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ATF3 and *EGR2* gene expression levels in sdLDL-treated macrophages of patients with coronary artery stenosis

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Abstract

Background: The metabolism of cholesteryl esters (CEs) is under the control of a gene network in macrophages. Several genes such as *ATF3* and *EGR2* are related to cholesterol metabolism.

Methods: In this study, the *ATF3* and *EGR2* gene expression levels were evaluated in differentiated macrophages of subjects undergoing coronary artery angiography [controls (<5% stenosis), patients (>70% stenosis)] after treatment with small dense low density lipoprotein (sdLDL) particles. Monocytes were isolated using a RosetteSep Kit and were differentiated into macrophages using the M-CSF factor. A modified heparin-$MgSO_4$-PEG method was used for the sdLDL preparation. The *ATF3* and *EGR2* gene expression levels were measured by the real-time quantitative polymerase chain reaction (RT-qPCR) technique.

Results: In contrast with the control group ($p = 0.4$), the *ATF3* expression level reduced significantly in the differentiated macrophages from all patients [single vessel disease (SVD), fold change 17 times, $p = 0.02$; two vessel disease (2VD), fold change 1.5 times, $p = 0.05$; three vessel disease (3VD), fold change 3.5 times, $p = 0.04$]. Also, the *EGR2* expression level reduced significantly in all groups ($p < 0.02$). The gene fold changes had no significant differences between the patients ($p > 0.8$).

Conclusions: We propose that the failure of *ATF3* gene expression improves the CE synthesis after sdLDL influx. Furthermore, the reduced *EGR2* gene expression level in the sdLDL-treated groups may be a negative factor in cholesterol homeostasis.

Keywords: *ATF3*; differentiation; *EGR2*; macrophage; small dense low density lipoprotein (sdLDL); stenosis.

Introduction

Atherosclerosis is an inflammatory disease strongly related to lifestyle [1, 2]. Obviously, the atherogenic lesions develop primarily on the formation of foam cells from macrophages in subendothelial space [3]. The molecular studies have been shown that the unfolded protein responses (UPR) in foam cells are involved in the apoptosis and necrosis so that the primary core of atherosclerosis plaques may be formed due to the aggregation of cells [4, 5].

Many studies have reported that M2 macrophages are characterized as the cells with high phagocyte power and may act in the healing of atherogenic lesions [6–9]. On the other hand, hyperlipidemia is considered as an important risk factor for atherosclerosis [10]. Some studies suggested that small dense low density lipoprotein (sdLDL) particles are dense lipoproteins penetrating into the vessel's subendothelial space [11]. These particles have a high half-life because of their contact with the subendothelial proteoglycans and, are easily scavenged by macrophages [12, 13]. The lipoprotein-derived and de-novo cholesterol levels are under tight control by

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several known pathways [14, 15]. However, the pathway failures may cause the excess cholesteryl esters (CEs) in the cytoplasm resulting in the formation of lipid droplets (LDs). The LD-derived CE hydrolysis is a limiting rate factor of free cholesterol (FC) efflux that is catalyzed by cholesteryl hydrolase [16, 17].

ATF3 (activating transcription factor 3) is one of the important genes acting in cholesterol metabolism and is involved in the foam-cell formation [18]. That is a transcription factor suppressing cholesterol 25-hydroxylase so that its function reduces the cellular cholesterol ester (CE) storage and lipid droplets (LDs) in the cytoplasm [19]. Furthermore, *ATF3* can directly downregulate LDL-receptor (LDLR) expression [20]. *EGR2* (early growth factor 2) is another gene involved in cholesterol metabolism. That contains a zinc finger domain that interacts with the DNA GC-rich region [21]. Some studies reported that *EGR2* and *SREBP2* coactivators induce the pathways involved in cholesterol/lipid biosynthesis [22].

Based on the roles of the genes and sdLDL in cholesterol homeostasis, we investigated the effects of sdLDL particles on the *ATF3* and *ERG2* gene expression levels in the differentiated M2-Macrophages of patients with coronary artery stenosis and healthy controls.

Materials and methods

Subjects

The subjects were selected from participants undergoing coronary artery angiography. The patients had coronary vessels with at least 70% stenosis. Based on the numbers of stenosed vessels, the patients were subdivided into three categories; single vessel disease (SVD, n = 6), two vessel disease (2VD, n = 6) and three vessel disease (3VD, n = 6). The controls (n = 6) were selected from subjects with symptoms of chest pain who were candidates for coronary artery angiography (<5% stenosis). The university Ethics Committee approved this study and, a questionnaire was completed by all subjects.

Monocyte isolation

Monocytes were isolated from whole blood using the RosetteSep technology Kit (STEMCELL Technologies, Vancouver, BC, Canada). A volume (50 μL) of tetramer antibodies was added to whole blood (1 mL) and was incubated for 20 min at room temperature. Then, it was diluted with phosphate-buffered saline (PBS) buffer (1:1 ratio, v/v), and was centrifuged with ficoll gradient (1000 *g*, 20 min). Finally, the monocytes were washed 3 times with PBS buffer containing 2% fetal bovine serum (FBS) (300 *g*, 10 min) (Figure 1A).

M2-macrophage differentiation

Monocytes (10^6 cells/mL) were cultured in Gipco™ RPMI 1640 medium containing 100 ng/mL human macrophage colony stimulating factor (hM-CSF, catalog number: #8929, Cell Signaling Technology, Beverly, MA, USA), 10% FBS, and 1% Gipco™ Pen Strep (catalog number: 10378016, Thermo Fisher Scientific, Waltham, MA, USA) in six-well plates for 6 days at 37 °C with 5% CO_2 (Figure 1B and C). The flow cytometry results showed that CD163 marker is positively considered for higher than 65% cells.

sdLDL separation

Heparin magnesium-sulfate (0.4 mL) was added to the pooled serum sample [controls (n = 3), 0.4 mL] and was incubated at 37 °C for 10 min. Then, it was incubated in cold water for 15 min and was centrifuged for 10 min at 12000 *g* (4 °C). Polyethylene glycol (PEG, 0.2 mL) was added to the supernatant (0.2 mL) containing sdLDL and high-density lipoprotein (HDL) particles. After incubation at room temperature (10 min), it was centrifuged at 8000 *g* for 20 min. The sediment was separated and dissolved in PBS buffer and finally, the sdLDL level was measured by spectrometric technique [23–25].

sdLDL treatment

Macrophages were seeded at a 96-well plate (10^4 cells in each well) and, were treated with different values of sdLDL (25, 50, 100 μg/mL). Then, the number of viable cells in each well was counted after 24 h the based on the trypan-blue staining method. A treatment dose (50 μg/mL) was applied in the study as the inhibition rate was not significant when compared with the control. The differentiated macrophages were divided into two groups (each 10^6 cells); treated with sdLDL and, treated with the separation

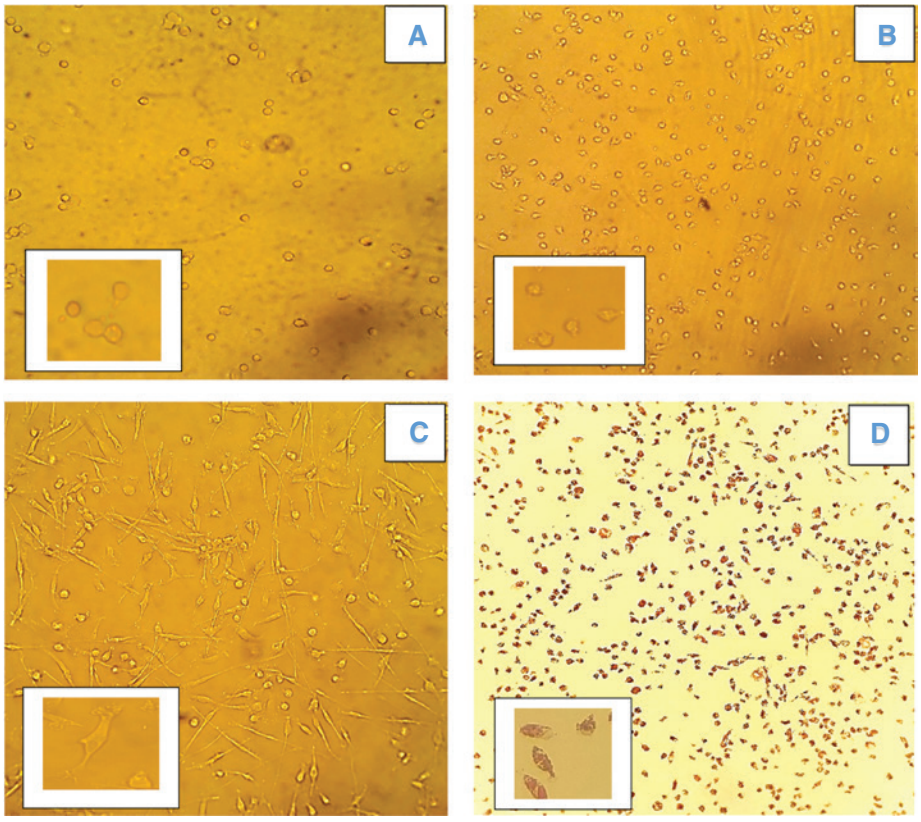

Figure 1: M2 macrophage differentiation.
The monocytes (A, 0-day) differentiated into macrophages using M-CSF factor (B, 3-day; C, 6-day). The stained macrophages with oil-red (D) after treatment with sdLDL.

matrix without sdLDL for 24 h in RPMI 1640, 10% FBS and 1% Pen Strep.

Oil-red staining method

The sdLDL uptake was evaluated using oil-red staining method. At first, the sdLDL-treated cells were washed with PBS buffer (3 times) and were fixed in formalin 10% for 10 min. Afterwards, the cells were washed with PBS buffer and were incubated with isopropanol 60% for 15 s. Then, they were incubated with oil-red for 1 min at 37 °C and were washed 3 times with PBS buffer [26] (Figure 1D).

RNA extraction and cDNA synthesis

The total RNA was prepared using the High Pure RNA Isolation Kit (catalogue number: 11 828 665 001, Roche) and, cDNA was synthesized using the cDNA synthesis kit (catalogue number: #9160, Takara, Japan) in according with the manufacturer's instructions. All samples were preserved at –80 °C.

Real-time quantitative polymerase chain reaction (RT-qPCR) technique

The RT-qPCR reactions were performed in 0.1 mL microtubes containing cDNA 1 µL, primer 0.2 µL from each (0.1 µM) and dNTP 1 µL (100 µM). The primers were designed by the primer-blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast) (*ATF3*; 5′-TCACAAAAGCCGAGGTAGCC-3′ and 5′-AGGCACTCCGTCTTCTCCTT-3′, *EGR2*; 5′-ACCATCTTTCCCAATGCCGA-3′ and 5′-GATCATGCCATCTCCGGCCA-3′, β-actin; 5′-TCCCTGGAGAAGAGCTACG-3′, 5′-GTAGTTTCGTGGATGCCACA-3′). The RT-qPCR temperature cycles were performed after incubation at 95 °C for 2 min containing 95 °C for 5 s followed 30 s at 63 °C for *ATF3* and *EGR2* genes and 59 °C for β-actin gene.

Statistical analysis

Data analysis was performed with the statistical software package (SPSS 20.0, Chicago, IL, USA). The data distribution was evaluated using the Kolmogorov-Smirnov

Table 1: Demographic and para-clinic parameters.

Group	Stenosis	Age, years (mean ± SD)	BMI, kg/m² (mean ± SD)	SBP, mmHg (mean ± SD)	DBP, mmHg (mean ± SD)	PR, bpm (mean ± SD)
Control		56.33 ± 9.2	29.49 ± 5.99	128.33 ± 2.88	78.30 ± 2.88	75.00 ± 5.09
Patient	SVD	54.03 ± 1.41	26.12 ± 1.22	120.00 ± 1.00	80.00 ± 1.12	85.01 ± 1.00
	2VS	51.34 ± 11.71	26.19 ± 0.94	120.00 ± 10.08	73.32 ± 5.77	76.33 ± 3.21
	3VS	67.00 ± 4.00	24.08 ± 1.25	125.66 ± 23.79	82.33 ± 13.65	78.31 ± 10.41
Total		57.45 ± 9.44	26.50 ± 3.50	123.81 ± 12.22	78.09 ± 7.64	78.09 ± 6.48
p-Value		0.20	0.33	0.85	0.6	0.41

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PR, pulse rate.

test. The demographic data was tested using Student's t test. In each group (containing controls and patients), $\Delta CT = CT_{ATF3\ (EGR2)} - CT_{\beta\text{-actin}}$ was identified. Then, the differences ($\Delta\Delta CT$) between $\Delta CT_{patient(s)}$ and $\Delta CT_{control}$ were calculated. The reaction efficiency was estimated equal to 2. $2^{-\Delta\Delta CT}$ values were calculated to compare the gene expression levels. p-Values of less than 0.05 were calculated to be significant.

Results

Table 1 shows several demographic and para-clinic characteristics of the participants. There were no significant differences in age, gender, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse rate (PR) and smoking parameters between the study groups ($p \geq 0.2$).

The *ATF3* gene expression level was measured in the differentiated M2-macrophages of controls (<5% stenosis) and patients (>70% stenosis) after treatment with sdLDL. In contrast with the estimated results from patients, the *ATF3* expression level increased no significantly in the controls ($p = 0.4$). However, the *ATF3* expression levels reduced significantly in all patient groups (SVD, fold change 17 times, $p = 0.02$; 2VD, fold change 1.5 times, $p = 0.05$; 3VD, fold change 3.5 times, $p = 0.04$) (Figure 2).

The *EGR2* expression level was also measured in the M2-macrophages treated with sdLDL. Data showed that sdLDL is able to reduce the *EGR2* expression level. The *EGR2* expression levels reduced 2.5 ($p = 0.02$), 16 ($p = 0.01$), 1.8 ($p = 0.03$) and 4 times ($p = 0.04$) in controls, SVD, 2VD and 3VD, respectively (Figure 3). However, the

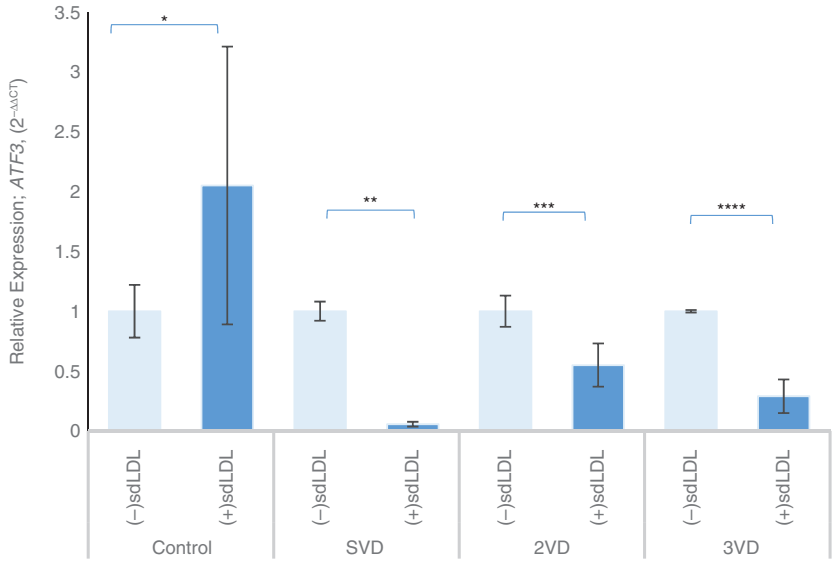

Figure 2: *ATF3* gene expression changes in differentiated M2 macrophages.
The subjects underwent coronary artery angiography [controls, <5% stenosis and patients (SVD, single vessel disease; 2VD, two vessel disease; 3VD, three vessel disease), >70% stenosis]. The monocytes isolated from the whole blood and differentiated into M2-macrophages. The *ATF3* gene expression levels evaluated after treatment with the sdLDL particles. *$p = 0.4$; **$p = 0.02$; ***$p = 0.05$; ****$p = 0.04$.

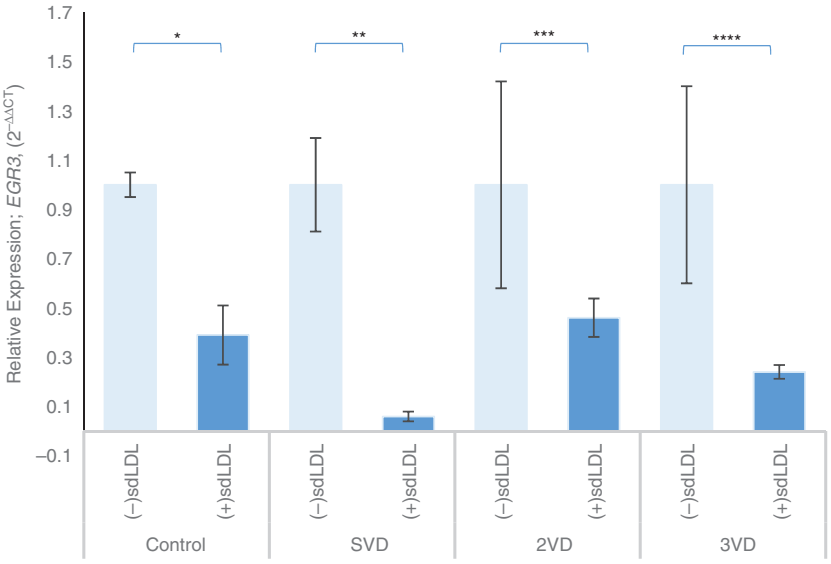

Figure 3: *EGR2* gene expression changes in differentiated M2-macrophages.
Controls, <5% stenosis and patients >70% stenosis containing; SVD, single vessel disease; 2VD, two vessel disease and 3VD, three vessel disease. The *EGR2* expression level evaluated in the differentiated M2-macrophages after treatment with the sdLDL particles. *p = 0.02; **p = 0.01; ***p = 0.03; ****p = 0.04.

ATF3 and *EGR2* expression fold changes had not the significant differences between the patient groups ($p > 0.8$).

Discussion

Atherosclerosis is a multifactorial disease related to lifestyle and, is developed by inflammatory events. Furthermore, macrophages are known as a central agent within atherosclerotic plaques. The phagocytic macrophages (M2) scavenge the non-functional components in vessel sub-endothelial space and are beneficial as compared with the apoptotic macrophages (M1). Obviously, the accumulation of LDs containing mostly cholesteryl esters (CEs) may cause the conversion of macrophages into foam cells [27]. However, the free cholesterol (FC) efflux can affect the LD storage [28]. The studies reported that the LDs bud off from the endoplasmic reticulum (ER) and include several enzymes such as hydroxylase and Acyl CoA: cholesterol acyl transferase (ACAT) involved in cholesterol metabolism [29, 30]. However, LD size is related to the amount of cytosolic CE incorporation [31]. It is also reported that the lysosome acid lipase (LAL) catalyzes the LDL-derived CEs into free cholesterol (FC) so that they are transported into cytosol by lysosomal LAMP and NPC proteins [32]. Furthermore, the cytosolic FC can be transported into circulation by ABC transporters or be diffused, re-esterified and stored in LDs (Figure 4). Studies also reported that some LD-associated proteins such as PLIN2 are highly expressed in macrophages [33]. The cytosolic FC may also be derived from LDs due to their fusion with lysosomes. These studies showed that the FC esterification often occurs in the cytosol and is the stored form of macrophages. As LDL particles and their modified forms are external sources of cellular CEs thus in this study, we investigated the *ATF3* and *EGR2* gene expression levels in differentiated macrophages after treatment with sdLDL particles.

Activating transcription factor, known as liver regenerating factor 1 (*ATF3*/LRF) is a transcription repressor expressed highly in vascular endothelial cells and macrophages. Inoue et al. [34] reported the *ATF3* induction in human umbilical vein endothelial cells (HUVECS) by well-known stimulating agents such as TNF-α, Ox-LDL and phosphatidylcholine. However, they reported that the *ATF3* expression is not highly increased after the treatment with native LDL particles. In this study, we showed that *ATF3* expression level does not decrease in the differentiated macrophages of subjects with normal coronary arteries. As it is a transcription repressor for 25-OH cholesterol hydroxylase we thus suggested its high level causes the substrate (25-OH cholesterol) of ACAT enzyme to decrease and the CE production. It suggests that the sdLDL influx into normal M2 macrophages may cause the inhibition of CE production and the increase of FC efflux. In the other hand, the *ATF3* expression level reduced in the differentiated macrophages of patients. The results, however, did not show the relationship between the severity of vessel stenosis and *ATF3*

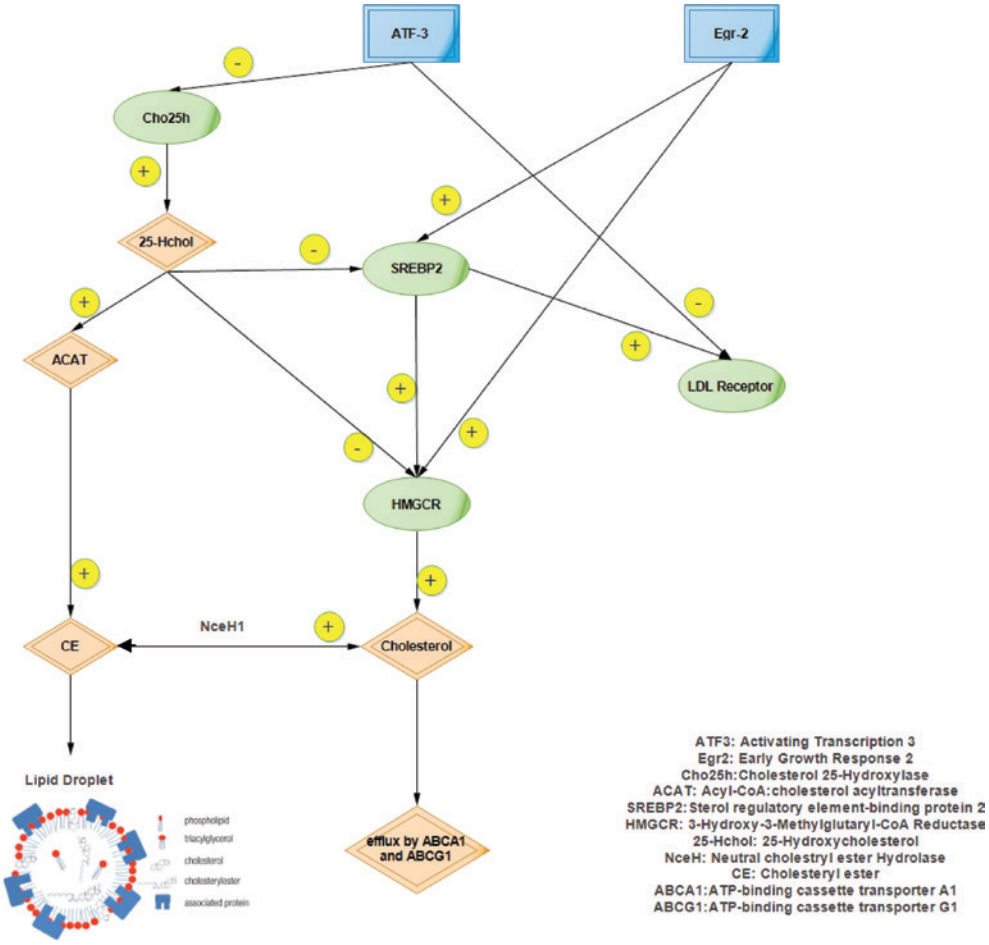

Figure 4: *ATF3* and *EGR2* in cholesterol metabolism.
The increase of *ATF3* inhibits the synthesis of 25-hydroxy cholesterol (25-Chol) and cholesteryl esters (CE) resulting in the reduction of lipid droplets (LDs). In contrast with the *ATF3* gene, *EGR2* increases the free cholesterol synthesis and the cholesterol efflux via the activation of *SREBP2* and *HMGCR* genes.

expression level but they suggested a high potential for LD formation in patients.

Some studies also reported that *EGR2* expression is related to cholesterol metabolism. Zhang et al. [35] reported the interaction of *EGR2* with the sterol-independent regulatory element (SIRE) located on the *LDLR* gene. Furthermore, Leblanc et al. [22] reported the *EGR2* and *SREBP2* transcription factors are able to induce the expression of *HMGCR*, *CYP51* and *SCD2* genes involved in cholesterol metabolism. They showed their downregulation simultaneously with the increase of SREBP2 due to *EGR2* gene knockout. Moreover, Nagarajan et al. [36] showed the induction of *HMGCR* and *SCD2* genes by adenovirus-mediated *EGR2* overexpression system in Schwann cells. These studies showed that *EGR2* is involved in FC synthesis and LD formation. In our study, the *EGR2* expression level reduced in all the groups treated with sdLDL. We proposed *EGR2* acts as a negative regulator of the cholesterol homeostasis.

In conclusion, we proposed that the failure of *ATF3* gene expression in differentiated macrophages of patients with the stenosis of coronary arteries may improve the cellular CE synthesis and LD formation after sdLDL influx. Furthermore, the results showed that the reduction of *EGR2* gene expression level in the sdLDL-treated groups may be a negative feedback on cholesterol influx.

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