Biochemical and Biophysical Research Communications 434 (2013) 534–540

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Vascular smooth muscle cells isolated from adipose triglyceride lipase-deficient mice exhibit distinct phenotype and phenotypic plasticity

Yanhui Lin [a], Shunmei Chiba [a], Akira Suzuki [b], Satoshi Yamaguchi [b], Takaya Nakanishi [a], Hirofumi Matsumoto [a], Yoshihiko Ikeda [c], Hatsue Ishibashi-Ueda [c], Ken-ichi Hirano [b], Seiya Kato [a,*]

[a] Department of Pathology and Cell Biology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan
[b] Laboratory of Cardiovascular Disease, Novel, Non-invasive and Nutritional Therapeutics (CNT), Osaka University, 6-2-3 Furuedai, Suita, Osaka 565-0872, Japan
[c] Department of Pathology, National Cerebral and Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan

ARTICLE INFO

Article history:
Received 28 February 2013
Available online 10 April 2013

Keywords:
Vascular smooth muscle cells
Phenotype
Adipose triglyceride lipase
Triglyceride-deposit cardiomyovasculopathy
Atherosclerosis

ABSTRACT

The alteration of triglyceride (TG) metabolism in vascular smooth muscle cells (SMC) is likely to be correlated with certain phenotype, though this has not been elucidated. Adipose triglyceride lipase (ATGL) exerts major TG catalytic activity in both adipotic and non-adipotic cells. In the present study, we isolated SMC from ATGL-deficient mice (ATGL$^{-/-}$mSMC). ATGL$^{-/-}$mSMC showed spontaneous TG accumulation with lower mitogenic response and smooth muscle actin (SMA) expression compared to ATGL $^{+/+}$mSMC. Percentage of senescence-associated β-galactosidase positive cells was also increased in ATGL$^{-/-}$mSMC. Real-time PCR followed by screening with focused DNA array analysis revealed up-regulated expression of glucokinase (1.7-fold), lipoprotein lipase (3.8-fold) and interleukin-6 (3.7-fold) and down-regulated expression of vascular endothelial growth factor-A (0.2-fold), type I collagen (0.5-fold), and transforming growth factor-β (0.4-fold) in ATGL$^{-/-}$mSMC compared to ATGL$^{+/+}$mSMC. Next, ectopic gene transfer of human ATGL was attempted using doxycycline (Dox)-regulatable myc-DDK-tagged adenovirus vector (AdvATGL). AdvATGL infection resulted in a reduction of TG accumulation with elevated mitogenic response and SMA expression, and decreased in senescent cell numbers in ATGL$^{-/-}$mSMC. Moreover, deviated gene expression pattern in ATGL$^{-/-}$mSMC was potentially corrected. Our data suggest that ATGL$^{-/-}$mSMC have a distinct phenotype that may be related to vascular pathogenesis. Plasticity of SMC phenotypes correlated to lipid metabolism could be a therapeutic target.

1. Introduction

Vascular insults induce phenotypic modulation of vascular smooth muscle cells (SMC), which further mediates myofibroblastic proliferation and vascular remodeling [1,2]. Classically, the phenotypic modulation of SMC has been understood as their switching from a contractile (quiescent) phenotype to a proliferative (synthetic) phenotype, such conversion is typically seen in atherosclerotic neointimal lesions [3]. Recently, SMC have been discovered to exhibit more diverse phenotypes, including inflammatory [4] and osteogenic [5] phenotypes, in vascular pathogenesis. Phenotypic plasticity is potentially regulated by transcriptional activation of intracellular signaling molecules [6], but the determinants of SMC phenotype and their regulatory mechanisms are still unclear. Improper regulation of cholesterol metabolism is deeply involved in atherogenesis [1,2], and SMC exposed to low-density lipoprotein acquire a pro-thrombotic phenotype [7]. In addition, it has been reported that foam cells located in the atheromatous plaque originate from macrophages and SMC, and that SMC have a great capacity to hydrolyze triglyceride (TG) [8]. Thus the alteration of TG metabolism in SMC is likely to be correlated with certain phenotype, though this has not been elucidated to date.

Hormone-sensitive lipase (HSL), which was first purified in the 1980s, was initially believed to be responsible for the major lipolytic activity related to TG [9]. More recently, however, the studies using HSL-null mice have revealed preserved TG lipase activity in these mice, anticipating the presence of alternative lipase [9]. Another enzyme, known as adipose triglyceride lipase (ATGL) discovered in 2004 [10], functions in the degradation of lipid droplets, not only in adipocytes but also in non-adipotic cells [11]. ATGL-deficient (ATGL$^{-/-}$) mice suffer from a fatal cardiomyopathy with massive TG accumulation in their cardiomyocytes [12]. Moreover, mutation of the ATGL gene causes human myopathy (neutral lipid storage disease) [13] and triglyceride-deposit cardiomyovasculopathy (TGCV) [14,15]. As patients with TGCV suffer from severe atherosclerosis [14,15], it could be anticipated that TG metabolism mediated by ATGL is involved in the regulation of vascular cell phenotype and atherogenesis.

* Corresponding author. Fax: +81 98 895 1407.
E-mail address: seikato@med.u-ryukyu.ac.jp (S. Kato).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.03.109

In the present study, we investigated phenotypic characteristics of SMC isolated from ATGL$^{-/-}$ mice. We found that ATGL$^{-/-}$ SMC have a distinct phenotype that is associated with alterations of specific gene expressions related to vascular function, and that these can be potentially corrected by an ectopic transfer of wild-type ATGL.

2. Materials and methods

2.1. Reagents

SMC growing media (SG2, containing 5% FBS, 0.5 ng/ml of EGF, 2 ng/ml of bFGF, and 5 μg/ml of insulin) and differentiation media (SD, containing 1% FBS and 30 μg/ml of heparin) were purchased from Kurabo (Osaka, Japan). Nile red and neutral buffered formalin were purchased from Wako (Osaka, Japan). Saponin, epinephrine, oleic acid and fatty acid–free bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO). Oleic acid was initially dissolved in pure ethanol and 5 mM of stock solution was prepared by further dissolving into 10% BSA/PBS, of which aliquots were sealed with nitrogen gas. Doxycycline (Dox) was purchased from Clontech (Mountain View, CA).

2.2. SMC culture from ATGL$^{-/-}$ mice

ATGL$^{-/-}$ mice were a generous gift from Prof. Rudolf Zechner (Institute of Molecular Biosciences, University of Graz, Austria) [12]. ATGL$^{-/-}$ and wild-type (ATGL$^{+/+}$) littermates were generated by breeding heterozygous mice. SMC were isolated from thoracic aortas of 8-week old male ATGL$^{+/+}$ mice (ATGL$^{+/+}$mSMC) and ATGL$^{-/-}$ mice (ATGL$^{-/-}$mSMC) by the explant method [16]. Less than 8 passages of cells were used in the present study. Animal handling and experiments were conducted according to the guidelines of the Directive 2010/63/EU of the European Parliament and the Council of the European Union. The animal experimental procedures were also approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

2.3. Assessments of lipid accumulation and lipase activity

Intracellular neutral lipid was stained with Nile red. Cells were fixed with 10% neutral buffered formalin for 10 min followed by permeabilization with 0.05% saponin for 30 min at room temperature. After several washes with PBS, cells were stained with 1 μg/ml of Nile red in PBS (1:1000 dilution of 1 mg/ml of stock solution prepared with DMSO). Nile red fluorescence was observed using an Olympus fluorescent microscope (Tokyo, Japan). Cellular TG content was measured using a Triglyceride Quantification Kit (K622-100, BioVision, Milpitas, CA). For the measurement of lipase activity, cells were lysed in cold PBS by sonication and lipase activity of whole cell extracts was measured using a QuantiChrom™ lipase assay kit (BioAssay Systems, Hayward, CA) according to the dimercaptopropanol tributyrate method [17].

2.4. Assays for proliferation and proportion of senescent cells

Viable cell number was measured by means of a WST-1 assay (Roche, Indianapolis, IN). Senescence-associated β-galactosidase (SA-β-Gal) activity was detected with a commercially available kit (Sigma–Aldrich).

2.5. Focused DNA array and real-time PCR

Total RNA was isolated with Trizol (Life Technologies, Carlsbad, CA). Focused DNA array was performed using a Genopal™ metabo-Tip system (Kurabo) which contains 195 genes related to major metabolic pathways, proteasomes, oxidative stress, cytokines, intracellular signaling, inflammation, angiogenesis and circadian rhythm, according to the information provided by the manufacturer. Alterations of specific gene expression were further tested by real-time PCR. Extracted RNA (1 μg) was exposed to PrimeScript II reverse transcriptase (Takara, Otsu, Japan) in a total volume of 20 μl. One microliter of RT reaction mixture was subjected to real-time PCR using Fast SYBR-Green fluorescence dye and a StepOne real-time PCR system (Applied Biosystems, Carlsbad, CA). Complementary DNAs were amplified with AmpliTaq Gold PCR Master Mix (Life Technologies). Amplification reactions were performed in duplicate and fluorescence curves were analyzed with included software. All PCR results were normalized for the expression of β-actin. PCR primer sets used in the present study are listed in Supplementary Table 1.

2.6. Recombinant adenoviruses

Full-length cDNA of human ATGL with myc-DDK tag (Origene, Rockville, MD) was subcloned into the recombinant adeovirus vector (AdvATGL) using an Adeno-X Tet-On System (Clontech) according to the manufacturer's instructions. AD293 cells (Agilent Technologies, Santa Clara, CA) were used as a packaging cell line and a ViraBind™ adenovirus purification kit (Cell Biolabs, San Diego, CA) was used for the amplification of the viruses. The adenovirus expressing bacterial β-galactosidase (AdvLZ) or Dox negative condition was used for the negative control. Titer was determined by means of a conventional plaque assay using a Noble Agar (Difco, Detroit, MI).

2.7. Western blotting

Western blotting was performed as previously described [18]. The membranes were probed with a 1:200 dilution of a rabbit polyclonal anti-ATGL (Cell Signaling Technology, Danvers, MA) or 1:1000 dilution of mouse monoclonal anti-Myc (9E10, Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with peroxidase-conjugated secondary antibodies. The proteins were subsequently developed using ImmunoStar LD reagents (Wako) and visualized with a luminescent imager (Ez-Capture, ATTO, Tokyo, Japan). Alternatively, some blots were incubated with Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA) and re-probed with a 1:2000 dilution of anti-β-actin antibody (AC-15, Sigma–Aldrich).

2.8. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Mean values were compared by means of ANOVA and unpaired Student's *t*-test. Probabilities of less than 0.05 were considered to be significant.

3. Results

3.1. ATGL$^{-/-}$mSMC showed spontaneous TG accumulation

Compared to ATGL$^{+/+}$mSMC, ATGL$^{-/-}$mSMC exhibited a hypertrophic morphology with increased cytoplasmic vacuoles, and Nile red staining revealed neutral lipid accumulation in ATGL$^{-/-}$mSMC (Fig. 1A). Cellular TG content was 0.73 ± 0.11 mM/μg · protein in ATGL$^{+/+}$mSMC vs. 1.56 ± 0.05 mM/μg · protein in ATGL$^{-/-}$mSMC ($P < 0.01$). Epinephrine-stimulated induction of lipase activity was observed in ATGL$^{+/+}$mSMC but not in ATGL$^{-/-}$mSMC (Fig. 1B).

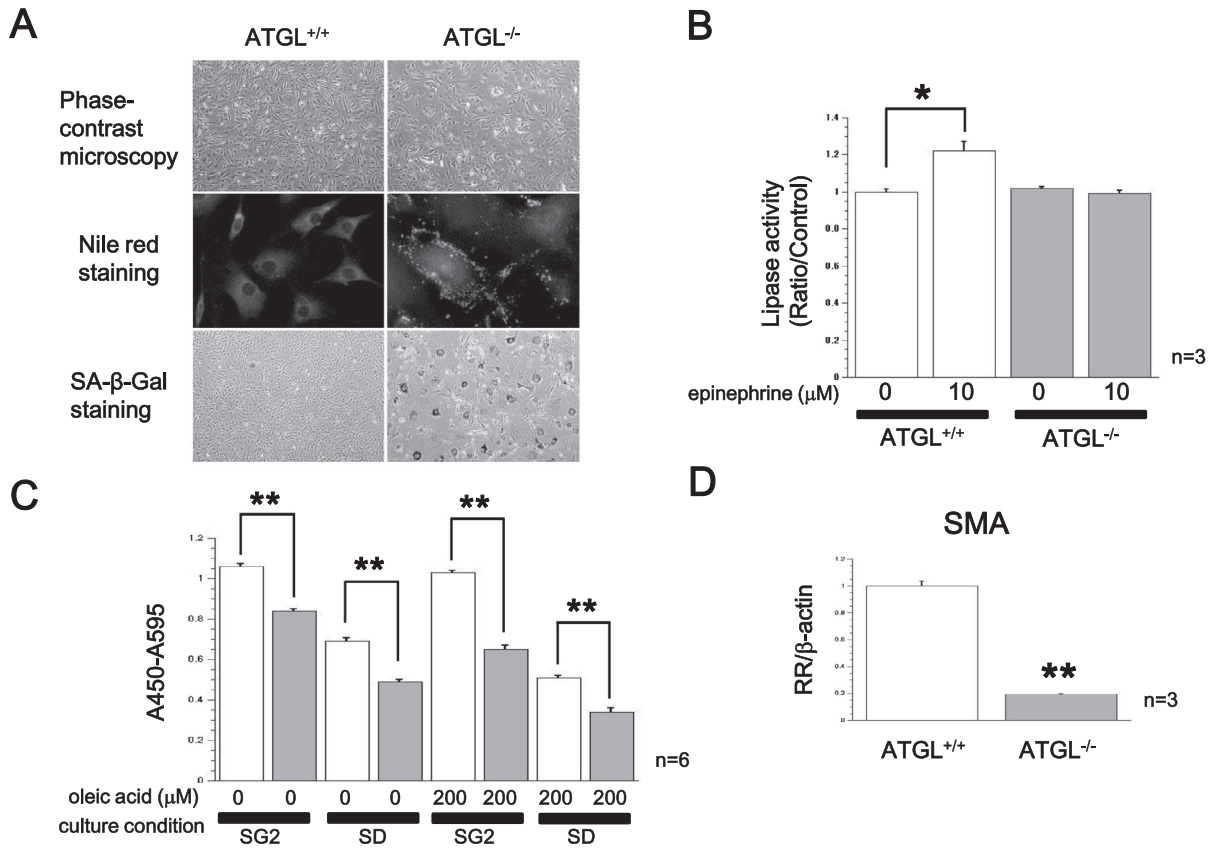

Fig. 1. ATGL$^{-/-}$mSMC showed distinct phenotype. ATGL$^{+/+}$mSMC or ATGL$^{-/-}$mSMC were seeded at a subconfluent density and cultured for 24 h with SG2 media. (A) Representative morphologies observed by phase-contrast microscopy (upper panels), with Nile red staining (middle panels), and with SA-β-Gal staining (bottom panels) are shown. (B) SMC were stimulated with 10 μM of epinephrine for one hour and lipase activity was measured. (C) ATGL$^{+/+}$mSMC or ATGL$^{-/-}$mSMC (5×10^3 cells/well) were seeded on a 96-well culture plate and were then incubated for 48 h with SG2 or SD media in the presence or absence of oleic acid (200 μM). Viable cell number was analyzed by WST-1 assay. (D) ATGL$^{+/+}$mSMC or ATGL$^{-/-}$mSMC were seeded at a subconfluent density and cultured for 24 h with SG2 media. Levels of SMA and β-actin mRNA were evaluated by real-time PCR (RR; relative ratio). *Bars*, mean ± SEM (*error bars*). $^{**}P < 0.01$, $^{*}P < 0.05$, *Open bars*, ATGL$^{+/+}$mSMC; *gray bars*, ATGL$^{-/-}$mSMC.

3.2. ATGL$^{-/-}$mSMC showed lower mitogenic response and SMA expression level with increased SA-β-Gal expression

In the presence of 200 μM of oleic acid, cellular TG content in ATGL$^{+/+}$mSMC (1.77 ± 0.13 mM/μg · protein) approached that in ATGL$^{-/-}$mSMC (2.05 ± 0.04 mM/μg · protein). Nevertheless, a WST-1 assay revealed that ATGL$^{-/-}$mSMC had a lower mitogenic response than ATGL$^{+/+}$mSMC had, irrespective of the presence or absence of oleic acid (Fig. 1C). As SMA expression is coordinately regulated with switching from the proliferative to the contractile phenotype [18], we tested SMA expression by real-time PCR. Unexpectedly, lower mitogenic response in ATGL$^{-/-}$mSMC was not correlated with the induction of SMA, the level of which was rather lower than that observed in ATGL$^{+/+}$mSMC (Fig. 1D). The proportion of SA-β-Gal-positive cells was very low in ATGL$^{+/+}$mSMC (0.46 ± 0.33%), while many SA-β-Gal-positive cells were observed in ATGL$^{-/-}$mSMC (30.2 ± 8.1%, $P < 0.01$) (Fig. 1A).

3.3. ATGL$^{-/-}$mSMC showed deviated expression of specific genes related to vascular function

To further investigate the phenotypic characteristics of ATGL$^{-/-}$mSMC, focused DNA array analysis was performed. In the present study, genes that had changed by a cut-off factor of 2 or 0.5 were defined as up-regulated (Supplemental Table 2) or down-regulated genes (Supplemental Table 3). Reliability of DNA array data was further tested by real-time PCR. We chose genes related to endothelial function (VEGF-A; vascular endothelial growth factor-A) [19,20], collagen synthesis (type I collagen, TGF-β; transforming growth factor-β) [18,21], metabolism (LPL; lipoprotein lipase, GCK; glucokinase) [22,23] and inflammation (IL-6; interleukin-6 [24]. ATGL$^{-/-}$mSMC exhibited higher expression levels of GCK (approximately 1.7-fold, $P < 0.05$), LPL (approximately 3.8-fold, $P < 0.05$) and IL-6 (approximately 3.7-fold, $P < 0.01$), and lower expression levels of VEGF-A (approximately 0.2-fold, $P < 0.01$), TGF-β (approximately 0.4-fold, $P < 0.01$) and type I collagen (approximately 0.5-fold, $P < 0.01$) compared to ATGL$^{+/+}$mSMC (Fig. 2).

3.4. Ectopic gene transfer of wild-type ATGL to ATGL$^{-/-}$mSMC reduced TG accumulation

Ectopic gene transfer of wild-type human ATGL gene was attempted using tetracycline-regulatable myc-DDK-tagged adenovirus vector (AdvATGL). In Dox-negative conditions, no significant differences in morphology were observed among the cells infected with 200 MOI (multiplicity of infection) of AdvLZ or AdvATGL, in comparison with mock-infected cells (data not shown). Treatment with Dox resulted in dose-dependent induction of human ATGL protein in ATGL$^{-/-}$mSMC (Fig. 3A). Nile red staining showed smaller proportion of lipid droplets in wild-type ATGL-induced ATGL$^{-/-}$mSMC (Fig. 3B). Consistently, cellular TG content was significantly reduced in these cells (by approximately 90% compared to the control, $P < 0.01$) (Fig. 3C). Epinephrine-stimulated induction of lipase activity was observed only in wild-type ATGL-induced ATGL$^{-/-}$mSMC and not in native ATGL$^{-/-}$mSMC (Fig. 3D).

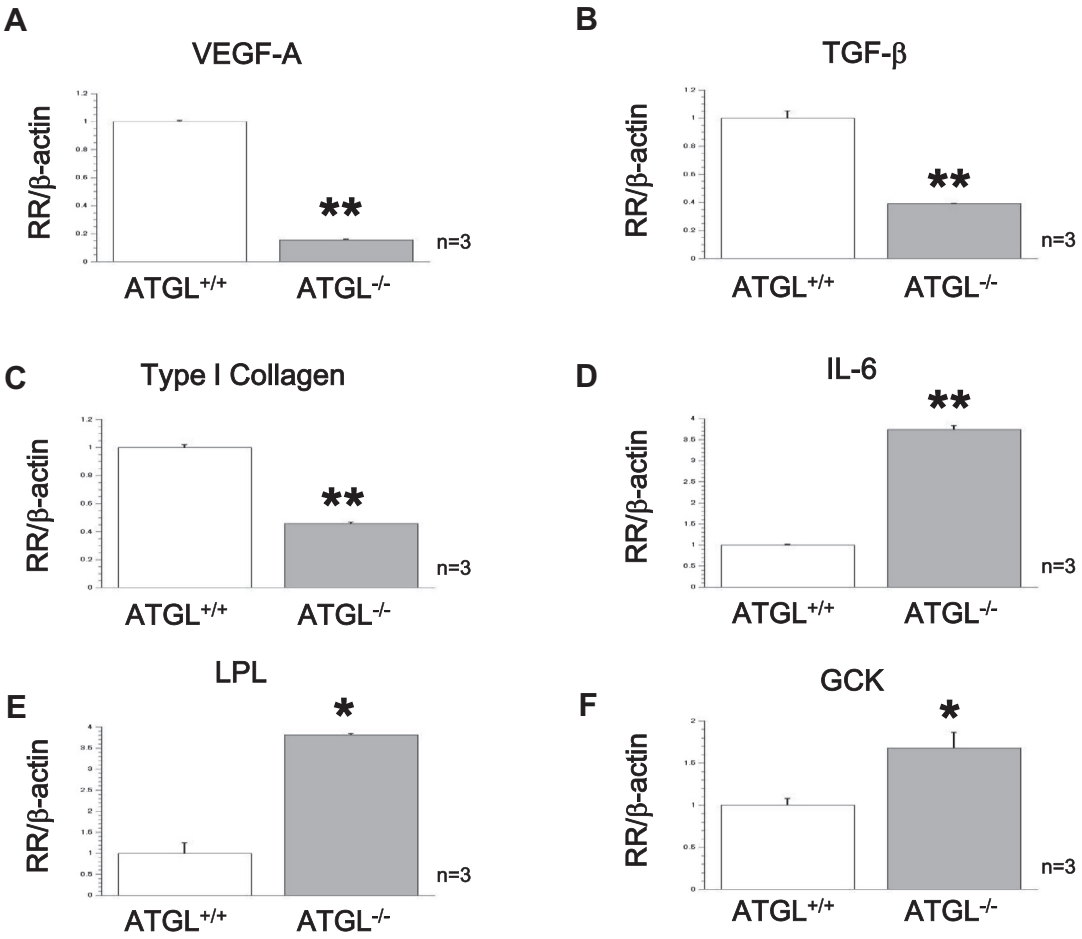

Fig. 2. ATGL$^{-/-}$mSMC showed distinct expression pattern of specific genes. ATGL$^{+/+}$mSMC or ATGL$^{-/-}$mSMC were seeded at a subconfluent density and cultured for 24 h with SG2 media. Levels of mRNAs corresponding to specific genes (A–F. gene names are indicated) and β-actin mRNA were evaluated by real-time PCR (RR; relative ratio). *Bars*, mean ± SEM (*error bars*). $^{*}P < 0.05$, $^{**}P < 0.01$, *Open bars*, ATGL$^{+/+}$mSMC; *gray bars*, ATGL$^{-/-}$mSMC.

3.5. Ectopic gene transfer of wild-type ATGL to ATGL$^{-/-}$mSMC potentially corrected distinct phenotype and specific gene expressions

In culture with differentiation media (SD), the cell numbers of ATGL$^{-/-}$mSMC were not changed irrespective of the induction of wild-type ATGL. In culture with mitogen-containing growth media (SG2), however, ectopic ATGL induction dose-dependently increased the cell numbers of ATGL$^{-/-}$mSMC (Fig. 3E). SA-β-Gal staining showed that ectopic ATGL induction reduced the percentage of senescent cells (by approximately 30% compared to the control, $P < 0.01$, Fig. 3F) and real-time PCR showed that ectopic ATGL induction increased in SMA expression (approximately by 80% against the control, $P < 0.01$, Fig. 3G). Moreover, real-time PCR revealed that the deviated gene expression in ATGL$^{-/-}$mSMC trended to be corrected by ectopic ATGL expression. The up-regulated genes were VEGF-A (approximately 1.5-fold, $P < 0.05$), TGF-β (approximately 1.2-fold, $P < 0.05$) and type I collagen (approximately 1.1-fold, $P < 0.05$), and the down-regulated genes were IL-6 (approximately 0.8-fold, $P < 0.05$) LPL (approximately 0.2-fold, $P < 0.01$) and GCK (approximately 0.3-fold, $P < 0.01$) compared to the controls (Fig. 4).

4. Discussion

In the present study, ATGL$^{-/-}$mSMC showed spontaneous TG accumulation with lower mitogenic response and SMA expression compared to ATGL$^{+/+}$mSMC. The proportion of senescent cells was also greater in ATGL$^{-/-}$mSMC. ATGL$^{-/-}$mSMC showed deviated expression of specific genes related to vascular function. Moreover, ectopic gene transfer of wild-type gene to ATGL$^{-/-}$mSMC potentially corrected the alterations in phenotype and gene expression. We concluded that ATGL availability may be a distinct determinant of SMC phenotype, which may be involved in vascular pathogenesis related to TGCV and atherosclerosis. Importantly, TG-accumulated ATGL$^{-/-}$mSMC still preserved phenotypic plasticity. Thus the TG-accumulated phenotype of SMC could be a therapeutic target.

TG is a principal energy reserve in mammalian cells and ATGL is a key catalytic enzyme for TG, involved in the hydrolysis of the first ester bond of lipid molecules [9–11]. As it is already known that SMC can also hydrolyze TG [8], it can be assumed that ATGL deficiency causes accumulation of lipid droplets (LDs) in these cells. Accordingly, we observed massive accumulation of LDs in ATGL$^{-/-}$mSMC, visualized using Nile red neural lipid staining. Consistently, cellular TG content was also elevated in ATGL$^{-/-}$mSMC with low epinephrine-stimulated lipase activity (Fig. 1).

In the classic interpretation of phenotypic modulation among SMC, the proliferative (synthetic) phenotype is characterized by high mitogenic activity, up-regulated collagen synthesis, and lower expression of contractile proteins such as SMA, whereas the opposite tendencies are seen in the contractile (quiescent) phenotype [3,18]. ATGL$^{-/-}$mSMC showed low mitogenic activity but also low SMA expression (Fig. 1). Thus the phenotype of ATGL$^{-/-}$mSMC

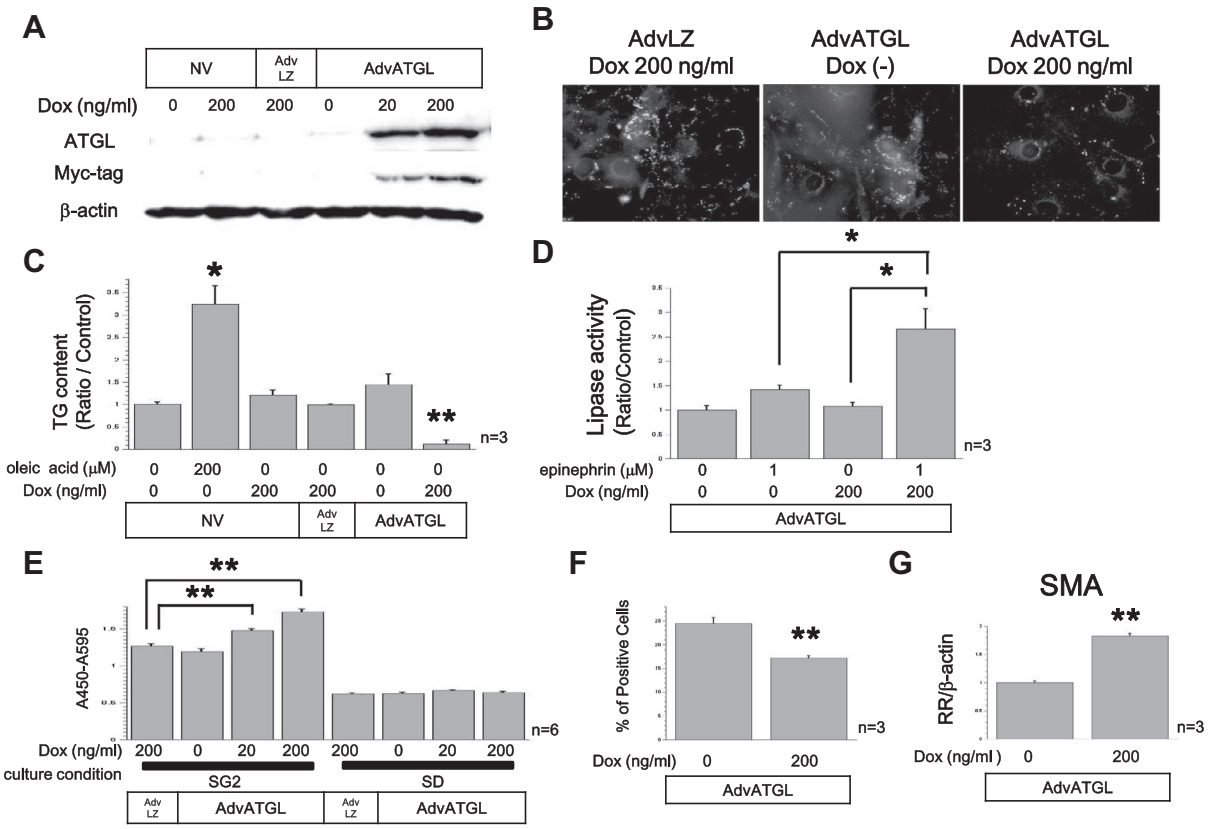

Fig. 3. Ectopic transfer of wild-type gene in ATGL$^{-/-}$mSMC reduced lipid accumulation with phenotypic corrections (A) ATGL$^{-/-}$mSMC were infected with 200 MOI of AdvLZ or AdvATGL, otherwise were mock-infected (NV), and were then treated with the indicated doses of Dox for 24 h. Cell lysates were subjected to Western blotting analysis for ATGL, myc-epitope tag and β-actin. (B) ATGL$^{-/-}$mSMC were infected with 200 MOI of AdvLZ or AdvATGL, and were then treated with Dox (200 ng/ml) for 24 h. Representative morphologies observed with Nile red staining are shown. (C) ATGL$^{-/-}$mSMC were treated in the same manner as those in panel A. Oleic acid (200 μM) was also added to the mock-infected cells as a positive control. Cellular TG content is expressed as a ratio to that in control-uninfected cells. (D) ATGL$^{-/-}$mSMC were infected with 200 MOI of AdvATGL and were then cultured with or without Dox (200 ng/ml) for 24 h. SMC were then stimulated with 1 μM of epinephrine for one hour and lipase activity was measured. (E) ATGL$^{-/-}$mSMC were infected with 200 MOI of AdvLZ or AdvATGL and were then cultured in SG2 or SD media with the indicated doses of Dox for 48 h. WST-1 assay was performed. (F) ATGL$^{-/-}$mSMC were infected with 200 MOI of AdvATGL, and were then cultured with or without Dox (200 ng/ml) for 24 h. Percentages of SA-β-Gal-positive cells are shown. (G) ATGL$^{-/-}$mSMC were treated in the same manner as those in panel F. Levels of SMA and β-actin mRNA were evaluated by real-time PCR (RR; relative ratio). *Bars*, mean ± SEM (*error bars*). $^{*}P < 0.05$, $^{**}P < 0.01$.

may not be consistent with the contractile (quiescent) phenotype. We found that ATGL$^{-/-}$mSMC were more likely to exhibit a senescent phenotype (Fig. 1). It has been reported that SMC isolated from aged rodents exhibit lower proliferative and migratory activities and lower levels of contractile expression compared to cells isolated from young animals [25]. Thus we assume that the ATGL-deficient or TG-accumulated phenotype shares some characteristics with the senescent phenotype. Further study is needed to clarify the phenotypic switching mechanism of SMC beyond the classic interpretation.

ATGL$^{-/-}$mSMC showed a unique pattern of gene expression (Supplemental Tables 2 and 3; Fig. 2). For example, VEGF-A expression was lower in ATGL$^{-/-}$mSMC than in ATGL$^{+/+}$mSMC. VEGF-A induces endothelial cell migration, survival, vascular development and angiogenesis [19]. Physiological angiogenesis may contribute to the restoration of normoxia in the vascular wall, resolution of inflammation, and regression of atherosclerosis [20]. Pathologic angiogenesis, on the other hand, enhances vascular inflammation, oxidative stresses, and risk of intramural hemorrhage, leading to plaque expansion and fragility [20]. We assume that the lower VEGF-A expression in ATGL$^{-/-}$mSMC is disadvantageous with regard to initial defense against the early stage of the atherogenic process, but we cannot deny that it is beneficial with regard to the inhibition of pathologic angiogenesis in the advanced stages of plaque development. Further study is needed to clarify *in vivo* plaque morphology in this context. ATGL$^{-/-}$mSMC also showed down-regulated expression of type I collagen and TGF-β. SMC is a major source of collagenous proteins in the vasculature [18] and TGF-β plays a pivotal role in vascular matrix accumulation [21]. Disability of TGF-β-mediated collagen synthesis may be related to the plaque venerability, though the role of TGF-β in atherogenesis remains the subject of debate [21]. Other genes were up-regulated in ATGL$^{-/-}$mSMC. GCK may allow ATGL$^{-/-}$mSMC to utilize glucose, compensating for their inability to use TG [23]. LPL is a key enzyme in the catabolism of TG-rich plasma lipoproteins such as chylomicrons and VLDL [22]. In addition, LPL functions in lipoprotein binding and selective uptake of cholesterol ester, a process regarded as pro-atherosclerotic. The principal sites of LPL production are the heart, skeletal muscle, and adipose tissue [22]; thus the contribution of SMC-derived LPL in the systemic lipid metabolism could be underestimated. The local pro-atherosclerotic effect of up-regulated LPL availability may be related to vascular pathogenesis. We also detected the up-regulation of IL-6. Accumulating evidence suggests that increased IL-6 expression is involved in various cardiovascular disease processes including atherosclerosis [24]. IL-6 signaling mediates the activation of NF-κB transcription factor in vascular cells, which subsequently induces the expression of adhesion molecules and chemotaxins and then exacerbates the inflammatory process [26]. We found a deviated gene expression pattern in ATGL$^{-/-}$mSMC, the significance of which with regard to vascular pathogenesis remains to be elucidated.

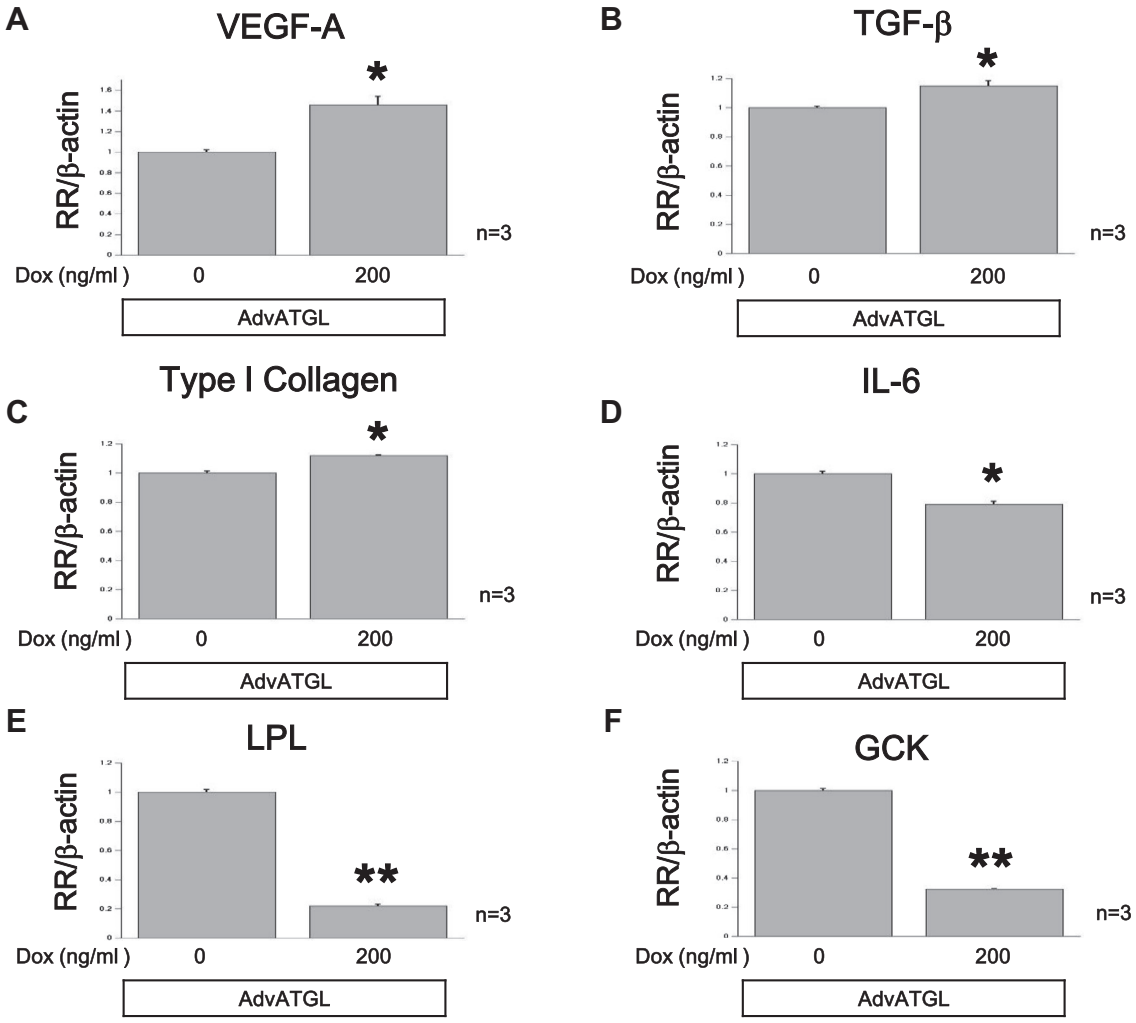

Fig. 4. Ectopic transfer of wild-type gene in ATGL$^{-/-}$mSMC corrected the deviated expression pattern of specific genes. ATGL$^{-/-}$mSMC were infected with 200 MOI of AdvATGL, and were then cultured with or without Dox (200 ng/ml) for 24 h. Levels of mRNAs corresponding to specific genes (A–F. gene names are indicated) and β-actin mRNA were evaluated by real-time PCR (RR; relative ratio). *Bars*, mean ± SEM (*error bars*). $^{*}P < 0.05$, $^{**}P < 0.01$.

Notably, the distinct phenotype of ATGL$^{-/-}$mSMC and the deviated gene expressions were corrected by an ectopic transfer of wild-type ATGL gene (Figs. 3 and 4). Thus we confirmed the plasticity of the phenotype of ATGL$^{-/-}$mSMC. Phenotypic plasticity is recognized as one of the most important functions of SMC, which also implies an opportunity for their use in therapeutic intervention [6]. Recently, it has been reported that RNA-interference-mediated down-regulation of ATGL results in the enhancement of TNF-α-induced ICAM-1 expression in human endothelial cells [27]. Thus the disability of ATGL may influence various vascular cell phenotypes.

The present study has several limitations that must be mentioned. First, the regulatory mechanism of ATGL$^{-/-}$mSMC phenotype is still unclear. Second, SMC phenotype of ATGL-deficient mice was not confirmed in *in vivo*. As reported elsewhere, ATGL$^{-/-}$ mice suffer from fatal cardiomyopathy and die around 12 weeks after birth [12]. Accordingly, we isolated SMC from 8-week old ATGL$^{-/-}$ mice. Rodents are atherosclerosis-resistant and prolonged administration of a high-fat diet is usually required to induce atherosclerosis [28]. More recently, it has been reported that macrophages with ATGL deficiency attenuates *in vivo* atherogenesis [29]. In addition, significant differences among species in lipid metabolism regulatory mechanisms have also been noted [30]. These could be reasons why we did not observe distinct atheromatous lesions in the isolated aortic tissues from ATGL$^{-/-}$ mice. An additional study using human SMC is currently in progress in our research group.

Acknowledgments

We would like to thank Prof. Rudolf Zechner (Institute of Molecular Biosciences, University of Graz, Austria) for the generous gift of the ATGL knockout mouse line. This work was supported in part by grants-in-aid for Scientific Research (C: 22590349) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of the Japanese Government and research grants-in-aid for rare and intractable diseases from the Ministry of Health, Labour, and Welfare of the Japanese Government.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.109.

References

[1] R. Ross, Atherosclerosis-an inflammatory disease, N. Engl. J. Med. 340 (1999) 115–126.

[2] P. Libby, P.M. Ridker, G.K. Hansson, Progress and challenges in translating the biology of atherosclerosis, Nature 473 (2011) 317–325.

[3] G.R. Campbell, J.H. Chamley-Campbell, Smooth muscle phenotypic modulation: role in atherogenesis, Med. Hypotheses 7 (1981) 729–735.
[4] A.W. Orr, N.E. Hastings, B.R. Blackman, B.R. Wamhoff, Complex regulation and function of the inflammatory smooth muscle cell phenotype in atherosclerosis, J. Vasc. Res. 47 (2010) 168–180.
[5] V.P. Iyemere, D. Proudfoot, P.L. Weissberg, C.M. Shanahan, Vascular smooth muscle cell phenotypic plasticity and the regulation of vascular calcification, J. Intern. Med. 260 (2006) 192–210.
[6] M.R. Alexander, G.K. Owens, Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease, Annu. Rev. Physiol. 74 (2012) 13–40.
[7] L. Badimon, R.F. Storey, G. Vilahur, Update on lipids inflammation and atherothrombosis, Thromb. Haemost. 105 (Suppl 1) (2011) S34–S42.
[8] L.K. Minor, G.H. Rothblat, J.M. Glick, Triglyceride and cholesteryl ester hydrolysis in a cell culture model of smooth muscle foam cells, J. Lipid Res. 30 (1989) 189–197.
[9] M.J. Watt, G.R. Steinberg, Regulation and function of triacylglycerol lipases in cellular metabolism, Biochem. J. 414 (2008) 313–325.
[10] R. Zimmermann, J.G. Strauss, G. Haemmerle, G. Schoiswohl, R. Birner-Gruenberger, M. Riederer, A. Lass, G. Neuberger, F. Eisenhaber, A. Hermetter, R. Zechner, Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase, Science 306 (2004) 1383–1386.
[11] E. Smirnova, E.B. Goldberg, K.S. Makarova, L. Lin, W.J. Brown, C.L. Jackson, ATGL has a key role in lipid droplet/adiposome degradation in mammalian cells, EMBO Rep. 7 (2006) 106–113.
[12] G. Haemmerle, A. Lass, R. Zimmermann, G. Gorkiewicz, C. Meyer, J. Rozman, G. Heldmaier, R. Maier, C. Theussl, S. Eder, D. Kratky, E.F. Wagner, M. Klingenspor, G. Hoefler, R. Zechner, Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase, Science 312 (2006) 734–737.
[13] J. Fischer, C. Lefèvre, E. Morava, J.M. Mussini, P. Laforêt, A. Negre-Salvayre, M. Lathrop, R. Salvayre, The gene encoding adipose triglyceride lipase (PNPLA2) is mutated in neutral lipid storage disease with myopathy, Nat. Genet. 39 (2007) 28–30.
[14] K. Hirano, Y. Ikeda, N. Zaima, Y. Sakata, G. Matsumiya, Triglyceride deposit cardiomyovasculopathy, N. Engl. J. Med. 359 (2008) 2396–2398.
[15] K. Hirano, A novel clinical entity: triglyceride deposit cardiomyovasculopathy, J. Atheroscler. Thromb. 16 (2009) 702–705.
[16] M. Kobayashi, K. Inoue, E. Warabi, T. Minami, T. Kodama, A simple method of isolating mouse aortic endothelial cells, J. Atheroscler. Thromb. 12 (2005) 138–142.
[17] I. Furukawa, S. Kurooka, K. Arisue, K. Kohda, C. Hayashi, Assays of serum lipase by the "BALB-DTNB method" mechanized for use with discrete and continuous-flow analyzers, Clin. Chem. 28 (1982) 110–113.
[18] S. Kato, J.R. Shanley, J.C. Fox, Serum stimulation, cell-cell interactions, and extracellular matrix independently influence smooth muscle cell phenotype *in vitro*, Am. J. Pathol. 149 (1996) 687–697.
[19] T. Matsumoto, H. Mugishima, Signal transduction via vascular endothelial growth factor (VEGF) receptors and their roles in atherogenesis, J. Atheroscler. Thromb. 13 (2006) 130–135.
[20] P.R. Moreno, M. Purushothaman, K.R. Purushothaman, Plaque neovascularization: defense mechanisms, betrayal, or a war in progress, Ann. NY Acad. Sci. 1254 (2012) 7–17.
[21] N.N. Singh, D.P. Ramji, The role of transforming growth factor-beta in atherosclerosis, Cytokine Growth Factor Rev. 17 (2006) 487–499.
[22] Y. Stein, O. Stein, Lipoprotein lipase and atherosclerosis, Atherosclerosis 170 (2003) 1–9.
[23] G. Mithieux, Role of glucokinase and glucose-6 phosphatase in the nutritional regulation of endogenous glucose production, Reprod. Nutr. Dev. 36 (1996) 357–362.
[24] M.Y. Abeywardena, W.R. Leifer, K.E. Warnes, J.N. Varghese, R.J. Head, Cardiovascular biology of interleukin-6, Curr. Pharm. Des. 15 (2009) 1809–1821.
[25] Z. Li, H. Cheng, W.J. Lederer, J. Froehlich, E.G. Lakatta, Enhanced proliferation and migration and altered cytoskeletal proteins in early passage smooth muscle cells from young and old rat aortic explants, Exp. Mol. Pathol. 64 (1997) 1–11.
[26] A.R. Brasier, The nuclear factor-κB-interleukin-6 signalling pathway mediating vascular inflammation, Cardiovasc. Res. 86 (2010) 211–218.
[27] T. Inoue, K. Kobayashi, T. Inoguchi, N. Sonoda, M. Fujii, Y. Maeda, Y. Fujimura, D. Miura, K. Hirano, R. Takayanagi, Reduced expression of adipose triglyceride lipase enhances tumor necrosis factor alpha-induced intercellular adhesion molecule-1 expression in human aortic endothelial cells via protein kinase C-dependent activation of nuclear factor-κB, J. Biol. Chem. 286 (2011) 32045–32053.
[28] B. Paigen, A. Morrow, C. Brandon, D. Mitchell, P. Holmes, Variation in susceptibility to atherosclerosis among inbred strains of mice, Atherosclerosis 57 (1985) 65–73.
[29] B. Lammers, P.G. Chandak, E. Aflaki, G.H. van Puijvelde, B. Radovic, R.B. Hildebrand, I. Meurs, R. Out, J. Kuiper, T.J. van Berke, L.D. Kolb, G. Haemmerle, R. Zechner, S. van Eck, M. Levak-Frank, D. Kratky, Macrophage adipose triglyceride lipase deficiency attenuates atherosclerotic lesion development in low-density lipoprotein receptor knockout mice, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 67–73.
[30] W.G. Bergen, H.J. Mersmann, Comparative aspects of lipid metabolism: impact on contemporary research and use of animal models, J. Nutr. 135 (2005) 2499–2502.