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MicroRNA-101 mediates the suppressive effect of laminar shear stress on mTOR expression in vascular endothelial cells

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ABSTRACT

Shear stress associated with blood flow plays an important role in regulating gene expression and cell function in endothelial cells (ECs). MicroRNAs (miRNAs) are highly conserved, small non-coding RNAs that negatively regulate the expression of target genes by binding to the mRNA 3′-untranslated region (3′UTR) at the posttranscriptional level involved in diverse cellular processes. This study demonstrates that microRNA-101 in response to laminar shear stress (LSS) is involved in the flow regulation of gene expression in ECs. qRT-PCR analysis showed that miR-101 expression was significantly upregulated in human umbilical vein endothelial cells (HUVECs) exposed to 12 dyn/cm^2 laminar shear stress for 12 h. We found that transfection of miR-101 significantly decreased the luciferase activity of plasmid reporter containing the 3′UTR of mammalian target of rapamycin (mTOR) gene. Western analysis revealed that the protein level of mTOR was significantly reduced in ECs transfected with miR-101. Furthermore, miR-101 overexpression induced cell cycle arrest at the G1/S transition and suppressed endothelial cell proliferation. Finally, transfection of miR-101 inhibitors attenuated the suppressive effects of LSS on mTOR expression, which identified the efficacy of loss-of-function of miR-101 in laminar flow-treated ECs. In conclusion, we have demonstrated that upregulation of miR-101 in response to LSS contributes to the suppressive effects of LSS on mTOR expression and EC proliferation. These studies advance our understanding of the posttranscriptional mechanisms by which shear stress modulates endothelial homeostasis.

1. Introduction

Shear stress, the frictional force created by blood flow, plays an essential role in endothelial homeostasis [1] and in the distribution of atherosclerotic lesions [2]. Regions of the arterial tree where low and oscillatory shear stress occurs due to disturbed flow such as vicinity of branch points and curvatures, are prone to atherogenesis. In contrast, steady laminar shear stress (LSS), which results in the reduction of inflammatory factors and inhibition of endothelial cell proliferation, tends to have an athero-protective effect [2].

The molecular mechanisms that mediate the effects of shear stress on endothelial cells remain to be elucidated. Current evidence suggests that shear stress can modulate endothelial biology by regulating gene expression [3]. The transcriptional mechanisms of flow-responsive gene expression have been extensively investigated, with many transcription factors such as NFκB, KLF2 and SIRT1 found to mediate the flow-induced effects on gene expression at the transcriptional level [4–6].

MicroRNAs (miRNAs) are highly conserved, small non-coding RNAs (19–22nt) that posttranscriptionally repress gene expression by inhibiting the translation or inducing the degradation of target mRNAs [7] involved in different cellular processes [8]. Recently, several studies reported that laminar shear stress regulated endothelial gene expression via miRNAs at the posttranscriptional level [9–11], indicating that miRNAs play critical roles in EC functions in response to shear stress. According to these studies, a group of miRNAs are upregulated or downregulated in ECs exposed to laminar flow [9]. This founding requires further investigation. In the present study, we report that miR-101 upregulated in ECs exposed to LSS mediated the suppressive effects of LSS on mammalian target of rapamycin (mTOR) expression and endothelial proliferation. These data advance our understanding of the posttranscriptional mechanisms by which shear stress modulates endothelial homeostasis.

Abbreviations: 3′UTR, 3′-untranslated region; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; LSS, laminar shear stress; miRNA, microRNA; mTOR, mammalian target of rapamycin; qRT-PCR, quantitative real-time polymerase chain reaction.

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2. Methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs, ScienCell) were grown in endothelial cell medium (ECM) supplemented with 10% fetal bovine serum and 1% endothelial growth factor. For all experiments, HUVECs within passage 6 were used. HEK-293T cells were grown in high-glucose DMEM supplemented with 10% FBS. All of cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Shear stress experiments

A parallel-plate flow system was used to impose shear stress on cultured HUVECs as previously described [12]. A glass slide seeded with a confluent monolayer of ECs was mounted on the bottom of the parallel-plate flow chamber (Glycotech, 2.5 cm in width, 5.0 cm in length and 0.025 cm in height). A laminar shear stress of 12 dyn/cm^2 imposed on ECs was generated by perfusing the culture media through the channel between the EC-containing glass slide and an acrylic plate in the flow chamber. The flow system was kept at 37 °C and ventilated with humidified 5% CO_2–95% air.

2.3. Transfection with miRNAs

The miR-101 mimic, negative control (NC), miR-101 inhibitor and inhibitor-negative control (I-NC) were obtained from GenePhama (see online supplement) and transfected into HUVECs at a final concentration of 50 nM using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

2.4. microRNA qRT-PCR

The microRNAs from ECs were isolated using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's protocol. Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription kit (Ambion). Real-time PCR was performed using the TaqMan universal master mix in the LightCycler480 qPCR detection system (Roche). Primers for RT and qPCR were included in TaqMan MicroRNA assays from Ambion (see online supplement). RNU6B was used as a reference for normalization. Relative levels of miRNA were defined from threshold cycle (Ct) values calculated by the $2^{-\triangle\triangle Ct}$ method.

2.5. Western blotting

Total protein extracted from ECs were loaded, subjected to SDS–PAGE and blotted on PVDF membranes. Blots were incubated with primary antibodies against mTOR (Cell Signaling, 1:2000) or beta-actin (Cell Signaling, 1:4000) and an HRP-conjugated secondary antibody, followed by chemiluminescence detection.

2.6. EdU assay

ECs were exposed to 10 μM EdU for 6 h at 37 °C, fixed with 4% formaldehyde for 30 min, and treated with 0.5% Triton X-100 for 10 min at room temperature. After washing with PBS, the cells were reacted with Apollo reaction cocktail for 30 min. Subsequently, the DNA contents of the cells were stained with Hoechst 33342 for 30 min. The images were acquired by fluorescence microscopy and overlapped using Image-Pro Plus 6.0. Apollo staining (red) and Hoechst staining (blue) were separately used to reveal the newly synthesized DNA and total DNA.

2.7. Cell cycle analysis

Cells were labeled with 1 × BrdU for 60 min, after which they were harvested, fixed, and stained with Alexa Fluor 488 and Propidium Iodide using the FlowCellect Bivariate Cell Cycle Kit (Millipore) according to the manufacturer's protocol. Cell cycle was detected using the EPICS XL-MCL flow cytometer (BECKMAN COULTER) and analyzed with WinMDI software.

2.8. Dual-luciferase assays

Bioinformatic analysis of microRNA target sites were performed using the TargetScan and miRanda software, which are available online. The mTOR-3′UTRs containing the miR-101-binding site were amplified by PCR from human genomic DNA using specific primers (see online supplement). The amplified products were restricted and ligated to the XhoI and NotI sites of psiCHECK vectors (Promega). The constructed reporter plasmid was confirmed by sequencing, and termed as psiCHECK-mTOR-3′UTR. HEK-293T cells were cotransfected with psiCHECK-mTOR-3′UTR (100 ng/ml) and either miR-101 or negative control (50 nM) using Lipofectamine 2000 (Invitrogen). Forty-eight hours following transfection, cells were assayed for both firefly luciferase (internal control) and renilla luciferase using the Dual-Luciferase Reporter Assay (Promega) following the manufacturer's protocol [13].

2.9. Statistical analysis

Data are expressed as means ± SEM from at least three independent experiments. Differences between treatment groups and control groups were tested using unpaired Student's *t* test. Analyses were performed using GraphPad Prism version 5.0, and *P* values < 0.05 were considered statistically significant.

3. Results

3.1. Laminar shear stress upregulates miRNA-101 expression

A group of miRNAs were significantly upregulated or downregulated in human umbilical vein endothelial cells (HUVECs) exposed to laminar flow using miRNA microarray approach in previous studies [9]. Here, to further examine the temporal dynamics of miR-101 expression, confluent HUVECs were exposed to 12 dyn/cm^2 laminar shear stress for various time durations. As detected by qRT-PCR, miR-101 level in ECs was significantly increased (about 1.5-fold) after 6 h of shear stress exposure and greatly increased (about 2.5-fold) after 12 h of that in comparison with the static control (Fig. 1 and Supplemental Fig. 1), indicating that miR-101 was upregulated by laminar flow in ECs.

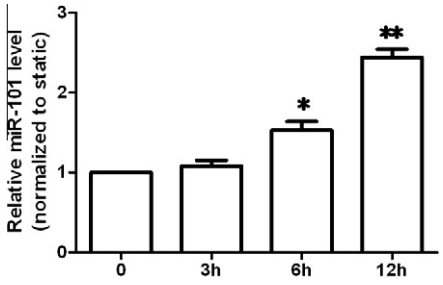

Fig. 1. Expression level of miR-101 in HUVECs exposed to 12 dyn/cm^2 laminar shear stress for various time durations as detected by real-time PCR. Data are presented as the mean ± SEM ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$ LSS versus static control.

3.2. miR-101. represses mTOR expression by targeting its binding site

The base pairing of miRNAs with 3′UTR of target mRNAs leads to the translational suppressive effects of miRNAs on target genes [7]. We performed bioinformatic analysis of the putative molecular targets of miRNA-101 using TargetScan and miRanda software. Sequence alignments of hsa-miR-101 with 3′UTR of human mTOR gene (mTOR) identified an miR-101 binding site (nucleotides 7902–7908 in mTOR) that is highly conserved among vertebrates (Fig. 2A), indicating that mTOR is a potential target gene of miR-101.

To determine whether miR-101 influences the expression of mTOR, miR-101 was transfected into HUVECs, Western blotting analysis revealed that the protein level of mTOR was markedly reduced in ECs overexpressing miR-101 compared with cells transfected with negative control (Fig. 2B). To further examine whether miR-101 directly targets mTOR, the luciferase reporter containing the mTOR-3′UTR fragment with the predicted miR-101 binding site (psiCHECK-mTOR-3′UTR) was co-transfected with miR-101 into HEK-293T cells. Luciferase assays showed that, compared to negative control, transfection with miR-101 resulted in significant decrease in renilla/firefly luciferase activity of reporter (Fig. 2C and Supplemental Fig. 2). Taken together, these results suggest that miR-101 repressed mTOR through a specific 3′UTR binding site.

3.3. Overexpression of miR-101 inhibits endothelial cell proliferation

Cell cycle progression is promoted by mTOR through its downstream effector S6K1, which accelerates the G1/S transition [14]. To examine the influence of miR-101 on endothelial proliferation, ECs transfected with miR-101 were exposed to EdU. Newly synthesized DNA can be indicated by EdU incorporation assay. Fluorescence microscopy showed that the ratio of EdU + cells to total cells in the ECs transfected with miR-101 was significantly reduced compared to the ECs transfected with negative control (Fig. 3A). To investigate the effects of miR-101 on cell cycle, transfected ECs were labeled with BrdU for flow cytometry. Cell cycle analysis showed that transfection of miR-101 led to a lower proportion of cells in S phase and a higher proportion in G0/G1 phase compared with negative control (Fig. 3B), indicating that miR-101 attenuated G1/S transition of cell cycle in ECs. These results suggest that miR-101 controlled cell cycle progression to inhibit the proliferation of ECs. Combined with the results in Part 2, our findings suggest that miR-101 may inhibit endothelial proliferation by repressing mTOR.

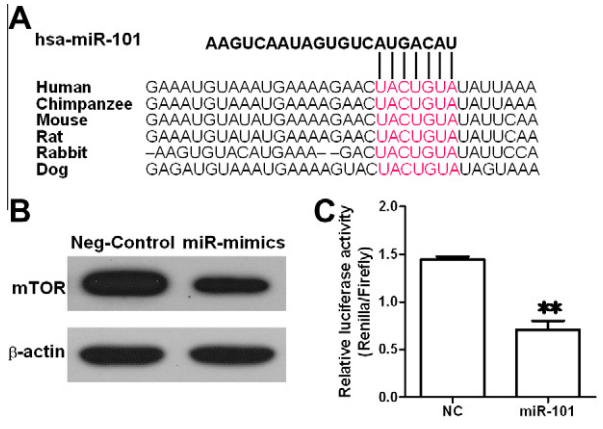

Fig. 2. miR-101 repressed mTOR by targeting its binding site. (A) Bioinformatic analysis shows putative miRNA-101 target sequences on mTOR-3′UTR are highly conserved among vertebrates. (B) Western blot analysis shows that protein level of mTOR decreased in ECs transfected with miR-101. (C) Dual-luciferase activity of the reporter of mTOR-3′UTR containing miR-101 binding site (psiCHECK-mTOR-3′UTR) decreased in ECs transfected with miR-101. Data are presented as the mean ± SEM (n = 3). $^{**}P < 0.01$ miR versus negative control.

3.4. Blockade of miR-101 attenuates the suppressive effect of laminar shear stress on mTOR expression

To investigate the effect of laminar shear stress on mTOR expression, confluent HUVECs were exposed to 12 dyn/cm^2 LSS for 12 h. Western blotting showed that LSS significantly decreased the expression level of mTOR compared with static control (Fig. 4). To examine the effect of loss-of-function of miR-101 in laminar flow-treated ECs, we transfected miR-101 inhibitors into HUVECs, which were subsequently treated with LSS as above. Transfection of the miR-101 inhibitor led to no significant difference in the mTOR expression level between the LSS-treated and static control groups (Fig. 4), indicating that blockade of miR-101 attenuated the suppressive effects of laminar flow on mTOR expression. The results of gain-and-loss of function of miR-101 demonstrate that LSS-induced inhibition of mTOR was under miR-101 control.

4. Discussion

The main findings of this study are that miR-101, one of the miRNAs in ECs regulated by laminar shear stress, mediates the anti-proliferative effect of LSS by directly targeting mTOR.

Shear stress, as an important regulator on ECs function, has been widely investigated in previous studies; however, these studies focused mostly on transcriptional mechanisms. In recent years, with the recognition of microRNAs, the posttranscriptional mechanisms of flow-responsive gene expression have been explored, with several microRNAs induced by shear stress, such as miR-19a [9], miR-21 [10], miR-10a [11], miR-92a [15], miR-23b [16] and miR-663 [17], found to regulate specific genes expression at post-transcriptional level. In this study, we have demonstrated a new approach of microRNAs mediating flow-regulation of EC functions. However, many flow-induced miRNAs biological functions that might be involved in endothelial cell proliferation, apoptosis, inflammation and oxidative stress remain to be further examined.

The suppressive effects of laminar shear stress on endothelial proliferation at posttranscriptional level were supported by the previous findings that LSS-induced miR-19a represses cyclin D1 expression [9] and miR-23b inhibits Rb phosphorylation [16]. Our results provide new evidence that LSS-induced miR-101 exerts its antiproliferative effects by targeting mTOR. Negative regulation of the cell cycle in ECs, that is, inhibition of the G1-phase progression, is the common downstream mechanism of the three approaches [9,16]. In addition, because inhibition of mTOR causes Rb hypophosphorylation [18], miR-101 might enhance the suppressive effect of miR-23b on Rb phosphorylation. Thus, the three miRNAs may act in a coordinated manner to contribute to the LSS-induced suppression of endothelial proliferation.

In addition to being involved in cell proliferation, mTOR also plays an essential role at the early stage of autophagy by negatively regulating autophagy upstream of the Atg1/Ulk complex [19]. Autophagy is a reparative, life-sustaining process involved in the degradation of long-lived proteins and excess or dysfunctional organelles [20]. Autophagy may have an athero-protective effect by safeguarding plaque cells against cellular distress and degrading damaged intracellular material [21]. Thus, the effect of flow-induced miR-101 on endothelial autophagy and the interaction of ECs proliferation and autophagy are worth further investigation, and are being assessed in our ongoing study.

In summary, we have demonstrated that upregulation of miR-101 in response to LSS contributes to the suppressive effects of

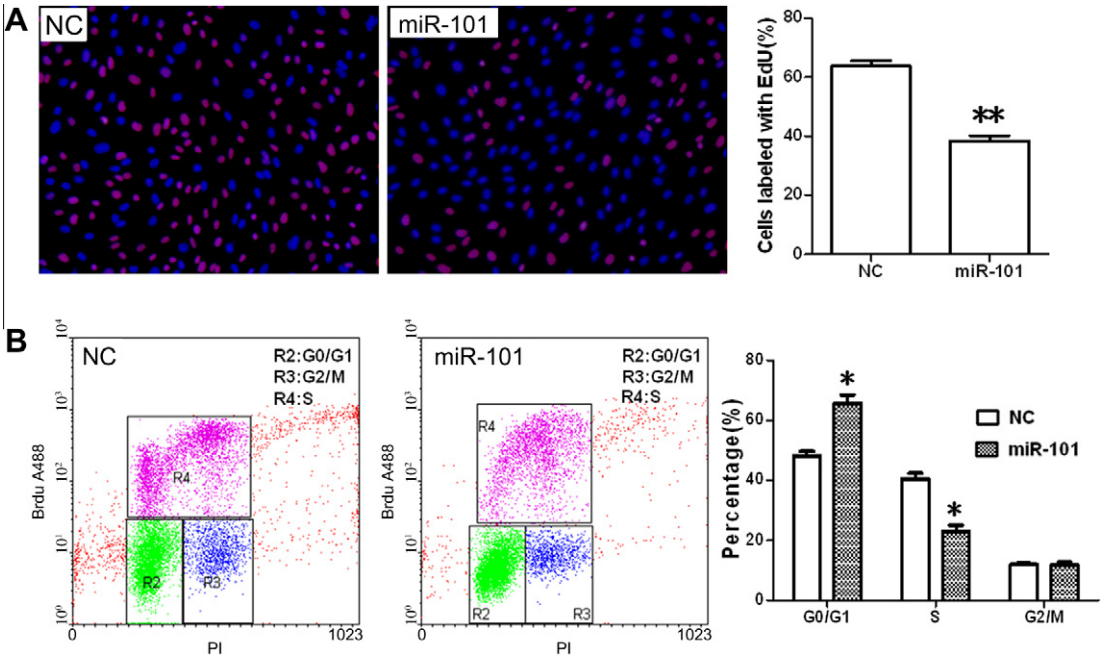

Fig. 3. Overexpression of miR-101 inhibited endothelial cell proliferation. (A) EdU positive cells percentage (EdU%) decreased in ECs transfected with miR-101 detected by fluorescent microscopy (100×). (B) Flow-cytometric analysis shows that the proportion of cells in S phase decreased and that in G0/G1 phase increased in ECs transfected with miR-101. Data are presented as the mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ miR versus negative control.

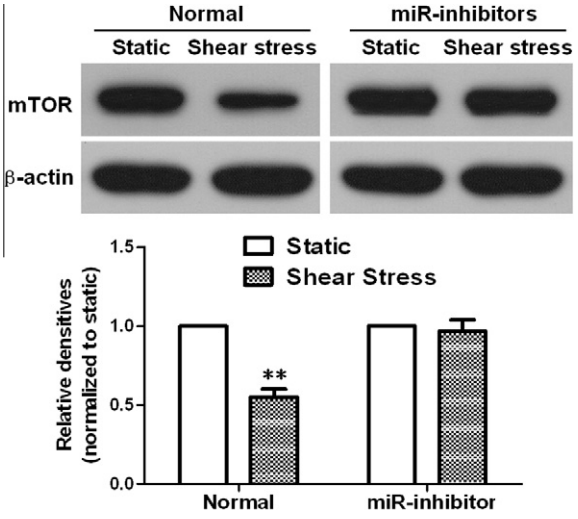

Fig. 4. LSS repressed the expression of mTOR in ECs and blockade of miR-101 attenuated the suppressive effect of LSS on mTOR. Normal: ECs transfected with null; miR-inhibitors: ECs transfected with miR-101 inhibitors. Data are presented as the mean ± SEM ($n = 3$). $^{**}P < 0.01$ LSS versus static control.

LSS on mTOR expression and EC proliferation. These results advance our understanding of the posttranscriptional mechanisms by which shear stress modulates endothelial homeostasis, and provide more evidence for the intervention of cardiovascular disease by biophysical methods such as enhanced external counterpulsation [22,23].

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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.2012.09.026.

References

[1] S. Chien, Mechanotransduction and endothelial cell homeostasis: the wisdom of the cell, Am. J. Physiol. Heart Circ. Physiol. 292 (2007) 1209–1224.
[2] Y.S. Chatzizisis, A.U. Coskun, M. Jonas, E.R. Edelman, C.L. Feldman, P.H. Stone, Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behavior, J. Am. Coll. Cardiol. 49 (2007) 2379–2393.
[3] O. Traub, B.C. Berk, Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force, Arterioscler. Thromb. Vasc. Biol. 18 (1998) 677–685.
[4] J. Partridge, H. Carlsen, K. Enesa, H. Chaudhury, M. Zakkar, L. Luong, A. Kinderlerer, M. Johns, R. Blomhoff, J.C. Mason, D.O. Haskard, P.C. Evans, Laminar shear stress acts as a switch to regulate divergent functions of NF-kappaB in endothelial cells, FASEB J. 21 (2007) 3553–3561.
[5] A.R. Kinderlerer, F. Ali, M. Johns, E.A. Lidington, V. Leung, J.J. Boyle, S.S. Hamdulay, P.C. Evans, D.O. Haskard, J.C. Mason, KLF2-dependent, shear stress-induced expression of CD59: a novel cytoprotective mechanism against complement-mediated injury in the vasculature, J. Biol. Chem. 283 (2008) 14636–14644.
[6] Z. Chen, I.C. Peng, X. Cui, Y.S. Li, S. Chien, J.Y. Shyy, Shear stress, SIRT1, and vascular homeostasis, Proc. Natl. Acad. Sci. USA 107 (2010) 10268–10273.
[7] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[8] J. Krol, I. Loedige, W. Filipowicz, The widespread regulation of microRNA biogenesis, function and decay, Nat. Rev. Genet. 11 (2010) 597–610.
[9] X. Qin, X. Wang, Y. Wang, Z. Tang, Q. Cui, J. Xi, Y.S. Li, S. Chien, N. Wang, MicroRNA-19a mediates the suppressive effect of laminar flow on cyclin D1 expression in human umbilical vein endothelial cells, Proc. Natl. Acad. Sci. USA 107 (2010) 3240–3244.
[10] M. Weber, M.B. Baker, J.P. Moore, C.D. Searles, MiR-21 is induced in endothelial cells by shear stress and modulates apoptosis and eNOS activity, Biochem. Biophys. Res. Commun. 393 (2010) 643–648.

[11] Y. Fang, C. Shi, E. Manduchi, M. Civelek, P.F. Davies, MicroRNA-10a regulation of proinflammatory phenotype in athero-susceptible endothelium in vivo and in vitro, Proc. Natl. Acad. Sci. USA 107 (2010) 13450–13455.

[12] N. Wang, H. Miao, Y.S. Li, P. Zhang, J.H. Haga, Y. Hu, A. Young, S. Yuan, P. Nguyen, C.C. Wu, S. Chien, Shear stress regulation of Kruppel-like factor 2 expression is flow pattern-specific, Biochem. Biophys. Res. Commun. 341 (2006) 1244–1251.

[13] M. Crawford, E. Brawner, K. Batte, L. Yu, M.G. Hunter, G.A. Otterson, G. Nuovo, C.B. Marsh, S.P. Nana-Sinkam, MicroRNA-126 inhibits invasion in non-small cell lung carcinoma cell lines, Biochem. Biophys. Res. Commun. 373 (2008) 607–612.

[14] D.C. Fingar, C.J. Richardson, A.R. Tee, L. Cheatham, C. Tsou, J. Blenis, mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E, Mol. Cell Biol. 24 (2004) 200–216.

[15] W. Wu, H. Xiao, A. Laguna-Fernandez, G.J. Villarreal, K.C. Wang, G.G. Geary, Y. Zhang, W.C. Wang, H.D. Huang, J. Zhou, Y.S. Li, S. Chien, G. Garcia-Cardena, J.Y. Shyy, Flow-dependent regulation of Kruppel-like factor 2 is mediated by microRNA-92a, Circulation 124 (2011) 633–641.

[16] K.C. Wang, L.X. Garmire, A. Young, P. Nguyen, A. Trinh, S. Subramaniam, N. Wang, J.Y. Shyy, Y.S. Li, S. Chien, Role of microRNA-23b in flow-regulation of Rb phosphorylation and endothelial cell growth, Proc. Natl. Acad. Sci. USA 107 (2010) 3234–3239.

[17] C.W. Ni, H. Qiu, H. Jo, MicroRNA-663 upregulated by oscillatory shear stress plays a role in inflammatory response of endothelial cells, Am. J. Physiol. Heart Circ. Physiol. 300 (2011) 1762–1769.

[18] N. Gao, Z. Zhang, B.H. Jiang, X. Shi, Role of PI3K/AKT/mTOR signaling in the cell cycle progression of human prostate cancer, Biochem. Biophys. Res. Commun. 310 (2003) 1124–1132.

[19] B. Ravikumar, C. Vacher, Z. Berger, J.E. Davies, S. Luo, L.G. Oroz, F. Scaravilli, D.F. Easton, R. Duden, C.J. O'Kane, D.C. Rubinsztein, Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease, Nat. Genet. 36 (2004) 585–595.

[20] D.J. Klionsky, S.D. Emr, Autophagy as a regulated pathway of cellular degradation, Science 290 (2000) 1717–1721.

[21] W. Martinet, G.R. De Meyer, Autophagy in atherosclerosis: a cell survival and death phenomenon with therapeutic potential, Circ. Res. 104 (2009) 304–317.

[22] Y. Zhang, X. He, X. Chen, H. Ma, D. Liu, J. Luo, Z. Du, Y. Jin, Y. Xiong, J. He, D. Fang, K. Wang, W.E. Lawson, J.C. Hui, Z. Zheng, G. Wu, Enhanced external counterpulsation inhibits intimal hyperplasia by modifying shear stress responsive gene expression in hypercholesterolemic pigs, Circulation 116 (2007) 526–534.

[23] Y. Zhang, X. He, D. Liu, G. Wu, X. Chen, H. Ma, Z. Du, Y. Dong, Y. Jin, W. He, K. Wang, W.E. Lawson, J.C. Hui, Z. Zheng, Enhanced external counterpulsation attenuates atherosclerosis progression through modulation of proinflammatory signal pathway, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 773–780.