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SREBP1c silencing reduces endoplasmic reticulum stress and related apoptosis in oleic acid induced lipid accumulation

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ABSTRACT

Objective: Sterol regulatory element binding protein 1c (SREBP1c) is one of the major transcription factors that is involved in non-alcoholic fatty liver disease (NAFLD) development by increasing hepatic fatty acid and triglyceride synthesis. Our study aimed to investigate the interaction of SREBP1c with endoplasmic reticulum (ER) stress in oleic acid (OA) induced lipid accumulation.

Material and Methods: Optimum lipid droplet (LD) formation and SREBP-1c induction were determined in alpha mouse liver 12 (AML12) hepatocytes following the incubation with different OA concentrations. To determine the effect of SREBP-1c, cells were transfected with siRNA specific for SREBP-1c. LD formation and SREBP-1c induction were determined via Oil Red O and immunblotting, respectively. Phospho IRE1, GRP78, CHOP, ATF6 and JNK levels were determined with immunofluorescence staining.

Results: Optimum LD formation and SREBP-1c induction were achieved at 0.5 mM oleat concentration. While SREBP-1c silencing decreased LD formation in non-OA treated cells, no significant effect of silencing was determined following OA administration. On the other hand, SREBP-1c silencing in OA treated cells reduced phospho IRE1, ATF6, JNK and CHOP expressions.

Conclusion: Our results suggest that the novel function of SREBP-1c can regulate ER stress response in OA induced lipid accumulation.

Keywords: Lipid accumulation, SREBP1c, ER stress, Apoptosis

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), one of the essential components of metabolic syndrome, is one of the major health problems today and is currently recognized as a disease entity by the World Health Organization and numerous professional societies. NAFLD is characterized by the formation of lipids in more than 5% of liver weight. Studies have shown that 25% of the population is suffering from fatty liver disease and 90% of these patients are obese individuals [1]. Therefore, understanding the mechanisms regulating lipid accumulation in the pathogenesis of NAFLD is extremely important, however, there are not published studies [2]. It has been shown that the abnormalities in the mechanisms regulating lipogenesis are major cause of triglyceride deposition in hepatocytes [3]. Sterol regulatory element binding proteins (SREBPs) are transcription factors which modulate lipogenesis. SREBPs consist of three isoforms, termed SREBP1a, SREBP1c and SREBP2, and are encoded by two genes: SREBF1 and SREBF2. They are synthesized as inactive precursor proteins attached to the ER membrane, and each isoform has a unique effect on lipid homeostasis. Proteolytic processing of precursors generates transcriptionally active forms that control the expression of a range of genes involved in cholesterol, fatty acid, phospholipid and triacylglycerol synthesis [4]. SREBP1c meditates the transcription of genes involved in fatty acid and triglycerides synthesis, including ATP citrate lyase, acetyl-CoA synthetase (ACS), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), steroyl-CoA desaturase-1 (SCD-1) and glycerol-3-phosphate acyltransferase (GPAT), while SREBP2 is specifically responsible for cholesterol synthesis and transport genes as well as LDL receptor gene transcription [5]. SREBP1a targets the genes of both transcription factors. SREBP1a targets the genes of both pathways.

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Another important issue in NAFLD pathogenesis is endoplasmic reticulum (ER) stress which is characterized by abnormalities in lipid synthesis and chaperones. ER is a crucial organelle responsible in the proper synthesis/folding of proteins, calcium storage, biosynthesis of sterols and phospholipids, and detoxification of toxic substances. Under basal conditions, glucose regulated protein 78 (GRP78) is attached to three major ER stress sensor proteins; inositol requiring kinase 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). In stress conditions that exceed the folding capacity, GRP78 binds to incorrectly folded or unfolded proteins, and dissociates from IRE1, PERK and ATF6 are activated to promote a physiological response called ER stress [6]. In addition to protein misfolding, irregularities in lipid homeostasis activate ER stress and the excessive ER stress response plays a role in the development and progression of NAFLD by enhancing lipid accumulation, inflammation, insulin resistance, autophagy and apoptosis [7].

Although, SREBP1c is a well-identified transcription factor that has been shown to play a role in disease progression by increasing hepatic fatty acid and triglyceride synthesis, its interaction with ER stress in oleic acid (OA) induced lipid accumulation has not been fully understood yet. In this study we aimed to contribute to literature by clarifying the association between ER stress and SREBP1c and their role in OA induced lipid accumulation.

2. MATERIALS and METHODS

Cell culture and treatments

Alpha mouse liver 12 (AML12) hepatocytes were maintained in Dulbeco's Modified Eagle's Medium (DMEM) (Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Massachusetts, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Thermo Fisher Scientific, Massachusetts, USA) at 37 °C with 5% CO_2. To stimulate intracellular lipid accumulation, cells were exposed to different concentrations of oleic acid:bovine serum albumin (BSA) complex (Sigma Aldrich, St. Louis, USA) for 24 h. Cells were seeded in 6/24-well plates for 48 h before treatment with vehicle (DMEM) or OA:BSA complex at the indicated concentrations and time periods. All experiments were applied according to protocols approved by Marmara University, School of Medicine Ethics Committee (protocol number 09.2019.188).

SREBP1c siRNA transfection

Alpha mouse liver 12 cells were seeded at a density of 8×10^5 in 6 well plate for 24 h and then transfected with siRNA specific for SREBP1c (Thermo Fisher Scientific, siRNA ID: 151861) by using Lipofectamine RNAiMax Reagent (Thermo Fisher Scientific, Massachusetts, USA, Catalog No: 13778075) according to manufacturer's instructions. Briefly, 80 pmol SREBP1c siRNA in 1:3 and 1:6 ratio of siRNA: Lipofectamine RNAiMax Reagent was prepared in OptiMEM (Thermo Fisher Scientific, Massachusetts, USA) and incubated for 5 min at room temperature. The mixture was gently added dropwise to the cells in OptiMEM and then incubated at 37 °C with 5% CO_2. 24 and 48 hours after the transfection, cells were harvested for immunoblot experiment to confirm that the SREBP1c was silenced.

Following the optimization of siRNA transfection, cells were seeded in 6/24-well plates for 48 h before pretreatment with SREBP1c siRNA and then treated with vehicle (DMEM) or OA:BSA to divide into four groups totally; i) Control, ii) SREBP1c siRNA, iii) OA, iv) SRBEP1c siRNA + OA.

Oil Red O staining

Following the siRNA and/or OA administrations, cells were fixed with 10% neutral-buffered formalin for 10 min, washed twice with PBS, and stained with 0.2% Oil Red O (Sigma Aldrich, St. Louis, USA) in isopropanol for 15 min. Cells were then washed in PBS, and visualized and photographed using light microscope (Zeiss, Amsterdam, Netherlands). Lipid accumulation was quantified by counting the number of lipid droplets in at least thirty cells for each group.

Immunoblot analysis

Cells were collected and lysed in radioimmunoprecipitation assay (RIPA) buffer (Cell Signalling Technology, Massachusetts, USA) in accordance with the manufacturer's instructions. Protein concentrations were measured with BCA assay (Thermo Fisher Scientific, Massachusetts, USA). Total 20 µg of protein samples were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. Following the blocking with 5% BSA in Tris-buffered saline with Tween TBST solution for 1 h, membranes were probed with primary antibodies against SREBP1c (Abcam, Cambridge, UK, Catalog No: ab3259) and β-actin (Cell Signaling Technology, Massachusetts, USA, Catalog No: 4967) overnight. After washing of unbound primary antibodies with TBST and use of HRP-conjugated secondary antibodies, blots were visualized with chemiluminescence kit (Cell Signaling Technology, Massachusetts, USA). The density of bands was quantified and normalized with β-actin using Image J software.

Immunofluoresence analysis

AML12 cells were seeded on twelve-well glass chamber slides and treated with OA for 24 h with or without SREBP1c silencing. After incubations, cells were fixed in 4% formaldehyde for 15 min, blocked in 10% goat serum, and incubated for overnight at 4°C with indicated antibodies; GRP78 (Cell Signaling, Massachusetts, USA, Catalog No: 3177), phospho IRE1 (Abcam, Massachusetts, USA, Catalog No: ab104157), CHOP (Aviva Systems Biology, California, USA, Catalog No: ARP31591_P050), JNK (Novus Biologicals, Colorado, USA, Catalog No: NBP2-25115) and ATF6 (Novus Biologicals, Colorado, USA, Catalog No: NBP2-76329). Following use of Alexa Fluor 488 or Alexa Fluor 594 secondary antibodies and DAPI, images were captured using a Zeiss LSM700 confocal microscope (Amsterdam, Netherlands) and analyzed using Image J software.

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The average number of fluorescence intensities per cell was recorded in at least thirty cells for each group.

Statistical analysis

Statistical analysis was performed using Prism 4 (GraphPad) software. For determination of statistical significances of differences, one-way ANOVA was performed followed by multiple comparisons using the Student-Newman-Keuls test. P-value less than 0.05 has been accepted to be statistically significant.

3. RESULTS

Inducing lipid accumulation in AML12 cells

Increased lipid droplet formation in hepatocytes is a typical finding of hepatic steatosis. The water-soluble OA:BSA complex has earlier been shown to efficiently stimulate lipid accumulation and used in the literature (8-10). Here in our study, we first confirmed this finding via Oil Red O staining and established an *in vitro* hepatic steatosis model in AML12 cells. As shown in Fig. 1A, AML12 cells were treated with concentrations of 0.06 mM, 0.5 Mm and 1 mM OA to stimulate lipid droplet formation. Analysis of the lipid droplet number per cell revealed a significant increase in all OA treated groups compared to control (Fig. 1B). In addition to Oil Red O data, SREBP1c protein expression was also observed. We determined that exposure of AML12 cells to 0.5 mM OA stimulated the expression of SREBP1c (Fig. 1C) as well as the formation of lipid droplets. In light of these findings, we decided to apply 0.5 mM OA for 24 h in our study, since the major effect to SREBP1c expression was detected in that concentration.

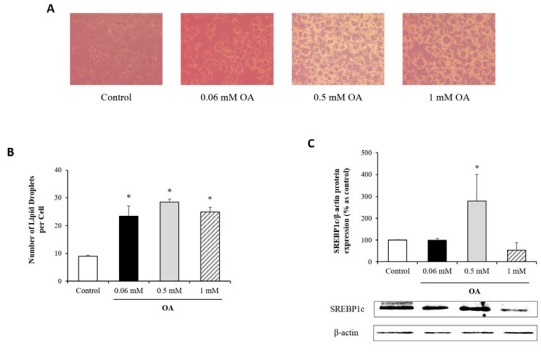

Figure 1. *Inducing lipid accumulation in AML12 cells*
AML12 cells were treated with different concentrations of OA:BSA complex (0.06-0.5-1 mM) for 24 hours. (A) Represantative light microscopic images show lipid droplet formation following Oil Red O staining (400X magnification). (B) Quantification of the numbers of lipid droplet formation per cell. (C) SREBP1c protein expression was analyzed by western blotting followed by densitometric analysis of protein bands and relative ratios were quantified and normalized relative to β-actin.
Data are expressed as mean ± S.D.
**p < 0.05 vs. control, (n=3).*

SREBP1c inhibition and its effect on lipid accumulation

To assess the role of SREBP1c in our *in vitro* hepatic steatosis model, we aimed to reduce SREBP1c levels via siRNA transfection. Therefore, we established different time and siRNA:transfection reagent ratio conditions to determine the most effective inhibition. Protein expression of SREBP1c was effectively reduced in 1:3 siRNA:transfection reagent ratio for 24 h compared to other conditions (Fig. 2A). To confirm the functional impact of SREBP1c knockdown in oleat induced lipid droplet generation, cells were also treated with OA at 0.5 mM concentration for 24 h and stained via Oil Red O (Fig. 2B). Analysis of the images revealed a decrease in lipid droplet formation in SREBP1c siRNA group compared to the control. Differently from non-OA treated ones, siRNA transfection followed by OA administration had no significant effect on lipid droplets compared to OA group (Fig. 2C).

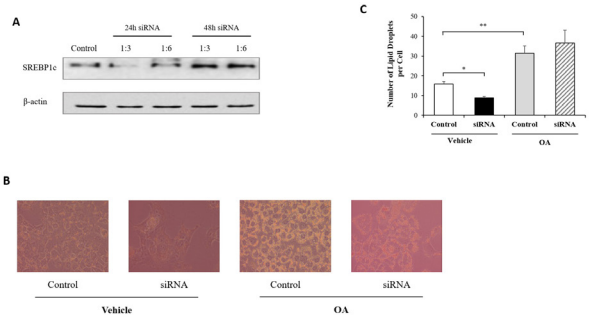

Figure 2. *SREBP1c inhibition and its effect on lipid accumulation*
(A) AML12 cells were transfected with 1:3 and 1:6 siRNA:transfection reagent ratios for either 24 h or 48 h. Protein bands of SREBP-1c and β-actin were visualized with chemiluminescence kit following the use of HRP-conjugated secondary antibodies. After SREBP1c silencing, cells were treated with vehicle (DMEM) or OA:BSA (0.5 mM) to divide into following groups; i) control, ii) SREBP1c siRNA, iii) OA, iv) SREBP1c siRNA + OA. (B) Represantative light microscopic images show lipid droplet formation following Oil Red O staining (400X magnification). (C) Quantification of the numbers of lipid droplet formation per cell.
Data are expressed as mean ± S.D.
***p < 0.01, and *p < 0.05, (n=3).*

Effect of SREBP1c silencing on ER stress and related signaling status following OA administration

After the verification of our steatosis model and SREBP1c silencing, we next tried to gain insight into the importance of SREBP1c signaling in ER stress and related apoptosis. We monitored well-identified parameters of ER stress and apoptosis, including phospho IRE1, GRP78, CHOP, ATF6 and JNK, using immunoflouresence. Microscopic images for selected parameters are shown in Fig. 3A. Anaylsis of images revealed that neither OA application nor siRNA tranfection had any effect on GRP78 expression (Fig. 3B). Activation status of ATF6 is presented as the ratio between nucleus and cytoplasmic levels

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[11]. To calculate ATF6 activation in each group, we measured the ratio between nuclear-and cytoplasmic fluorescence intensities to quantify nuclear localization (N/C). As shown in Fig. 3B, the ratio of nuclear-to-cytoplasmic ATF6 fluorescence intensity was increased in siRNA transfected cells under normal conditions, which was reduced in siRNA transfected cells after OA application. However, the expression of ER stress-related proteins, phospho IRE1 and JNK was up-regulated following OA application, while transfection with SREBP1c siRNA reversed OA induced activation of phosho IRE1 and JNK. CHOP levels was also reduced in SREBP1c silenced cells following OA in spite of no change in OA treated control cells (Fig. 3B).

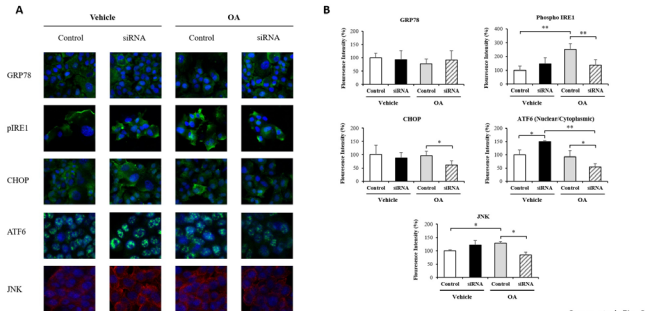

Figure 3. *Effect of SREBP1c silencing on ER stress and related signaling status following OA administration*

AML12 cells were first transfected with SREBP1c siRNA at 1:3 siRNA:transfected reagent ratio for 24 h and then with vehicle (DMEM) or OA:BSA (0.5 mM) to divide into following groups; i) control, ii) SREBP1c siRNA, iii) OA, iv) SREBP1c siRNA + OA. Cells were fixed, and stained for GRP78, phospho IRE1, ATF6, CHOP (green), JNK (red) and nuclei by DAPI (blue). (A) Representative confocal microscopic images of cells from each group (400x magnification). (B) Levels of GRP78, phospho IRE1, nuclear (N) and cytoplasmic (C) forms of ATF6, JNK and CHOP were examined via quantification of relative fluorescence intensities.

Data are expressed as mean ± S.D.

***p < 0.01, and *p < 0.05, (n=3).*

4. DISCUSSION

Non-alcoholic fatty liver disease is one the most common diseases of the metabolic disorder, which is defined by the presence of lipids in more than 5% of liver weight. As a result of increase in obesity and type 2 diabetes mellitus (T2DM) worldwide, the prevalence of NAFLD is rising continuously and is progressing from steatosis to non-alcoholic steatohepatitis (NASH) and liver cirrhosis [12, 13]. According to the literature, in vitro models of steatosis in NAFLD studies can be categorized into four groups; i) primary cell cultures (Hepatocytes, Kupffer cells, stellate cells), ii) immortalized cell lines (AML12, HepG2), iii) co-culture models, and iv) three-dimensional cell cultures [14]. Ethical issues and isolation problems of the primary cell cultures make the immortal cell lines more suitable [14]. In the present study we used AML12 cells in establishing *in vitro* steatosis model due to their extended replicative activity and stable phenotype. Palmitic acid (PA) and OA are two most abundant long-chain free fatty acids in the normal and fatty liver specimens. It has been identified that different combinations of PA and OA induce the steatosis in a manner of dose – and time-dependent [15]. Accordingly, induction of steatosis in human hepatocellular carcinoma (HepG2) cells was established after OA administration [8]. In an another *in vitro* study using rat primary hepatocyte cells, administration of PA and OA combination induced lipid accumulation and cytotoxicity [16]. In our study, we established an *in vitro* hepatic steatosis model by treating AML12 cells with different OA concentrations for 24 hours. We also checked the SREBP1c protein expression to determine the most effective OA dose in inducing SREBP1c activation. Despite the finding of lipid accumulation in all OA administrated cells, only 0.5 mM OA was significantly induced the SREBP1c expression. Therefore, we decided to apply 0.5 mM OA in our further experiments in determining the effect of SREBP1c silencing in a manner of lipid accumulation and ER stress.

Sterol regulatory element binding proteins are important players of lipid metabolism, involved in pathological and physiological conditions, including nutrition, cell growth, energy stress, inflammation. SREBP1c is a transcription factor that is highly expressed in a variety of tissues, including liver, adipose, and skeletal muscle. Its ability to increase the transcription of genes involved in fatty acid and triacylglycerol synthesis has been well identified. SREBP1c overexpression has been shown to induce lipid accumulation insulin resistance, diabetes and NAFLD, in both *in vitro* and *in vivo* studies [17-19]. Conversely, SREBP1c inhibition is reduced the risk of metabolic disorders, such as obesity, atherosclerosis, and fatty liver [20]. In our study, SREBP1c silencing (without OA administration) in AML12 cells resulted in a decrease in lipid droplet formation. Although, inhibition of SREBP1c had no effect against OA induced lipid droplet accumulaiton.

A wide range of cellular disturbances, including high fat diet, impair the efficacy of protein folding in the ER leading to accumulation of misfolded and unfolded proteins [21, 22]. Liver cells cope with ER stress by an adaptive response mechanism, known as unfolded protein response (UPR) [23]. UPR has both cytoprotective and cytotoxic effects. The activity of UPR maintains homeostasis during transient ER stress in normal circumstances. However, under prolonged or excessive ER stress conditions in hepatocytes, UPR fails to recover the normal function of the ER and leads to apoptosis via the upregulation of the pro-apoptotic CHOP and JNK [24-26]. Studies have reported that CHOP is essential for the secretion of proinflammatory factors and hepatocyte apoptosis. In a study, CHOP deletion has been shown to provide resistance in HepG2 cells following palmitate administration [27]. The contribution of ER stress in NAFLD progression was determined either in rats fed a high fat diet [22] or in the livers of patients with NASH [28, 29]. Both IRE1 and ATF6 are well-identified and sensitive ER stress indicators found in ER membrane bound to chaperones [30]. Accumulation of unfolded proteins results in the dissociation of GRP78 from luminal domain of IRE1, thus IRE1 is oligomerized and auto-phosphorylated [31]. Beside its endonuclease activity

that mediates the activation of XBP-1 via splicing the inactive transcript, IRE1 activates apoptosis signal-regulating kinase 1 (ASK1) and phosphorylates JNK by its kinase domain [25, 32]. Apart from IRE1, ATF6 itself acts as a transcription factor that mediates a number of target genes, including GRP78, GRP94 and CHOP [33]. Following the dissociation from GRP78, ATF6 translocates to golgi, undergoes a cleavage, and transports to nucleus [34]. In this regard, increase in nucleus to cytoplasm ratio of ATF6 is accepted as an indicative for ER stress associated ATF6 activation [35]. In our study, we observed the status of ER stress activation via forming OA induced steatosis in AML12 cell line. In this context, OA mediated lipid accumulation induced ER stress in a manner of IRE1 phosphorylation and its downstream JNK without affecting the ratio of nuclear-to-cytoplasmic ATF6 and GRP78 levels, indicating the role of OA in UPR.

Steatosis can be either a cause or consequence in the context of ER stress. Lipid induced ER stress has been shown in several studies [16, 36, 37]. Conversely, it has been demonstrated that chronic ER stress has an important role in lipostasis and transition from steatosis to steatohepatitis [38, 39]. There are a number of well-identified mechanisms which ends up lipogenesis through ER stress, including IRE1 mediated XBP1 activation [40, 41]. There are also many evidences that ER stress induced lipogenesis drives with SREBPs which are main transcription factors of de novo lipogenesis [42, 43]. It has been shown that ER stress initate SCAP independent SREBP1/2 activation in the ER via caspase 2 mediative S1P activation that drives the cell to lipogenesis [44]. Recent studies using *in vitro* and *in vivo* fatty liver models have identified the effect of ER stress in inducing lipid accumulation and SREBP1c activation [45]. Specifically, Damiano et al. [46], observed that tunicamycin-mediated ER stress induction in HepG2 cells was related with SREBP1c activation. In another study using HepG2 and L02 cells, it was found that thapsigargin – induced ER stress increased SREBP1c, FASN, ACC protein expressions along with triglyceride levels, resulting in hepatic steatosis [47]. Despite these studies underline the association between ER stress related de novo lipogenesis and SREBP activation in steatosis progression, the effect of SREBP1c silencing in ER stress activation during OA induced steatosis has not been investigated yet. We now demonstrated that SREBP1c has effect on ER stress in a positive fashion in OA induced steatosis. According to our results, SREBP1c silencing in OA induced lipid steatosis lead to a significant decrease in ER stress (phosho IRE1, ATF6) and ER stress triggered apoptosis (JNK, CHOP) parameters.

In conclusion, SREBP1c might have an individual effect that changes state of affairs in OA-induced lipid accumulation via altered ER stress response. Interestingly, our ATF6 finding suggests that a complex and potential relationship exists between ATF6 and SREBP1c. The discrepancy in the ATF6 intensity following SREBP1c silencing among vehicle and OA groups can be a consequence of response diversity of cells in normal and OA-induced conditions. There are some studies which claimed that ATF6 reduce hepatic steatosis by antagonizing SREBPs [48]. Our results can be an evidence for presumptive bidirectional involvement of SREPB1c which should be investigated with further investigations. Decrease in CHOP and JNK levels in SREBP1c siRNA+OA group also indicated the crucial role of SREBP1c silencing in reducing ER stress mediated apoptosis, the ultimate response to UPR. It is also highlighted that other ER stress sensors, including PERK-ATF4 signaling, might involve in CHOP activation. Therefore, further investigations are needed to clarify whether oleat induced CHOP activation is related to ATF6 or not. Our data collected so far in OA-induced steatosis model gain insight for therapeutic potential of SREBP1c to inhibit the impact of fatty liver by means of ER stress and related apoptosis.

Compliance with Ethical Standards

Ethical Approval

The study protocol was approved by the Marmara University, School of Medicine Ethics Committee (February 01, 2019, protocol number 09.2019.188).

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Conflict of Information

The authors declare no conflict of interest to declare.

Author Contributions

E.S., T.D. and N.K.O. generated the initial idea and conducted experimental design. E.S., T.D., D.D.D. and B.O. performed experiments and analyzed data. T.D. conducted cell culture experiments. E.S. conducted confocal microscopy experiments. All authors performed critical revision of the manuscript and gave final approval of the submitted version.

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