Differentially expressed microRNAs in TGFβ2-induced epithelial-mesenchymal transition in retinal pigment epithelium cells

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Abstract. The epithelial-mesenchymal transition (EMT) of retinal pigment epithelium (RPE) cells plays a key role in proliferative vitreoretinopathy (PVR) and proliferative diabetic retinopathy (PDR), both of which lead to severe loss of vision. Recently, microRNAs (miRNAs) have been found to be involved in the regulation of various physiological and pathological processes, such as embryogenesis, organ development, oncogenesis and angiogenesis. However, the expression profile and function of miRNAs in the EMT of RPE cells remain to be clarified. In this study, human miRNA expression profiles were identified using microarrays and 304 miRNAs were found to be differentially expressed in TGFβ2-induced EMT in human RPE cells. Of these differentially expressed miRNAs, 185 miRNAs were downregulated and 119 miRNAs were upregulated at least 2-fold in TGFβ2 treatment samples. Similar alterations of miRNA expression were validated for 35 representative miRNAs by quantitative polymerase chain reaction analysis. Therefore, these results suggested that differentially expressed miRNAs play potential roles in TGFβ2-induced EMT in RPE cells. This is an essential step in the identification of miRNAs associated with PVR and PDR progression, and in the identification of potential therapeutic targets for these diseases.

Introduction

Intraocular fibrotic disorders, such as proliferative vitreoretinopathy (PVR) and proliferative diabetic retinopathy (PDR), are major causes of severe visual impairment in patients with diabetic retinopathy (DR) and rhegmatogenous retinal detachment (RRD). Fibrotic lesions on the retina reduce the flexibility of retina, induce retinal detachment, and result in difficulty in retinal reattachment and aggravation of visual acuity (1). Despite the improvement of surgical techniques and the development of anti-angiogenic agents, there is still no satisfactory therapy for PVR and PDR. Thus a better understanding of the mechanism involved in these diseases is critical for the development of effective treatments.

Mounting evidence shows that epithelial-mesenchymal transition (EMT) is a major pathophysiologic change in the development and progression of fibrotic lesions, including PDR and PVR. Excessive wound healing and stimulation of inflammatory cytokines lead to EMT, thereby resulting in the formation of pre- or sub-retinal fibrous membranes (2). It is widely recognized that retinal pigment epithelial (RPE) cells are the main contributors to the development of fibrosis on the retina (3), although other cell types including hyalocytes, retinal Müller glial cells, fibroblasts and macrophages are also involved in this process (4). This process is initially triggered by a variety of cytokines, typically transforming growth factor β (TGFβ), which has been well documented to promote various types of fibrotic diseases (5), including PVR and PDR (6,7). Once activated, trans-differentiated RPE cells are capable of migrating into the intraretinal layers or vitreous body, produce extracellular matrix (ECM) components, and transform into fibroblast-like cells, which results in the formation of epiretinal membranes that can contract and cause retinal detachment and visual impairment (6,7). Therefore, agents that are able to prevent the EMT of RPE cells may be of great therapeutic value in preventing retinal fibrosis for RRD and DR patients.

microRNAs (miRNAs) have been found to be involved in the regulation of complex physiological and pathological processes, such as embryogenesis (8), organ development (9), oncogenesis (10) and angiogenesis (11). miRNAs are an extensive class of 18-24 nt non-coding RNAs that regulate gene expression at the post-transcriptional level. By interacting with multiple mRNAs, miRNAs are able to induce translation suppression or degradation of mRNA (12,13). miRNAs have emerged as potent regulators of EMT and

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mesenchymal-epithelial transition (MET), with their abilities to target multiple components involved in the EMT transcription factors and epithelial integrity. Several miRNAs have been shown to directly target families of EMT transcription factors. For instance, the downregulation of miR-200 leads to the upregulation of ZEB1 and ZEB2 expression, and promotes EMT progression. Conversely, the forced expression of miR-200 prevents EMT and enhances MET (14). Snail, a classical transcription factor promoting EMT, is targeted by several miRNAs, including miR-29b and miR-34a. Enhanced expression of miR-29b in metastatic prostate cancer cells can reverse EMT and inhibit the invasive phenotype (15). miR-34a also downregulates Snail and induces MET, while the suppression of miR-34a/b/c leads to the upregulation of Snail and EMT markers conversely (16). In addition to Snail, miR-34a represses the expression of Slug and ZEB1 (16). Moreover, miRNAs affect the integrity of epithelial architecture during EMT progression. During TGFβ-induced EMT in rat kidney epithelial cells, miR-491-5p targets Par3, an epithelial polarity complex protein, and then contributes to the destabilization of tight junctions, a major step in the initiation of EMT (17). By affecting EMT and MET processes, miRNAs are involved in the regulation of stem cell pluripotency, tumor metastasis and progression, as well as fibrosis.

Despite the increasing evidence of miRNAs in EMT and fibrosis in several organs, the role of miRNAs in RPE cells EMT is largely unknown. In the current study, we determined the miRNA expression profile in TGFβ2-induced EMT in RPE cells by microarray. A total of 35 representative miRNAs were confirmed by quantitative polymerase chain reaction (qPCR). The results suggested that miRNAs play critical roles in TGFβ2-induced EMT in human RPE cells and may contribute to the development of PVR and PDR. This is an essential step in the identification of miRNAs associated with PVR and PDR progression, and important for investigation of the function of these differentially expressed miRNAs in PVR and PDR.

Materials and methods

Cell culture and treatment. The APRE-19 human RPE cell line was kindly provided by Professor Fu Shang from the Laboratory for Nutrition and Vision Research (Boston, MA, USA) and cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA, USA). The cells were grown to confluence at 37˚C in a humidified atmosphere containing 5% CO_2 and dissociated with 0.25% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA) solution. For TGFβ2 treatments, cells were cultured in six-well plates and treated with 5 ng/ml recombinant human TGFβ2 (Cell Signaling Technology, Danvers, MA, USA) for 24 h.

qPCR analysis for gene expression. Total RNA was extracted from cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized with a reverse transcription kit (Takara, Siga, Japan), using conditions recommended by the manufacturer. For the quantitative analysis of mRNA expression, SYBR® PrimeScript™ RT-PCR kit (Takara) was used to amplify the target genes by the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Western blot analysis for protein expression. For total protein extraction, cells were lysed in 100 μl of lysis buffer with protease inhibitor cocktail. The protein samples mixed with 5X SDS sample buffer were subjected to SDS-PAGE, and then electroblotted onto the PVDF membrane. Membranes were blocked in 5% non-fat milk and incubated with different primary antibodies at 4˚C overnight. After washing with PBST, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The bands on the membranes were visualized using chemiluminescence detection reagents. Three independent experiments were performed.

Microarray analysis. Total RNA was isolated using TRIzol (Invitrogen) and miRNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, which efficiently recovered all the RNA species, including miRNAs. RNA quality and quantity were measured using a Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity was determined by gel electrophoresis. The isolated miRNAs from the ARPE-19 cell line were then labeled with Hy3™/Hy5™ using the miRCURY™ Array Power Labeling kit (Exiqon, Vedbaek, Denmark) and hybridized on a miRCURY LNA miRNA Array (v.18.0, Exiqon) according to the array manual. Following hybridization, the slides were removed, washed several times using wash buffer kit (Exiqon), and then dried by centrifugation for 5 min at 15 x g. The slides were then scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA).

Data analysis. Scanned images were imported into GenePix Pro 6.0 software (Axon Instruments) for grid alignment and data extraction. The microarray assays were repeated three times for each group. Replicated miRNAs were averaged and miRNAs with intensities ≥30 in any of the samples were selected for calculating the normalization factor. Hierarchical clustering was performed using MEV software (v4.6, TIGR).

miRNA real-time reverse transcription-PCR (RT-qPCR). To verify the alterations in the expression of specific miRNAs that were found to be altered in the miRNA microarray analysis, we selected representative miRNAs (miRNA-let-7a, let-7b, let-7c, let-7d, let-7i, 15a, 15b, 106a, 106b, 34a, 26a, 26b, 29a, 29b, 29c, 16, 21, 19a, 135a, 223, and miRNA-1909) for RT-qPCR. qPCR was performed by using one step PrimeScript miRNA cDNA synthesis kit and a SYBR Premix Ex Taq™ II real-time PCR kit (Takara) according to the manufacturer's instructions. The reaction products were analyzed by the ABI Prism 7000 sequence detection system (Applied Biosystems). Experiments were repeated three times. Data were analyzed according to the comparative Ct method and normalized to RNU6B expression in each sample, which served as an internal control.

Statistical analysis. Each experiment was repeated at least three times. Numerical data are presented as mean ± SD. The difference between means was analyzed with independent

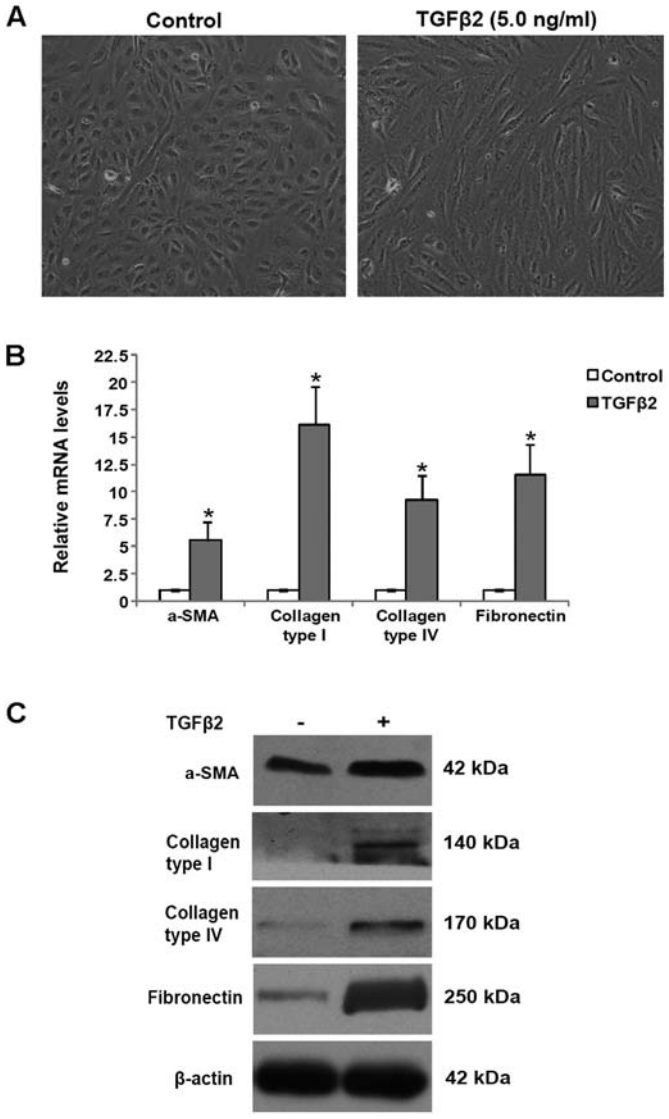

Figure 1. TGFβ2-induced EMT in RPE cells. RPE cells were cultured in the absence or presence of TGFβ2 (5 ng/ml) for 24 h. (A) Cell morphology was examined using a phase contrast microscope at a magnification of x100. (B) The mRNA expression levels of α-SMA, collagen type I, collagen type IV, and fibronectin were determined by quantitative PCR. Gene expression levels were normalized to the GAPDH control. *P<0.05 vs. the control group. (C) The protein expression levels of α-SMA, collagen type I, collagen type IV, and fibronectin were detected by western blotting. All experiments were repeated three times with similar results.

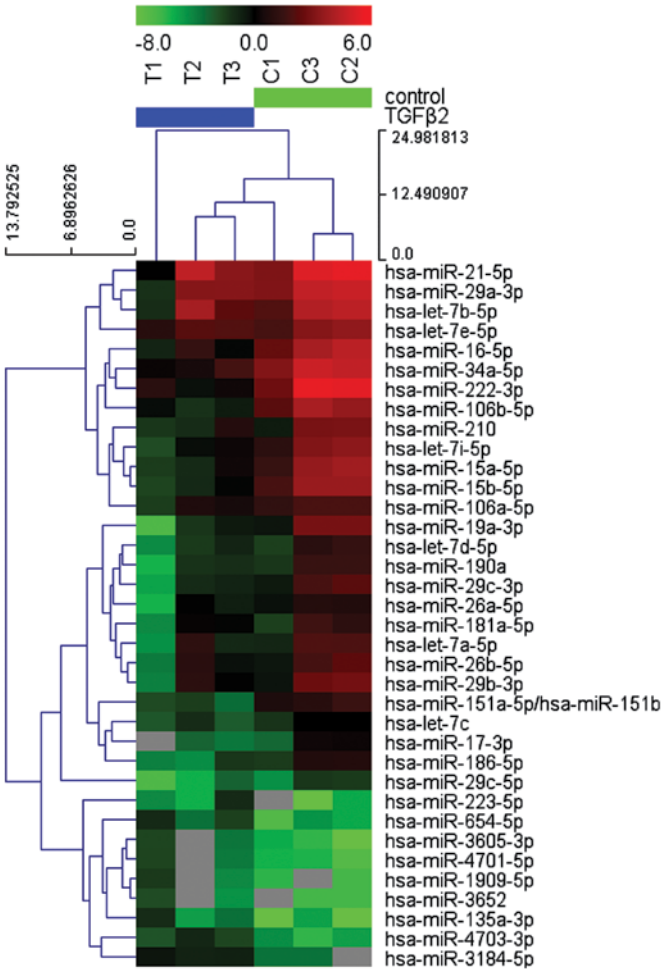

Figure 2. Hierarchical clustering for differentially expressed miRNAs in TGFβ2-induced RPE cells EMT. Microarray assays were used to determine the genome-wide miRNA expression in RPE cells cultured in the presence or absence of TGFβ2 (5 ng/ml) for 24 h. Three separate microarray assays were performed for each group. Colors indicate the expression relative to the mean for each miRNA. The red color indicates a higher expression level, and the green color indicates a lower expression level relative to the level in the control samples.

samples t-test. Differences were considered significant when P<0.05. Statistical analyses were performed with the software SPSS 15.0 (SPSS Inc. Chicago, IL, USA).

Results

TGFβ2-induced EMT in RPE cells. To examine the effect of TGFβ2 on the EMT of RPE cells, cell morphology and EMT markers such as α-SMA, collagen type I, collagen type IV, and fibronectin were investigated. As shown in Fig. 1A, stimulation of RPE cells with 5 ng/ml of TGFβ2 resulted in a significant change in cell morphology, presenting as a marked transition from an epithelial to a more mesenchymal phenotype. In addition, the qPCR results showed that the expression of α-SMA, collagen type I, collagen type IV, and fibronectin were upregulated ~5.4-, 16.3-, 9.3- and 11.5-fold in TGFβ2-induced RPE cells (Fig. 1B, P<0.05 vs. the control group). A similar effect of TGFβ2 was observed by the western blot analysis results (Fig. 1C). Thus, these data suggested that 5 ng/ml of TGFβ2 can strongly induce EMT in RPE cells.

miRNA expression profiles in TGFβ2-induced EMT in RPE cells. To investigate the difference of miRNA expression during EMT, we performed array-based miRNA profiling in human RPE cells after treatment with TGFβ2 for 24 h. Expression levels of all human miRNAs (1,223 miRNAs) were analyzed and 304 miRNAs were significantly differentially expressed with the presence of TGFβ2. It was found that 185 of the 304 miRNAs were downregulated in the TGFβ2 treatment group. miRNA-15b had the most marked change in expression (17.70 times lower in the TGFβ2 treatment group, Fig. 2 and Table I). A total of 119 miRNAs were upregulated in TGFβ2 treatment cells. miRNA-135a exhibited the greatest increase in expression where the expression in TGFβ2 treatment samples was ~14.27 times higher than that in the control samples (Fig. 2

Table I. Summary of TGFβ2-regulated microRNAs in RPE cells.

MicroRNA	Symbol	Fold change
hsa-let-7a-5p	Down	3.01
hsa-let-7b-5p	Down	2.20
hsa-let-7c	Down	3.44
hsa-let-7d-5p	Down	6.00
hsa-let-7e-5p	Down	2.21
hsa-let-7i-5p	Down	9.21
hsa-miR-15a-5p	Down	14.63
hsa-miR-15b-5p	Down	17.70
hsa-miR-106a-5p	Down	2.98
hsa-miR-106b-5p	Down	17.30
hsa-miR-34a-5p	Down	10.29
hsa-miR-181a-5p	Down	2.63
hsa-miR-210	Down	6.29
hsa-miR-29a-3p	Down	3.20
hsa-miR-29b-3p	Down	4.38
hsa-miR-29c-5p	Down	4.20
hsa-miR-29c-3p	Down	9.42
hsa-miR-26a-5p	Down	2.79
hsa-miR-26b-5p	Down	3.09
hsa-miR-16-5p	Down	10.56
hsa-miR-151a-5p	Down	11.56
hsa-miR-21-5p	Down	2.70
hsa-miR-19a-3p	Down	15.44
hsa-miR-186-5p	Down	9.00
hsa-miR-17-3p	Down	8.96
hsa-miR-190a	Down	8.17
hsa-miR-1909-5p	Up	12.83
hsa-miR-3652	Up	10.88
hsa-miR-3605-3p	Up	10.82
hsa-miR-654-5p	Up	10.20
hsa-miR-3184-5p	Up	7.06
hsa-miR-223-5p	Up	11.32
hsa-miR-135a-3p	Up	14.27
hsa-miR-4701-5p	Up	9.69
hsa-miR-4703-3p	Up	8.99

and Table I). Table I lists 35 differentially expressed miRNAs with at least a 2-fold change in expression.

Validation of differentially expressed miRNAs. Representative miRNAs which were identified by microarray were further validated by qPCR analysis. Similar alterations of miRNA expression were observed by qPCR analysis, although the fold change in the expression level was not exactly the same between the two different analytic methods. The results showed that miRNA-29b-3p, 26b-5p, 16-5p, let-7d-5p, 19a-3p, 15b-5p, 106b-5p, let-7a-5p, 106a-5p, 21-5p, let-7i-5p, and miRNA-15a-5p were downregulated >2-fold (7.7-, 7.5-, 6.8-, 4.1-, 3.5-, 3.5-, 3.3-, 2.5-, 2.3-, 2.0-, 2.0-, and 2.0-fold, respectively) in the

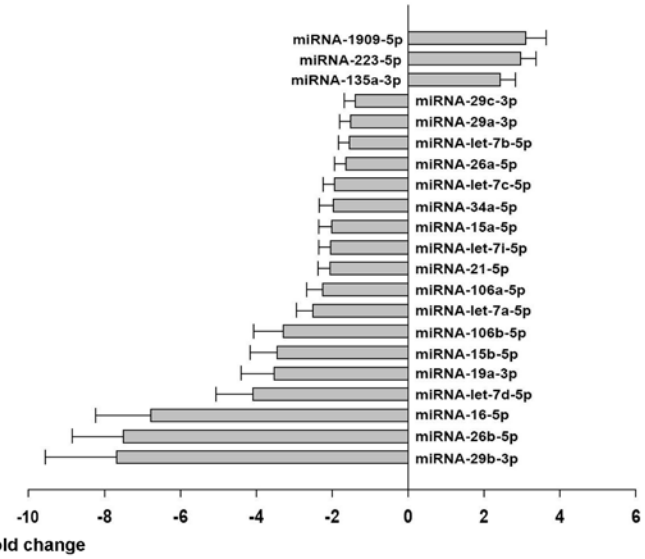

Figure 3. Validation of microarray data by qPCR. qPCR analysis was used to verify the representative miRNAs. Triplicate assays were performed for each RNA sample and the data were normalized to RNU6B as an endogenous control. Fold changes for these miRNAs from qPCR are shown as mean ± SD. The fold change is shown on the x-axis, and the miRNAs screened are shown on the y-axis. A negative number (-) indicates that the expression levels in the TGFβ2 treatment samples are lower than those in the control samples, and a positive number (+) indicates that the expression levels in the TGFβ2 treatment samples are higher than those in the control samples. miRNA-29b-3p exhibits the greatest decrease in expression, whereas miRNA-1909-5p exhibits the greatest increase in expression.

TGFβ2 treatment group (Fig. 3). Conversely, miRNA-1909-5p, miRNA-223-5p, and miRNA-135a-3p were upregulated (3.1-, 3.0- and 2.4-fold, respectively).

Discussion

miRNAs are emerging as extremely important factors in the regulation of gene expression and their dysregulation has been shown to be involved in a wide range of processes including cell differentiation (18,19), proliferation (20), metabolism (21) and apoptosis (22). In this study, the known EMT regulators such as miRNA-let-7 family, miRNA-34a, and miRNA-29 were downregulated in TGFβ2-induced EMT in RPE cells, which is consistent with the EMT phenotype of RPE cells. Furthermore, we identified a group of miRNAs that are seldom reported in EMT. These data suggest that miRNAs actively participate in TGFβ2-induced EMT in RPE cells. Thus our results provide a basis for further investigation of the biological function of these altered miRNAs in EMT.

The most downregulated miRNA in our study, miRNA-29b, has been reported in many types of fibrotic disorders (23-25) and cancers (15,26-28). Evidence has confirmed that miRNA-29 participates in the formation of extracellular matrix (ECM) and regulates organ fibrosis (23,24). Downregulation of miRNA-29b in C57BL/6 mice induced Col1a1, Col1a2 and Col3a1 mRNA overexpression in cardiac tissue (24). When miRNA-29b was knocked down in kidney of salt-induced hypertensive renal medullary fibrosis rats, a large number of collagen genes (Col1a1, Col3a1, Col4a1, Col5a1, Col5a2, Col5a3, Col7a1, Col8a1, MMP2 and ITGB1) was upregulated. Subsequently, a reporter gene assay validated these

genes as direct targets of miR-29b (29). In cancer research, miRNA-29b suppresses prostate cancer metastasis by regulating EMT signaling (15). Overexpression of miRNA-29b results in epithelial cell marker E-cadherin expression being enhanced, while N-cadherin, Twist and Snail expression are downregulated in prostate cancer cells (15). Other miRNA-29 family members, such as miRNA-29a and 29c, are also involved in fibrotic disorders (30). Both were downregulated in systemic sclerosis fibroblasts and skin sections, particularly miRNA-29a. Overexpression of miRNA-29a significantly decreases the levels of type I and III collagen expression in systemic sclerosis fibroblasts (30). Those studies are in line with our finding that miRNA-29a/b/c expression is downregulated in TGFβ2-induced EMT in human RPE cells, and that miRNA-29 is a potential and appealing therapeutic target for fibrotic disorders. However, further investigation is required to understand the role of miRNA-29 in regulating PVR and PDR.

Our second most downregulated miRNA, miRNA-26b, has been reported to be a tumor suppressor in breast cancer (31,32) and glioma development (33). miR-26b expression was decreased in breast cancer specimens and the overexpression of miR-26b inhibits cell growth and induces cell apoptosis by targeting PTGS2 and SLC7A11, respectively (31,32). Furthermore, the level of miR-26b was inversely correlated with the grade of glioma. Ectopic expression of miR-26b inhibited the proliferation, migration and invasion of human glioma cells by directly regulating EphA2 expression (33). Moreover, it was demonstrated that the level of miR-26 is significantly decreased in idiopathic pulmonary fibrosis (34), which suggests that miR-26 is a key contributor of EMT. However, the function of miR-26 in the regulation of EMT has not been determined. Therefore, it is crucial to determine its possible role in EMT in future studies.

miRNA-106b, another miRNA of interest in our study, belongs to the miRNA-106b-25 cluster, which is composed of the highly conserved miRNA-106b, miRNA-93 and miRNA-25. This cluster has been reported to play an important role in EMT and contribute to the metastasis of cancer cells. The miRNA-106b-25 cluster can target Smad7, resulting in overproduction of TGFβ type I receptor, activation of TGFβ signaling and induction of EMT in breast cancer cells (35). The miRNA-106b-25 cluster also increases the expression of Snail and enhances cell migration and invasion in H1299 non-small cell lung cancer cells by targeting β-TRCP2 (36). In addition, accumulating data have shown that the miRNA-106b-25 cluster plays an oncogenic role in various types of cancer, by influencing tumor growth, cell survival and angiogenesis (37,38). Members of this cluster are overexpressed in various types of malignancies, including esophageal adenocarcinoma gastric cancer, prostate cancer, laryngeal carcinoma and hepatic cell cancer (38). It has been identified as a promising biomarker of early metastasis following nephrectomy in patients with renal cell carcinoma, since its expression level is significantly lower in patients with metastasis (39). In contrast to the findings in oncology, our results have demonstrated that miRNA-106b was clearly downregulated in TGFβ2-induced EMT in human RPE cells. These discordant results may be due to the different function of miRNA-106b in different tissues and diseases. Therefore, it may be valuable to determine its possible role in EMT of RPE cells in future studies.

Other miRNAs, including miRNA-15a and -16, are members of a single miRNA family located in the13q14 locus. Previous studies (40,41) have indicated that the miR-15a-miR-16 locus may behave as tumor suppressors. Recent evidence has shown that the miR-16 family negatively regulates cell cycle progression by inducing G0/G1-cell accumulation (42). However, the function of miR-15a-miR-16 locus in EMT is unknown. In the current study, we determined that both miRNA-15a and miRNA-16 were downregulated significantly in TGFβ2-induced EMT in human RPE cells, indicating their possible role in regulating the EMT process. miRNA-223, which in one of the upregulated miRNAs in the present study, is identified as an oncogene in cancer and modulates a variety of cell events, including cell differentiation (43,44), proliferation (45), and migration (46). However, the role of miRNA-223 in EMT remains to be determined, thus further investigation is required to understand the role of miRNA-223 in regulating EMT.

In summary, to the best of our knowledge, this study has described the miRNA expression profile for the first time in the TGFβ2 induced EMT in RPE cells by microarray. A group of differentially expressed miRNAs were documented and may play critical roles in TGFβ2-induced EMT in human RPE cells as well as the development of PVR and PDR. These data provide new insights into the molecular mechanisms underlying PVR and PDR. However, further studies should shed more light on evaluating the function of these differentially expressed miRNAs and identify the potential therapeutic targets in PVR and PDR based on miRNAs.

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