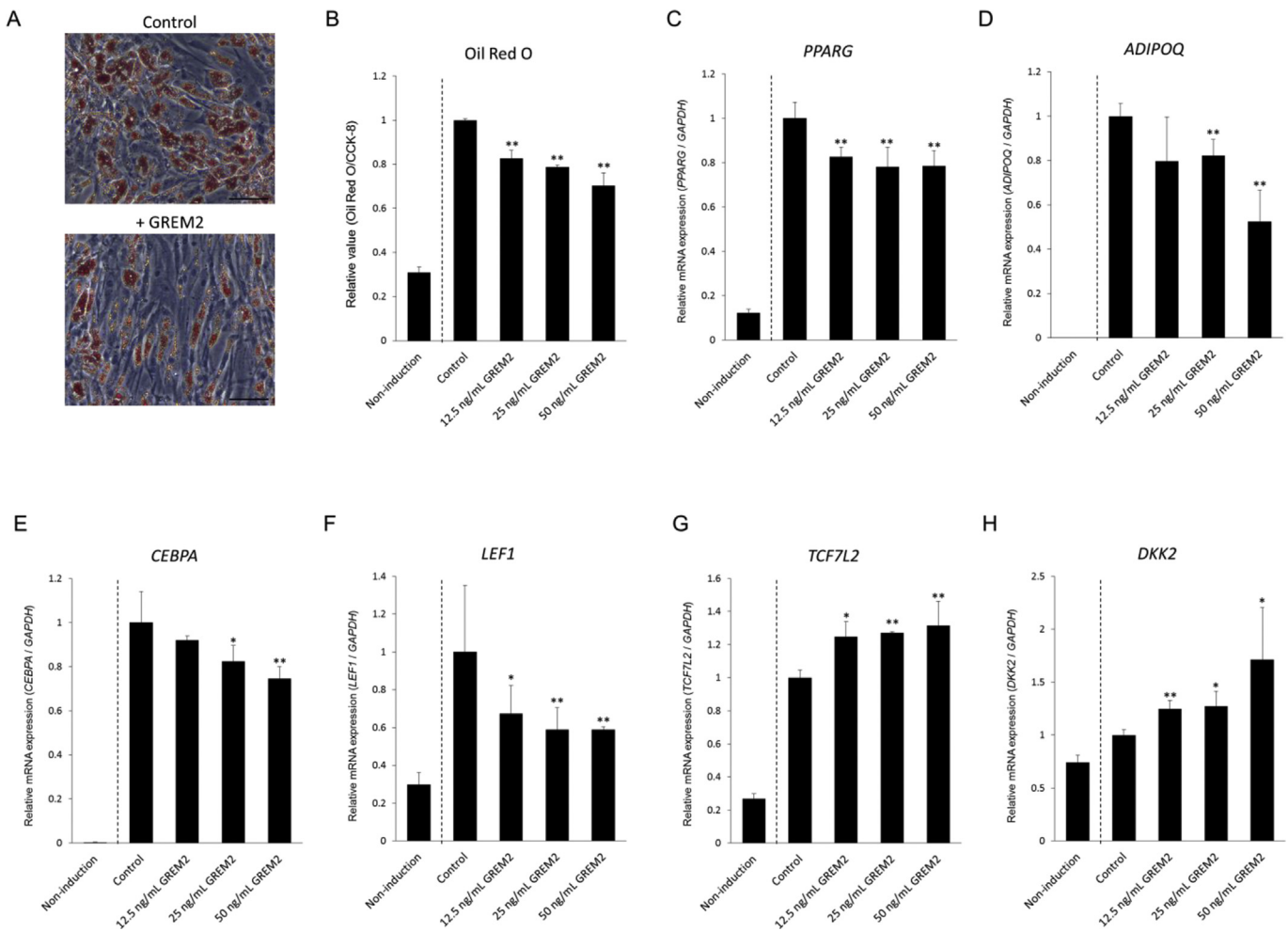
Fig. 4. Inhibitory effect of GREM2 on adipogenesis of ADSCs. ADSCs purchased from KURABO were cultured to induce adipogenesis with or without human recombinant GREM2 (A) Images of cells stained with Oil Red O after adipogenesis induction. Oil Red O-stained neutral fat was less observed in the cultured cells with GREM 2 at 50 ng/mL (+GREM2), compared with that in cells cultured without GREM2 (Control) (B) To evaluate adipogenesis, Oil Red O was dissolved out from stained cells. The absorbance of the eluate was measured at 490 nm and adjusted by cell number using CCK-8. The value of cells cultured without GREM2 was set as 1 (Control). **p < 0.01 (C−H) Total mRNA was collected from cultured cells after adipogenesis induction, and the expression levels of an adipogenic transcription factor gene *PPARG* (C) and of a lipogenic gene *ADIPOQ* (D) as well as those of the genes affected by Wnt/β-catenin signaling, *CEBPA* (E), *LEF1* (F), *TCF7L1* (G) and *DKK2* (H) were analyzed. *GAPDH* was used as an internal control of gene expression level. The relative mRNA expression levels to the value when culturing without GREM2 (Control), set as 1, were compared. **p < 0.01, *p < 0.05.
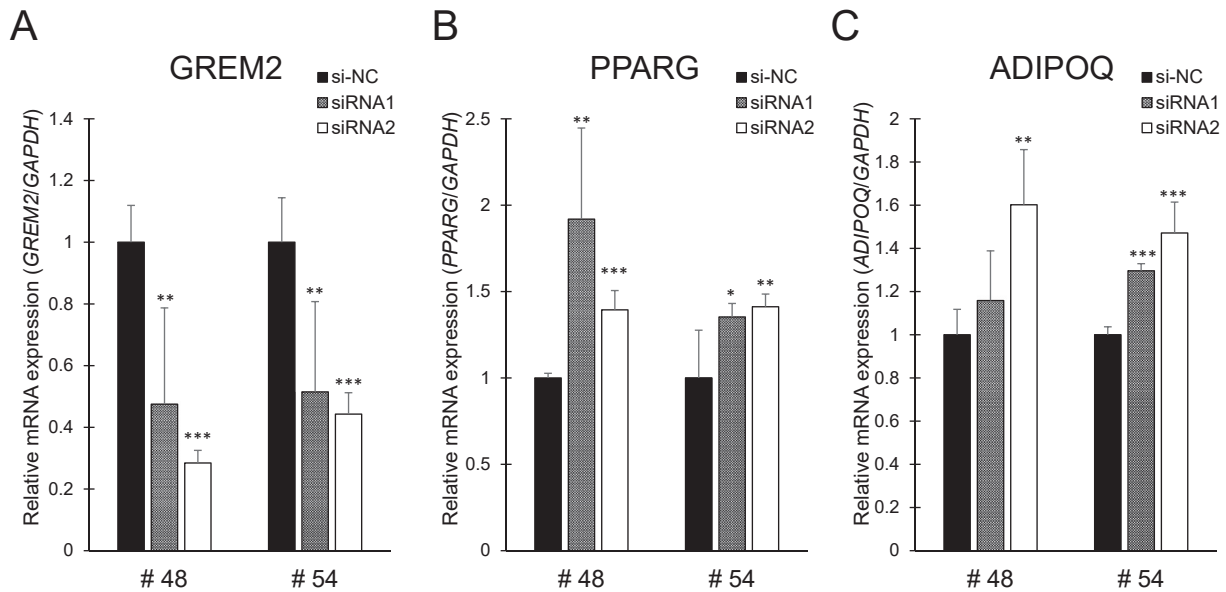
Fig. 5. siRNA-induced GREM2 knockdown promotes adipogenesis. ASCs from aged subjects (#48 and #54 in Table S1), which had a low adipogenic potential, were transfected with siRNA against GREM2 (siRNA1 and siRNA2) or a negative control siRNA (si-NC) (A–C) The ASCs were cultured in adipogenic medium for four days and total RNA was collected. The gene expression level of *GREM2* (A) and of adipogenic markers, *PPARG* (B) and *ADIPOQ* (C) were examined. *GAPDH* was used as an internal control of gene expression level. The relative value of the expression level of each gene in the presence of GREM2 siRNA to the expression level in the presence of si-NC (set as 1) was plotted. The difference between the gene expressions in the presence of GREM2 siRNA and those in the presence of si-NC was analyzed. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

[28,29] and *DKK2* [31,32] genes known to be up-regulated by Wnt/β-catenin signaling were found to be increased when GREM2 was added (Fig. 4G and H). These findings suggested that GREM2 inhibits the adipogenesis of ADSCs through Wnt/β-catenin signaling.

3.5. GREM2 knockdown restored adipogenesis in human aged ASCs

We next assessed if age-related increase in GREM2 expression is involved in a lower adipogenic potential of ASCs. ASCs from aged subjects (#48 and #54 in Table S1), which highly expressed GREM2 were selected, and these cells were transfected with GREM2 siRNA, then cultured in adipogenic medium for four days. We observed that GREM2 gene expression was down-regulated in the presence of GREM2 siRNA compared with when NC siRNA was treated (Fig. 5A). In aged ASCs in which GREM2 was knocked down, the expressions of adipogenic markers, PPARG and ADIPOQ, were increased (Fig. 5B and C). These results suggested that in aged ASCs with high GREM2 expression levels the adipogenic potential is low due to the inhibitory effect of GREM2 on adipogenesis.

4. Discussion

GREM2 was reported to be highly expressed in and around the developing heart in model organisms, and it is required for proper heart differentiation and atrium development [8,10,33]. Recently, GREM2 was found to inhibit the differentiation of BMSCs into osteoblasts [15,17]. However, the detailed expression pattern of GREM2 in human adipose tissues and the effects of GREM2 on ADSCs remained unknown. In our previous report, the analysis of the differentiation potential of ASCs obtained from subjects 5–97 years of age into three different lineages revealed that the individual differences in differentiation potential increased and the overall adipogenic potential decreased with age [23]. Here, we presented a novel role of GREM2 in human adipose tissues. Immunohistological analysis revealed that GREM2 expression per cell increased with age (Fig. 1D). In addition,

GREM2 expression increased with age in ASCs isolated from adipose tissues (Fig. 2A). Furthermore, we demonstrated that the GREM2 expression level is negatively correlated with the adipogenic potential of ASCs (Fig. 3). From these findings, we hypothesized that the age-related increase in GREM2 plays a role in the reduction of the adipogenenic potential of ASCs. We evaluated the effects of GREM2 on the adipogenic potential of ADSCs in vitro as well as on some signaling molecules that might be relevant to their adipogenesis and found that GREM2 significantly suppressed adipogenesis through Wnt/β-catenin signaling (Fig. 4), We also found that the expression of adipogenic marker genes were increased in aged ASCs in which GREM2 was knocked down compared with a negative control, suggesting that increase in GREM2 expression is involved in age-related decrease in the adipogenic potentials of ASCs.

In addition, GREM2 expression levels in the adipose tissues from 11 subjects were correlated with the expression levels of the gene in the corresponding ASCs (at passage 5) isolated and cultured from those tissues (Fig. S1). In adipose tissues, epigenetic changes which affect GREM2 expression may occur in ASCs with age, and the level of such changes might produce the individual difference in the differentiation potentials of ASCs. GREM2 was found to be involved in suppressing the differentiation of stem cells in adipose tissues, and further investigation will be needed to clarify the mechanism how GREM2 expression is increased with age. Currently, ASCs are being used as materials for regenerative therapy, and the differentiation potential of the stem cells used has a significant impact on the treatment efficacy. Elucidation of the causes of the age-related increases in GREM2 gene expression and the mechanism by which GREM2 regulates differentiation may provide insights into regenerative medicine, anti-aging, and anti-obesity research, but further investigations are needed.

Declaration of competing interest

All authors declare no conflict of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2019.09.002.

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