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Involvement of tazarotene-induced gene 1 in proliferation and differentiation of human adipose tissue-derived mesenchymal stem cells

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Abstract

Objective: Mesenchymal stem cells (MSC) have both self-renewal and multilineage differentiation potential, and bone marrow-derived MSC have been applied for tissue regeneration and repair. Although adipose tissue-derived MSC (ASC) have emerged as an alternative cell source, little information is available regarding the biologic difference between ASC derived from visceral and subcutaneous fat. Therefore, we aimed to compare the proliferation and gene expression profile of cultured human visceral ASC (VASC) and subcutaneous ASC (SASC), and to identify a novel gene involved in proliferation and differentiation of ASC.

Materials and methods: We performed microarray analysis of cultured VASC and SASC, and investigated the role of tazarotene-induced gene 1 (TIG1), a most differentially expressed gene, in the proliferation and differentiation of ASC.

Results: SASC proliferated faster than VASC for over 10 passages, and TIG1 expression was consistently up-regulated in VASC of humans, rats and mice. Overexpression of the TIG1 gene in human SASC inhibited cell proliferation, whereas knockdown of TIG1 expression by siRNA promoted cell proliferation. In addition, overexpression of the TIG1 gene in SASC enhanced their differentiation into adipocytes, and promoted up-regulation of peroxisome proliferators-activated receptor γ and CCAAT/enhancer binding protein α. On the other hand, TIG1 overexpression in SASC inhibited their differentiation into osteocytes and the expression of osteocalcin.

Conclusion: TIG1 plays an important role in regulating proliferation and differentiation of ASC.

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Introduction

Mesenchymal stem cells (MSC) reside within bone marrow, fat and many other tissues, and can differentiate into various types of cells such as adipocytes, osteoblasts, chondrocytes, neurones, skeletal muscle cells, endothelial cells and vascular smooth muscle cells (1,2). MSC can be easily isolated from bone marrow and rapidly expanded *in vitro*, and thus these features make MSC an attractive therapeutic tool for tissue regeneration and repair (1,2). However, because an invasive procedure is required to obtain bone marrow cells, adipose tissue-derived MSC (ASC) have emerged as an alternative source of MSC (3).

Adipose tissue-derived MSC can be obtained from visceral and subcutaneous fat tissue; however, little information is available regarding biologic differences between MSC obtained from each tissue. In addition, the molecular mechanisms that explain the differences in cell proliferation and differentiation between visceral ASC (VASC) and subcutaneous ASC (SASC) remain unknown.

Accordingly, we compared the gene expression profile of human VASC and SASC, and identified tazarotene-induced gene 1 (TIG1) as a most up-regulated gene in VASC. Although TIG1 has been recognized as a tumour suppressor gene (4,5), its role in adipose tissue and ASC is not known. Therefore, we investigated the role of TIG1 in ASC proliferation and differentiation.

Materials and methods

Isolation and expansion of ASC

Subcutaneous and omental fat (< 2 g) were isolated from male patients undergoing open surgery for the treatment of aortic aneurysm ($n = 3$, Table 1), and male Lewis rats (200–250 g) and male C57/BL6 mice (25–30 g). Fat tissue was minced with scissors, treated with 2 mg/ml type II

Table 1. Patients' characteristics

Patient no.	Age	Sex	Body weight (kg)	Body mass index (kg/m^2)
1	74	Male	61	25.1
2	78	Male	59	19.7
3	77	Male	70	20.3

collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 1 h, and filtered with a nylon mesh (Netwell, Costar, Cambridge, MA, USA). Cells were then centrifuged at 200 ***g***, resuspended with α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), and plated onto plastic dishes and incubated at 37 °C under 5% CO_2. Five days after plating, non-adherent cells were removed, and adherent cells were further propagated. Surface antigen expression of human VASC and SASC was verified as positive for CD29, CD44, CD90 and HLA-ABC, and negative for CD14, CD31, CD34, CD45, CD117 and HLA-DR, characteristic of MSC (data not shown) (6). The experimental protocols were approved by the ethical committee and by the animal care committee of the National Cardiovascular Center. All of the patients gave written informed consent.

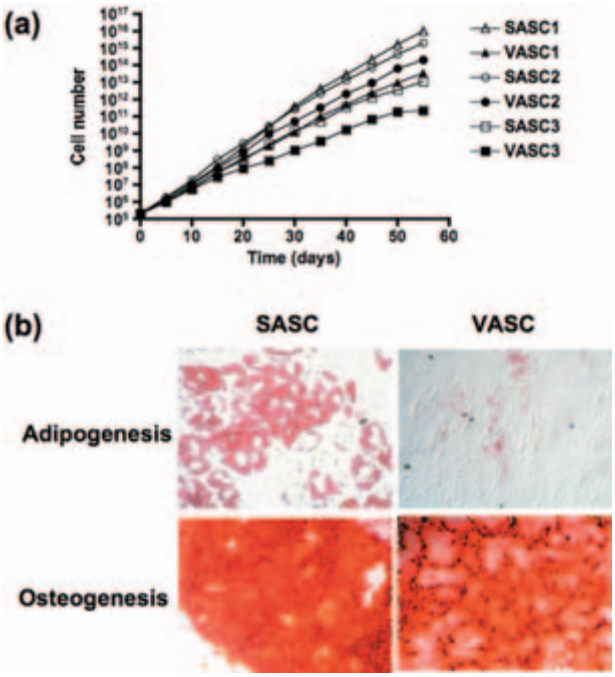

Figure 1. Characterization of human ASC. Growth kinetics of cultured human VASC and SASC obtained from three patients. Differentiation of human VASC and SASC into adipocytes and osteocytes. Adipocytes were stained with Oil Red O, whereas osteocytes were stained with Alizarin Red S. Scale bars = 50 µm.

Microarray analysis

Microarray analysis was performed as described previously (7). Briefly, total RNA was extracted from cultured human VASC and SASC using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified by spectrometry and the quality confirmed by gel electrophoresis. Double-stranded cDNA was synthesized from 6 µg total RNA, and *in vitro* transcription was performed to produce biotin-labelled cRNA using GeneChip One-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. After fragmentation, 10 µg cRNA was hybridized with GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) containing over 47 000 transcripts. GeneChips were then scanned in a GeneChip Scanner 3000 (Affymetrix). Normalization and filtering of the data were performed with GeneSpring GX 7.3.1 software (Agilent Technologies, Palo Alto, CA, USA). The raw data from each array were normalized as follows; each CEL file was preprocessed with RMA, and each measurement for each gene was divided by the 80th percentile of all measurements. Genes with an at least 10-fold change on average in two patients (patients 1 and 2) were then selected.

Quantitative real-time reverse transcription–polymerase chain reaction

Quantitative real-time reverse transcription–polymerase chain reaction was performed as described previously (7). Briefly, PCR amplification was performed in 50 µl containing 1 µl cDNA and 25 µl Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The oligonucleotides used in qRT-PCR analysis were purchased from Qiagen (QuantiTect Primer Assay). β-Actin (ACTB, for human) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, for rat and mouse) mRNA amplified from the same samples served as an internal control. After an initial denaturation at 95 °C for 10 min, a two-step cycle procedure was used (denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min) for 40 cycles in a 7700 sequence detector (Applied Biosystems). Gene expression levels were normalized according to that of the internal control. The data were analysed with Sequence Detection Systems software (Applied Biosystems).

Plasmids and siRNAs

The open reading frame of human TIG1 in pENTR221 vector was purchased from Invitrogen, and cloned into

Table 2. Differentially expressed genes in human VASC vs. SASC (> 10-fold)

Accession no.	Gene name	Fold change
Highly expressed genes in VASC		
AI669229	Tazarotene induced gene 1 (TIG1)	59.5
NM_002402	Mesoderm specific transcript (MEST)	33.9
NM_000900	Matrix Gla protein (MGP)	33.0
NM_000204	Complement factor I (CFI)	30.9
AW089415	Secreted frizzled-related protein 4 (SFRP4)	23.5
AF063591	CD200 antigen (CD200)	23.2
NM_025226	Regulator of G-protein signalling 5 (RGS5)	19.9
NM_005824	Leucine rich repeat containing 17 (LRRC17)	19.1
AK026415	Chimerin 2 (CHN2)	19.0
NM_003326	Tumour necrosis factor superfamily, member 4 (TNFSF4)	18.5
BF982174	Serum deprivation response (SDPR)	18.2
NM_024426	Wilms tumour 1 (WT1)	15.9
NM_002202	ISL LIM homeobox 1 (ISL1)	11.8
Highly expressed genes in SASC		
AI433463	Membrane metallo-endopeptidase (CD10)	86.2
U41813	Homeobox A9 (HOXA9)	30.8
NM_016588	Neuritin 1 (NRN1)	28.5
NM_017409	Homeobox C10 (HOXC10)	26.6
NM_003956	Cholesterol 25-hydroxylase (CH25H)	25.5
U90304	Iroquois homeobox 5 (IRX5)	22.9
AI478455	Empty spiracles homolog 2 (EMX2)	19.6
AI928035	Iroquois homeobox protein 2 (IRX2)	17.3
NM_005584	Mab-21-like 1 (MAB21L1)	15.3
NM_001999	Fibrillin 2 (FBN2)	14.4
NM_001884	Hyaluronan and proteoglycan link protein 1 (HAPLN1)	12.0
AI681917	Iroquois homeobox protein 3 (IRX3)	11.3
AF311912	Secreted frizzled-related protein 2 (SFRP2)	10.7
BF792917	Homeo boxA10 (HOXA10)	10.5
AF056085	G protein-coupled receptor 51 (GPR51)	10.4
AI345957	Leucine rich repeat and fibronectin type III domain containing 1 (LRFN1)	10.4

a destination vector pcDNA6.2/GFP-DEST (Invitrogen), according to the manufacturer's instructions. pcDNA6.2/GFP-GW/p64^{TAG} (Invitrogen) was used as a control vector. The plasmids were amplified by LB culture, and purified with an EndoFree Plasmid Maxi Kit (Qiagen). The siRNA targeting human TIG1 (siTIG1, NM_002888) was purchased from Qiagen, and Negative Control siRNA (Qiagen) was used as a control (siControl).

In vitro *transfection*

Transfection of plasmids and siRNAs into ASC was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Briefly, cells were plated 24 h before transfection, and plasmids or siRNAs were mixed with Lipofectamine 2000 in serum-free medium and incubated at room temperature for 20 min. The mixture was transferred to the culture plate, and incubated at 37 °C under 5% CO_2 for 24 h. Culture medium was changed to complete culture medium, and replaced every 2–3 days.

Cell proliferation assay

For cell proliferation assay, 2×10^3 cells were plated onto 96-well plates, and the cellular level of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), indicative of the mitochondrial function of living cells and cell viability, was measured with a CellTiter96 AQueous One Solution Kit (Promega, Madison, WI, USA) and a Microplate Reader (490 nm, Bio-Rad, Hercules, CA, USA), 5 days after *in vitro* transfection.

Adipogenic and osteogenic differentiation

To induce differentiation into adipocytes, cells were cultured with adipocyte differentiation medium: 0.5 mM 3-isobutyl-1-methylxanthine (Wako Pure Chemical Industries, Osaka, Japan), 1 μM dexamethasone (Wako Pure Chemical Industries), 50 μM indomethacin (Wako Pure Chemical Industries) and 10 μg/ml insulin (Sigma-Aldrich) in α-MEM. After 14 days of differentiation, cells were stained with Oil Red O (Sigma-Aldrich). For quantitative analysis

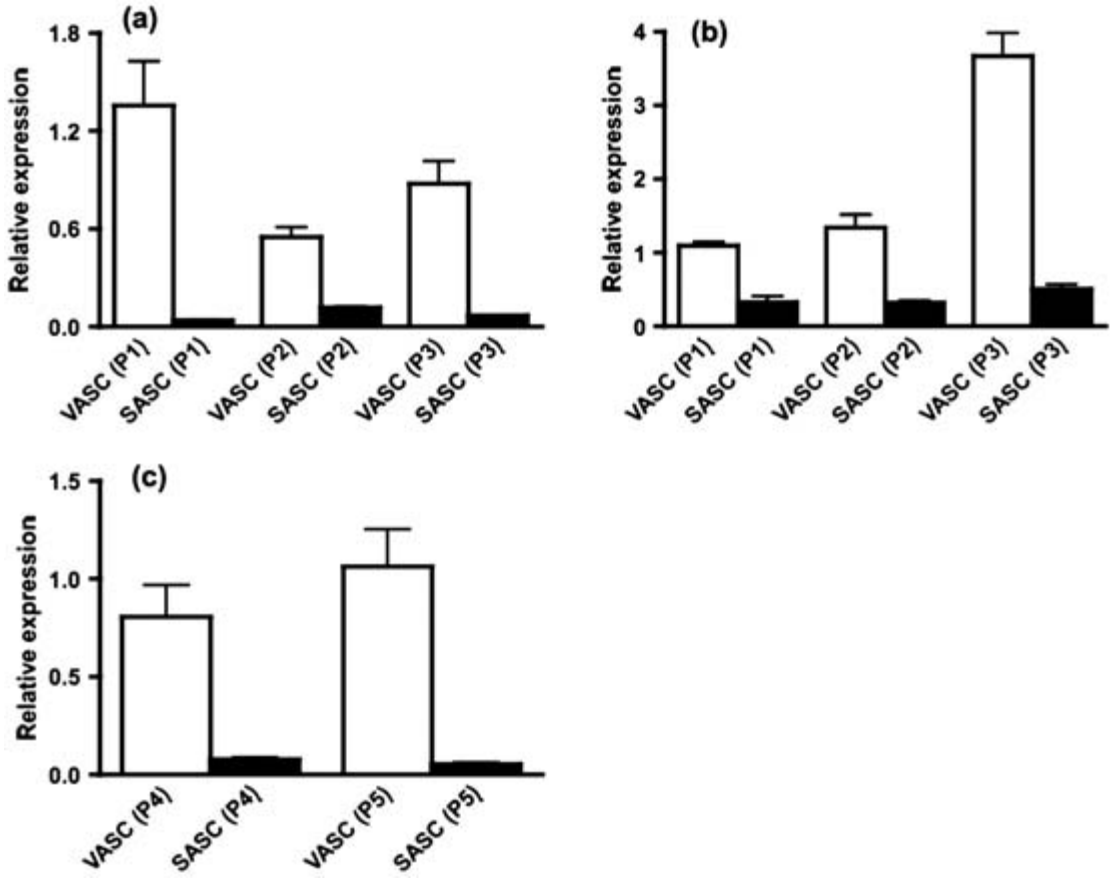

Figure 2. Tazarotene-induced gene 1 gene expression in VASC and SASC obtained from humans, rats and mice. Relative expression of TIG1 in cultured human VASC and SASC at passage 1 (P1) through passage 3 (P3). Relative expression of TIG1 in cultured rat VASC and SASC at P1 through P3. Relative expression of TIG1 in cultured mouse VASC and SASC at P4 and P5.

of adipogenesis, cells were stained with 10 μg/ml BODIPY 493/503 (Invitrogen) and 10 μM Hoechst33342 (Invitrogen), and visualized with an IN Cell Analyser (GE Healthcare, Piscataway, NJ, USA). Lipid area (green fluorescence) was measured and divided by the number of cells (blue fluorescence) in the same field (×200, 20 fields), using Multi Target Analysis software (GE Healthcare).

To induce differentiation into osteocytes, cells were cultured in α-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma, Osaka, Japan), according to the manufacturer's instructions. After 21 days of differentiation, cells were stained with Alizarin Red S (Sigma-Aldrich).

Statistical analysis

All data were expressed as mean ± standard error (SE). Comparisons of parameters among groups were made by one-way ANOVA, followed by Newman–Keuls' test. Comparisons of parameters between two groups were made by Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Proliferation and differentiation of cultured human ASC

We investigated proliferation and differentiation of human VASC and SASC obtained from three patients. In all cases, SASC proliferated more rapidly than VASC for over 10 passages (Fig. 1a). VASC and SASC differentiated into adipocytes and osteocytes, verifying the multipotency of these cells (Fig. 1b).

Differentially expressed genes in VASC vs. SASC

Of over 47 000 transcripts analysed, 13 genes including TIG1 and mesoderm specific transcript (MEST) were highly expressed in VASC more than tenfold, whereas 16

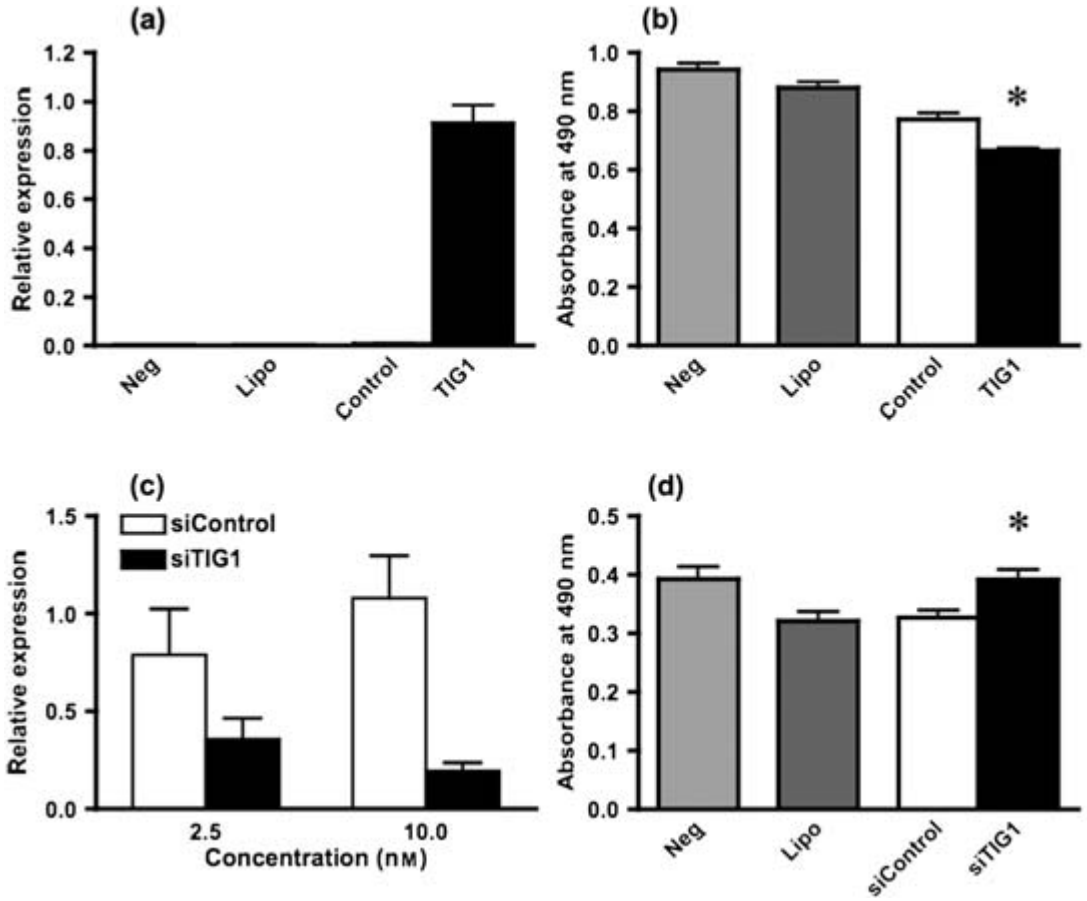

Figure 3. Effect of TIG1 gene expression on ASC proliferation. Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) for TIG1 mRNA after transfection of TIG1 plasmid into human SASC. Neg, cells without lipofection; Lipo, cells with lipofection alone; control, cells transfected with control plasmid by lipofection; TIG1, cells transfected with TIG1 plasmid by lipofection. MTS assay after transfection of TIG1 plasmid into human SASC and 5 days of culture. $*P < 0.05$ vs. control plasmid. qRT-PCR for TIG1 mRNA after transfection of siTIG1 into human VASC. MTS assay after transfection of siTIG1 into human VASC and 5 days of culture. Neg, cells without lipofection; Lipo, cells with lipofection alone; siControl, cells transfected with control siRNA by lipofection; siTIG1, cells transfected with siTIG1 by lipofection. $*P < 0.05$ vs. siControl.

genes including CD10 antigen and homeobox A9 (HOXA9) were highly expressed in SASC (> 10-fold, Table 2). Because TIG1 was the most highly up-regulated gene in VASC, we focused on TIG1 for further analysis.

Expression of TIG1 gene in VASC and SASC obtained from various species

To verify the expression of TIG1, qRT-PCR was performed using total RNAs separately obtained from cultured human ASC from human (Fig. 2a), rat (Fig. 2b) and mouse (Fig. 2c) fat tissue at various passages (P1–5). The results showed that TIG1 expression was markedly and consistently higher in VASC than in SASC in all species and at all passages examined.

Effect of TIG1 gene overexpression on ASC proliferation

To investigate the role of TIG1 in ASC proliferation, plasmid DNA containing the human TIG1 gene was transfected into cultured human SASC, in which TIG1 was not expressed. Transfection of TIG1 plasmid resulted in overexpression of TIG mRNA, as determined by qRT-PCR (Fig. 3a). MTS assay demonstrated that the viable cell number 5 days after transfection was significantly lower when TIG1 was overexpressed, as compared to the control vector (Fig. 3b). On the contrary, transfection of siTIG1 efficiently down-regulated the expression of TIG1 as determined by qRT-PCR (Fig. 3c), and MTS assay demonstrated that the viable cell number was significantly higher when siTIG1 was transfected (Fig. 3d). These results suggest that TIG1 regulates proliferation of cultured ASC.

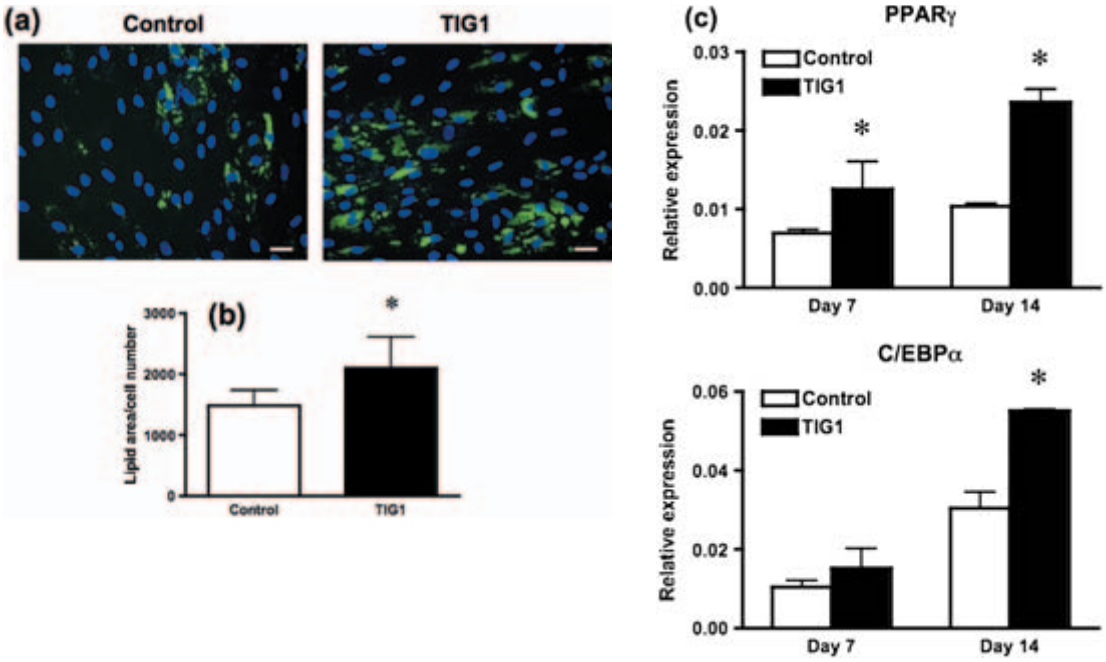

Figure 4. Effect of TIG1 gene overexpression on ASC differentiation into adipocytes. Adipocyte differentiation of human SASC after overexpression of TIG1, followed by induction of adipogenesis for 14 days. Cells were stained with BODIPY 493/503 (green) and DAPI (blue). Scale bars = 50 μm. Quantitative analysis of (a). Lipid area was divided by the number of cells in the same field. *$P < 0.05$ vs. control plasmid. qRT-PCR for PPARγ (upper) and C/EBPα (lower) after TIG1 gene overexpression, followed by induction of adipogenesis for 7 and 14 days. *$P < 0.05$ vs. control plasmid.

Effect of TIG1 gene overexpression on ASC differentiation into adipocytes

To examine the effect of TIG1 overexpression on cell differentiation into adipocytes, TIG1 plasmid was transfected into human SASC, and cells were induced to differentiate into adipocytes for 14 days. Lipid area after induction of adipogenesis was significantly higher than control (Fig. 4a,b), and the expression of peroxisome proliferators-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα) was significantly enhanced 14 days after induction of adipogenesis (Fig. 4c). These results suggest that TIG1 promotes ASC differentiation into adipocytes.

Effect of TIG1 gene overexpression on ASC differentiation into osteoocytes

Finally, human SASC were induced to differentiate into osteocytes after TIG1 overexpression. After 21 days of osteogenic differentiation, calcium deposition was lower than control (Fig. 5a), and the expression of osteocalcin mRNA was significantly lower (Fig. 5b). These results suggest that TIG1 inhibits differentiation of ASC into osteocytes.

Discussion

In this study, we compared the proliferation and gene expression of cultured human VASC and SASC, and showed that (i) human SASC proliferated faster than VASC, and (ii) TIG1 expression was most highly up-regulated in VASC. We also demonstrated that TIG1 (i) regulated proliferation of ASC (ii) promoted differentiation of ASC into adipocytes, and (iii) inhibited differentiation of ASC into osteocytes.

Consistent with our observations, it has been demonstrated that SASC proliferated at a higher rate than VASC (8); however, only the proliferation of primary culture for 7 days was observed, and not serial passages. In the present study, we verified that SASC proliferated faster than VASC for over 10 passages in all three patients examined. Therefore, in view of cell quantity and invasiveness, SASC may be more beneficial as a cell source for tissue regeneration and repair.

In microarray analysis, several of the up-regulated genes in VASC or SASC have been demonstrated to be involved in cell proliferation and differentiation. For instance, MEST, the second most highly up-regulated gene in VASC, has been reported to be markedly up-regulated in adipose tissue of obese mice, and transgenic

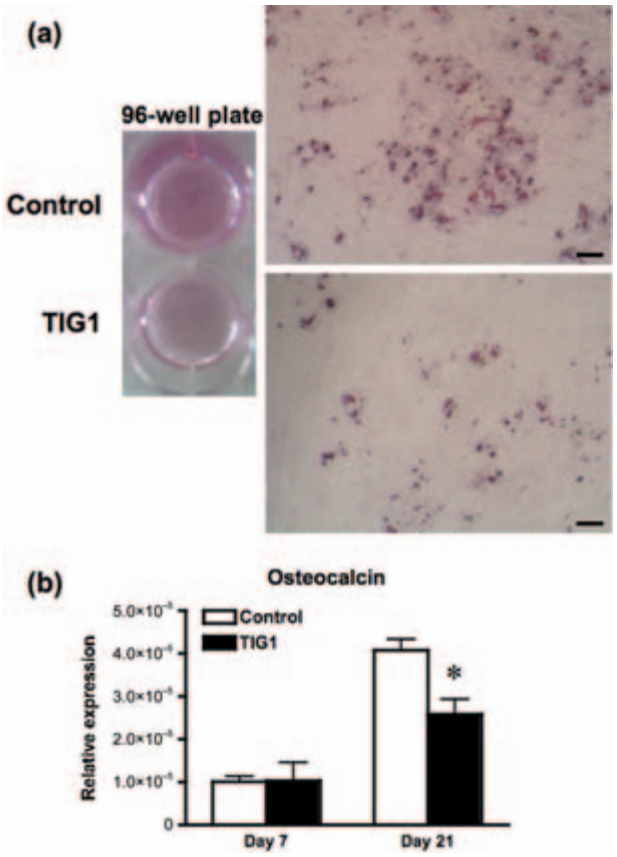

Figure 5. Effect of TIG1 gene overexpression on ASC differentiation into osteocytes. Osteocyte differentiation of human SASC after overexpression of TIG1, followed by induction of osteogenesis for 21 days. Cells were stained with Alizarin Red S. Scale bars = 50 μm. qRT-PCR for osteocalcin after overexpression of TIG1, followed by induction of osteogenesis for 7 and 21 days. *$P < 0.05$ vs. control plasmid.

overexpression of MEST in adipose tissue resulted in enlargement of adipocytes (9,10). In contrast, the highly expressed genes in SASC included several homeobox genes such as HOXA9, HOXA10, HOXC10, IRX2, IRX3 and IRX5. Homeobox genes encode transcription factors that play essential roles in controlling cell growth and differentiation (11), and it has been recently demonstrated that HOXA9, HOXA10 and HOXC9 were down-regulated in human omental ASC (12), which is consistent with our observations. Furthermore, secreted frizzled-related protein 4 (SFRP4) was highly up-regulated in SASC, whereas SFRP2 was highly expressed in VASC. SFRPs are decoy receptors for the Wnt signalling pathway (13), and SFRP4 has been shown to inhibit proliferation of prostate cancer cells (14,15), whereas SFRP2 has been demonstrated to play a major role in mediating the survival signal of MSC overexpressing Akt, an antiapoptotic gene (16). Taking these findings together, the difference in proliferation between VASC and SASC may be explained in part by these factors, although the precise mechanism remains to be elucidated.

TIG1 is one of the genes induced by tazarotene, a synthetic retinoid that binds retinoic acid receptor β (RARβ) and RARγ (17); however, its role in RAR-mediated biology is not known. Putative TIG protein appears to be a transmembrane protein with a small N-terminal intracellular region, a single membrane-spanning hydrophobic region, and a large C-terminal extracellular region containing a glycosylation signal (17,18), thus, TIG1 may function as an adhesion molecule. In fact, overexpression of TIG1 in prostate cancer cell line resulted in increased cell-cell contact *in vitro* and reduced tumorigenicity *in vivo* (18). In addition, it has been reported that silencing of the TIG1 promoter by hypermethylation is common in human cancers (4,5,19–21). These findings suggest TIG1 as a potential tumour suppressor; however, the role of TIG1 in adipose tissue and ASC is not known. In the present study, TIG1 was highly expressed in VASC, and proliferation of ASC was regulated by modulation of TIG1 expression. Moreover, our differentiation study demonstrated that TIG1 promotes differentiation of ASC into adipocytes, but inhibits their differentiation into osteocytes. Although involvement of the key transcription factors for MSC differentiation into osteocytes and adipocytes, such as Runx2 (22,23) and PPARγ (24,25), respectively, has been well-established, whether TIG1 associates with these molecules should be further analysed. It is possible that these transcription factors regulate TIG1 expression; however, this is under investigation.

In summary, TIG1, which is highly expressed in VASC, modulates ASC proliferation and differentiation, and these findings may provide information on biological aspects relating to the difference in cell proliferation and differentiation between VASC and SASC.

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