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Identification of MicroRNAs with Altered Expression Profiles in a Rat Model of Experimentally Induced Early Cerebral Aneurysms

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Objective: Structural adaptation of the vascular wall may occur due to various factors, such as shear stress, pressure, injury or inflammation. The role of microRNAs (miRNAs) in the development of vascular remodeling has been investigated in several studies. Recently, the authors reported altered expression profiles of miRNAs in late stage of experimentally induced giant cerebral aneurysm (CA) in rat models. But, early biologic roles of miRNAs in CA formation have not been explained yet. We employed microarrays analysis to identify miRNA expression profiles in early stage of CA in rat model and to compare with those in late stage of giant CA.

Methods: Seventy, 7-week-old male Sprague-Dawley rats underwent a CA induction procedure. The control animals (n=11) were fed a regular diet, and the experimental animals (n=59) were fed a regular diet with 1% normal saline for two months. Then, the rats were killed, their cerebral arteries were dissected, and the 13 regions of early aneurysmal change on the right olfactory artery-anterior cerebral artery bifurcation were cut for miRNA microarrays analysis. Six miRNAs (miRNA-1, miRNA-448, miRNA-352, miRNA-551b, miRNA-431, and miRNA-485) were randomly chosen for validation using real-time quantitative polymerase chain reaction.

Results: Among a set of differentially expressed miRNAs, 15 miRNAs were up-regulated more than 200% and five miRNAs were down-regulated less than 50% in the early CA tissues.

Conclusions: This study provides an overall view of miRNA expression profiles in experimentally induced early CAs and strongly supports the idea that some miRNAs, such as miR-31 and miR-27a, play an important role in pathological processes in early CA formation. Further investigations to detect their exact roles of these miRNAs in the pathogenesis of CA are needed.
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KEY WORDS: Intracranial aneurysm · MicroRNAs · Vascular remodelling · Smooth muscle cell · Endothelial cell · Macrophage.

Introduction

MicroRNAs (miRNAs) are endogenous small (19–24 nucleotide) non-coding sequences of RNA that negatively control gene expression at a post-transcriptional level either through translational inhibition or degradation of target mRNAs.[3] Specialized sets of these miRNAs play an essential part in regulating cell fate and tissue homeostasis including in smooth muscle cells (SMCs), endothelial cells (ECs), macrophage and extracellular matrix (ECM).[8,18]

Vascular remodeling is adaptive alterations of arterial wall architecture caused by a various sequence of stimuli, such as injury, inflammation, pressure, or shear stress. Vascular remodeling is formed in all vascular cell types, including SMCs and ECs and involves the reconstruction of the ECM. For instance, wavering up and down in shear stress contrib-

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utes to miRNA-regulated differential gene expression in ECs, which is essential for maintenance of vascular physiology.[14] Vascular remodeling results in differential expression of numerous miRNAs, triggering the balance between susceptibility and resistance to cardiovascular diseases. Furthermore, inflammatory cell recruitment, particularly of monocytes and macrophages, plays a key role in vascular remodeling by controlling SMC function and ECM turnover.[17]

Further elucidation of the regulation of miRNAs that have specific functions in vascular remodeling may allow future clinical applications of miRNAs as new class of targets in diagnostic and therapeutic tools and prevention of vascular diseases.

In this study, we used microarrays analysis to identify differential expression of miRNAs in early stage of experimentally induced cerebral aneurysm (CA) in rat model. We also compare with those in late stage of CA to detect possible targets of miRNAs for further proving their exact roles in the pathogenesis of CA.

Materials and Methods

Induction of experimental cerebral aneurysms

For inducing CAs, ligation of the left common carotid artery (CCA) and the posterior branches of both renal arteries was performed in the 70 male 7-week-old Sprague-Dawley strain rats (200 to 300 g). These procedures were done under intraperitoneal zoletil anesthesia (30 mg/kg) with xylazin (10 mg/kg) and additional injections if necessary. After the operation, 1% normal saline was fed for the drinking water to promote the degree of hypertension.

The rats were divided into two group as control group (n=11) receiving, no procedure with regular diet and aneurysm group (n=59) receiving ligation of the left CCA and the posterior branches of both renal arteries with regular diet and 1% normal saline.

Animal care and experiments in this study were done according to institutional animal care and use committee standards on the care and use of laboratory animals.

Tissue preparation

Two months after the induction procedures, all rats were euthanized with CO gas. Dissection and strip of cerebral arteries from their brains was performed under a surgical microscope. We obtained the samples from the right olfactory artery-anterior cerebral artery (OA-ACA) bifurcation area in the control group (n=11) and from 13 regions of early aneurysmal change on the right OA-ACA bifurcation in the aneurysm group (Figure 1).

RNA isolation

Thirteen early aneurysm samples and eleven control samples were homogenised with tissue lyser2 (Qiagen, Germantown, MD). Trizol (Invitrogen, Carlsbad, CA) was used for total RNA extraction in accordance with the manufacturer's instructions. For microarray and quantitative PCR (qPCR) analysis, low molecular weight RNA (<200 nucleotide) was isolated from the total RNA with mirVana miRNA Isolation Kit (Ambion Applied Biosystems, Austin, TX) in accordance with the manufacturer's instructions. The quality and quantity of each RNA preparation were assessed by a Nanodrop ND-1000 spectrophotometer (Agilent Technologies UK Ltd., West Lothian, UK).

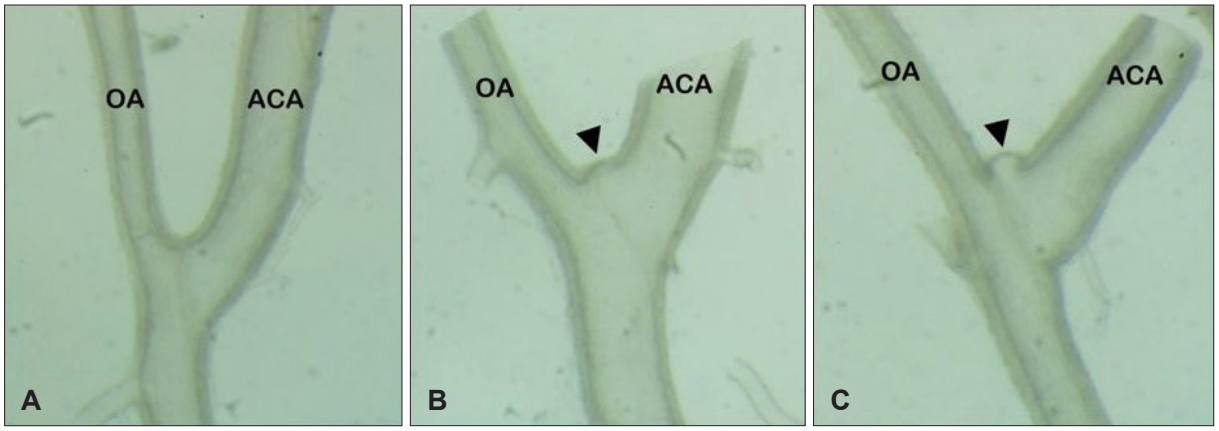

FIGURE 1. Two months after the induction procedures. The aneurysm samples of different stages were obtained from the right olfactory artery-anterior cerebral artery (OA-ACA) bifurcation area. A: Normal OA-ACA bifurcation. B: Early cerebral aneurysm at OA-ACA bifurcation (black arrowhead). C: Advanced cerebral aneurysm at OA-ACA bifurcation (black arrowhead). ACA: anterior cerebral artery, OA: olfactory artery (×400 magnification).

Microarray study

Each total RNA sample (700 ng) was labeled and hybridized using FlashTag™ Biotin HSR RNA Labeling kit (Genisphere LLC, Hatfield, PA). Total RNA was labeled using poly A polymerase. Biotin-labeled RNA were hybridized for 16−18 hr at 45C on Affymetrix miRNA v2.0 array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450, and then were scanned using the Affymetrix GeneChip Scanner 3000 7G. The data were analyzed with RMA-DABG using normalization method.

The normalized, and log transformed intensity values were analyzed using Expression Console (Affymetrix, Inc.). Fold change filters included the requirements that the genes be present in more than 200% of controls for over-expressed miRNA and lower than 50% of controls for down-expressed miRNA.

Reverse transcription and real-time quantitative polymerase chain reaction

Six miRNAs including three up-regulated (miRNA-1, miRNA-448, and miRNA-352) and three down-regulated (miRNA-551b, miRNA-431, and miRNA-485) miRNAs which showed significant differences in their levels with adjusted $p<0.05$ in the microarray experiment were randomly selected for real-time qPCR validation. Total RNA was extracted from early aneurysm part using NucleoSpin RNA II (Marcherey-Nagel, Düren, Germany). Reverse transcription was performed with mature miRNA-specific primer sets (Applied Biosystems, Foster City, CA) and miRNA reverse transcription kit (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. The miRNA-specific Taqman-based probes were purchased from Ambion Applied Biosystems and real time PCR was performed with the 7500 Fast Real Time PCR System (Ambion Applied Biosystem, Foster City, CA). The relative expression levels of the miRNA were calculated using the comparative Ct (2-DDCt) method with U6 small nuclear RNA as an internal control. All reactions were performed in triplicate for each sample.

Results

Expression profiles of microRNAs in cerebral aneurysms and control arteries

The relative expression levels of the miRNA were found to be significantly different between these two groups as shown in Figure 2. Fold change filters included the requirements that the genes be present in more than 200% of controls for over-expressed miRNA and less than 50% of con-

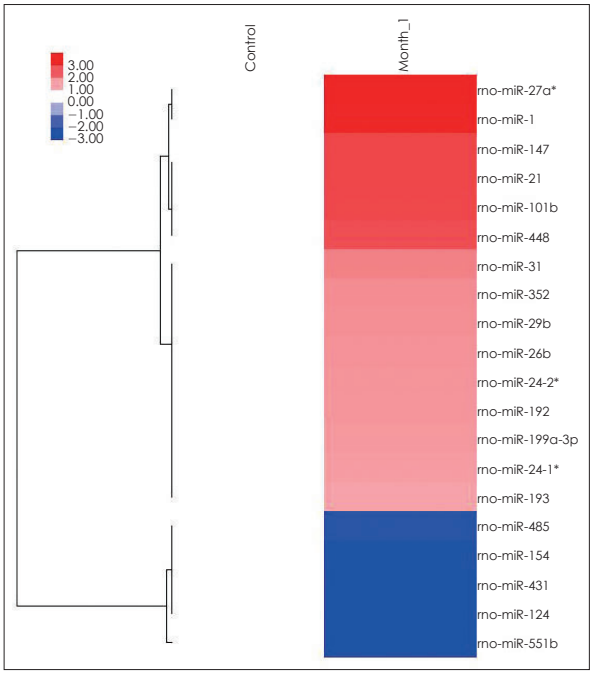

FIGURE 2. Hierarchical clustering analysis of miRNA expression of early cerebral aneurysms and control cerebral arteries. miRNAs are presented in rows and samples are presented in columns. Colors indicate relative signal intensities; red and blue colors indicate up-expressed and down-expressed miRNAs, respectively.

TABLE 1. The list of altered microRNA expression in the early cerebral aneurysm tissue

miRNA	Fold change
Up-expressed miRNAs	
rno-miR-27a*	8.05
rno-miR-1	7.43
rno-miR-147	4.85
rno-miR-21	4.84
rno-miR-101b	4.74
rno-miR-448	4.45
rno-miR-31	2.70
rno-miR-352	2.42
rno-miR-29b	2.36
rno-miR-26b	2.30
rno-miR-24-2*	2.26
rno-miR-192	2.24
rno-miR-199a-3p	2.19
rno-miR-24-1*	2.09
rno-miR-193	2.02
Down-expressed miRNAs	
rno-miR-551b	14.93
rno-miR-124	9.21
rno-miR-431	8.52
rno-miR-154	8.40
rno-miR-485	6.54

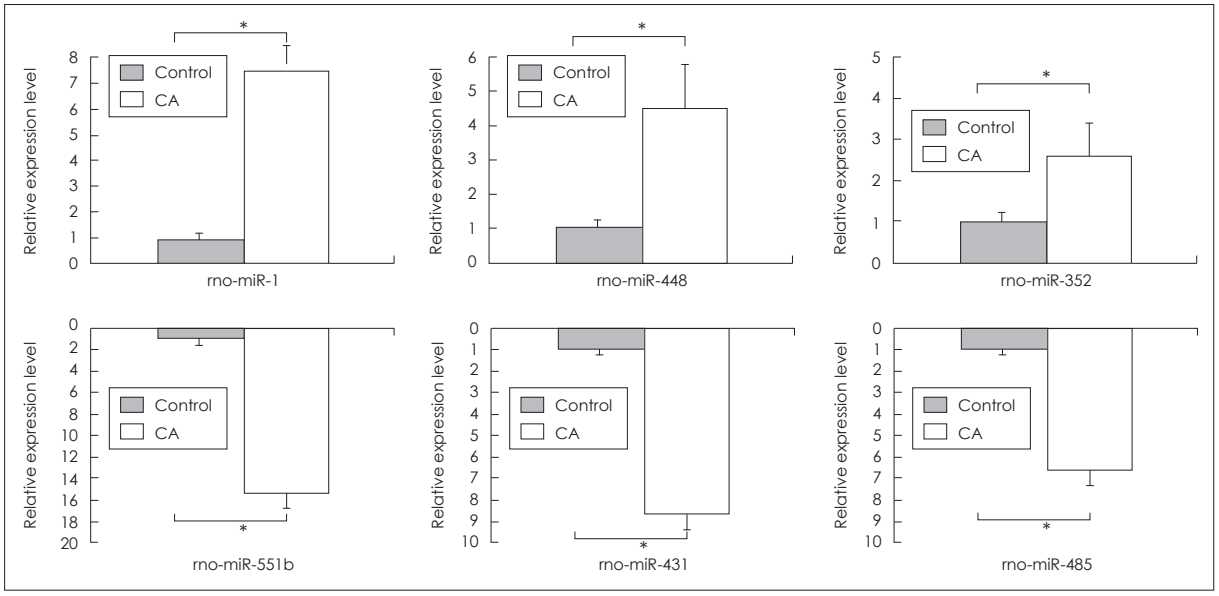

FIGURE 3. Summary of real-time quantitative polymerase chain reaction (qPCR) analysis for the up-expressed miRNAs (miRNA-1, miRNA-448, and miRNA-352) and the down-expressed miRNAs (miRNA-551b, miRNA-431, and miRNA-485). Six randomly selected miRNAs are listed on the x-axis, and relative expression levels are placed on the y-axis as up and down direction. *significant difference between CAs and control arteries (p<0.05). CA: cerebral aneurysm.

trols for down-expressed miRNA (Table 1). Among a set of differentially expressed miRNAs, 15 miRNAs were significantly up-regulated and five miRNAs were significantly down-regulated in the early CA tissues as compared with that in the arteries of the control group.

Validation of the microarray analysis data by real-time quantitative polymerase chain reaction

The relative expression levels of these miRNA analyzed by real-time qPCR were consistent with the microarray analysis results, as shown in the histogram ($p<0.05$) in Figure 3.

Statistical analysis

The p values were calculated using Student's t-test using statistical program SPSS version 13 (SPSS Inc., Chicago, IL). Differences were considered statistically significant at $p<0.05$.

Discussion

In recent miRNAs and arterial remodeling studies, it has been reported that many miRNAs drive phenotypic changes of SMCs, oscillating between a synthetic and contractile phenotype. These miRNAs are miR-145, miR-143, miR-133, miR-21, miR-1, miR-221, miR-222, miR-31, miR-146a, miR-26a, and miR-24.[1,4,6,7,9,12,13,16,21] In current study, miR-31 (2.7 fold), miR-1 (7.4 fold), miR-21 (4.8 fold), miR-24 family [miR-24-2* (2.3 fold) and miR-24-1* (2.1 fold)], and miR-26 family [miR-26b (2.3 fold)] were over-expressed.

Increased miR-21 expression may be caused by anti-apoptotic and a pro-proliferative response of SMCs within the vascular wall, most likely in an attempt to keep the vascular wall from ultimate rupture and further expansion. But, miR-21 expression is also up-regulated in dedifferentiated SMCs and represses SMC differentiation markers.[9,21] These studies back up the idea that the accurate role of miR-21 in the differentiation of SMCs is debatable. Up-regulated expression of miR-24 negatively controlled the TGF-β signaling pathway and induced myogenic activity by SMCs phenotype switch.[4]

In our former study about late staged giant CA in rat model, up-regulated expression of miR-1, miR-21, miR-24 family (miR-24-1-5p), and miR-26 family (miR-26b) were identified.[10] However, over-expression of miR-31 was identified only in early aneurysm sample. miR-31 is also profusely expressed in SMCs and up-regulated during proliferation of SMCs and neointima formation.[12] Therefore, these studies support the idea that miR-31 may play a protective role by a miRNAs network that promotes SMC proliferation in early aneurysm formation upon hemodynamic stress.

The recent studies show that a miRNAs networks in ECs modulate the response to atherogenic stimuli and hemodynamic stress.[5,15,21] ECs are placed at the border between the circulatory flow and the vascular wall. As a result, they are highly adapted to several forms of hemodynamic force, such as shear stress, circumferential wall stress and cyclic

stretch, generated by circulating blood. Moreover, they act as a trigger of the vascular response to these stimuli as effecter cell type. Low endothelial shear stress causes miRNAs that enhance the activation of endothelial cells and inflammatory gene expression. These enhanced gene expression progress towards formation of atherosclerotic plaques at curvatures or bifurcations of vascular wall.[5] On the contrary, high endothelial shear stress (HESS) is atheroprotective and up-regulates miRNAs that inhibit the inflammatory response and endothelial proliferation.

Up-regulated expression of miR-21 by HESS regulates apoptosis and induces atheroprotective nitric oxide production through control of endothelial nitric oxide synthase (eNOS) activity.[20] The deficiency of eNOS could be made compensation by neuronal NOS up-regulation in cerebral arteries. The eNOS and nNOS had a protective role in CA formation.[2] These experimental findings have suggested that up-regulated expression of miR-21 had a protective role in early CA formation. In the latest study, miR-101 has been established that it is induced by HESS mediating an atheroprotective role.[14]

In comparison with results of late giant CA samples, highly up-regulated expression of miR-27a (8.1 fold) was identified only in early CA sample. The latest study showed that miR-27a expression was significantly increased in vascular endothelial growth factor-treated breast cancer stem like cells. It means that miR-27a promotes angiogenesis by mediating endothelial differentiation.

The miR-147 has been identified to limit the inflammatory response following toll-like-receptors stimulation in stimulated macrophages and monocytes that make up a negative feedback loop.[11] In this study, up-regulated expression of miR-147 (4.9 fold) was identified. Our previous study about late staged giant CA in rat model showed up-regulated expression of miR-147 (6.6 fold) and miR-223 (2.8 fold).[10] These results have suggested that over-expression of miR-147 may play a protective role in early and late giant CA formation through anti-inflammatory effect.

Among the down-regulated miRNAs proved in this study, down-regulation of miR-124 promotes the growth and metastasis of glioblastoma cells.[22] This result suggests that down-regulated miR-124 and miR-154 can suppress the apoptosis and promote the cell proliferation.

In recent study, highly down-regulated miR-551b, miR-431 and miR-485 and highly up-regulated miR-448 and miR-352 have been revealed but accurate roles of these miRNAs in vascular remodeling have not been fully investigated till now.

Conclusion

This study provides an overall view of miRNA expression profiles in experimentally induced early CAs and strongly supports the idea that some miRNAs, such as miR-31 and miR-27a, play an important role in pathological processes in early CA formation compared with late stage of giant CA formation. Further investigations are needed to detect possible targets of miRNAs for further proving their exact roles in the pathogenesis of CA.

■ The authors have no financial conflicts of interest.

REFERENCES

1) Albinsson S, Suarez Y, Skoura A, Offermanns S, Miano JM, Sessa WC. MicroRNAs are necessary for vascular smooth muscle growth, differentiation, and function. **Arterioscler Thromb Vasc Biol 30**:1118-1126, 2010
2) Aoki T, Nishimura M, Kataoka H, Ishibashi R, Nozaki K, Miyamoto S. Complementary inhibition of cerebral aneurysm formation by eNOS and nNOS. **Lab Invest 91**:619-626, 2011
3) Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. **Cell 116**:281-297, 2004
4) Chan MC, Hilyard AC, Wu C, Davis BN, Hill NS, Lal A, et al. Molecular basis for antagonism between PDGF and the TGFbeta family of signalling pathways by control of miR-24 expression. **EMBO J 29**:559-573, 2010
5) Chatzizisis YS, Coskun AU, Jonas M, Edelman ER, Feldman CL, Stone PH. 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**:2379-2393, 2007
6) Cheng Y, Liu X, Yang J, Lin Y, Xu DZ, Lu Q, et al. MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. **Circ Res 105**:158-166, 2009
7) Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, et al. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. **Nature 460**:705-710, 2009
8) Fazi F, Nervi C. MicroRNA: basic mechanisms and transcriptional regulatory networks for cell fate determination. **Cardiovasc Res 79**:553-561, 2008
9) Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, et al. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. **Circ Res 100**:1579-1588, 2007
10) Lee HJ, Yi JS, Lee HJ, Lee IW, Park KC, Yang JH. Dysregulated Expression Profiles of MicroRNAs of Experimentally Induced Cerebral Aneurysms in Rats. **J Korean Neurosurg Soc 53**:72-76, 2013
11) Liu G, Friggeri A, Yang Y, Park YJ, Tsuruta Y, Abraham E. miR-147, a microRNA that is induced upon Toll-like receptor stimulation, regulates murine macrophage inflammatory responses. **Proc Natl Acad Sci U S A 106**:15819-15824, 2009
12) Liu X, Cheng Y, Chen X, Yang J, Xu L, Zhang C. MicroRNA-31 regulated by the extracellular regulated kinase is involved in vascular smooth muscle cell growth via large tumor suppressor homolog 2. **J Biol Chem 286**:42371-42380, 2011
13) Liu X, Cheng Y, Zhang S, Lin Y, Yang J, Zhang C. A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia. **Circ Res 104**:476-487, 2009
14) Neth P, Nazari-Jahantigh M, Schober A, Weber C. MicroRNAs in

flow-dependent vascular remodelling. **Cardiovasc Res 99**:294-303, 2013
15) Nicoli S, Standley C, Walker P, Hurlstone A, Fogarty KE, Lawson ND. MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. **Nature 464**:1196-1200, 2010
16) Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. **Physiol Rev 84**:767-801, 2004
17) Schober A, Zernecke A. Chemokines in vascular remodeling. **Thromb Haemost 97**:730-737, 2007
18) Small EM, Olson EN. Pervasive roles of microRNAs in cardiovascular biology. **Nature 469**:336-342, 2011
19) Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. **Dev Cell 15**:261-271, 2008
20) Weber M, Baker MB, Moore JP, Searles CD. MiR-21 is induced in endothelial cells by shear stress and modulates apoptosis and eNOS activity. **Biochem Biophys Res Commun 393**:643-648, 2010
21) Yang G, Pei Y, Cao Q, Wang R. MicroRNA-21 represses human cystathionine gamma-lyase expression by targeting at specificity protein-1 in smooth muscle cells. **J Cell Physiol 227**:3192-3200, 2012
22) Zhao WH, Wu SQ, Zhang YD. Downregulation of miR-124 promotes the growth and invasiveness of glioblastoma cells involving upregulation of PPP1R13L. **Int J Mol Med 32**:101-107, 2013