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Review

Biological Involvement of MicroRNAs in Proliferative Vitreoretinopathy

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Even with a high surgical success rate for retinal detachment and proliferative vitreoretinopathy (PVR) supported by the robust improvement in vitrectomy surgery and its related devices, certain questions still remain for the pathogenesis and treatment of PVR. One of the important biological events in PVR is epithelial–mesenchymal transition (EMT) of the retinal pigment epithelial (RPE) cells. MicroRNAs are noncoding, small, single-strand RNAs that posttranscriptionally regulate gene expression and have essential roles in homeostasis and pathogenesis in many diseases. Recently, microRNAs also had a critical role in EMT in many tissues and cells. One main purpose of this brief review is to describe the knowledge obtained from microRNA research, especially concerning vitreoretinal diseases. In addition, the potential role of microRNAs in prevention of PVR by regulating EMT in RPE cells is described. Understanding microRNA involvement in PVR could be helpful for developing new biological markers or therapeutic targets and reducing the rate of visual disability due to PVR.

Introduction

Vitrectomy surgery and related devices have improved greatly over the last few decades, and they enable us to reach higher successful rates after the surgeries for retinal detachment (RD). However, failure to reattach the retina sometimes causes the pathogenesis of proliferative vitreoretinopathy (PVR).[1–3] Biologically, among many types of cells, for example, glial cells, fibroblasts, macrophages, and lymphocytes, involved in the pathogenesis of PVR,[4–6] an important concept in PVR is epithelial–mesenchymal transition (EMT) of the retinal pigment epithelial (RPE) cells. In an eye with RD, RPE cells float into the vitreous cavity through the retinal breaks, adhere to the surface of the sensory retina, and undergo transformation from epithelial to fibrotic cells. Many types of growth factors and cytokines, such as platelet-derived growth factor, fibroblast growth factor (FGF), and epidermal growth factor, have been reported to be upregulated in the eyes with PVR; transforming growth factor β (TGFβ), a strong inducer of EMT, also is upregulated in the eyes with PVR and has a pivotal role in PVR.[4,7,8] Glucocorticoid is a potent inhibitor of TGFβ signal; therefore, steroid treatment theoretically is potent in preventing PVR.[9,10] However, previous studies showed insufficiency of the corticosteroid treatment; thus, an additional approach is required urgently.[11–13] On the other hand, the explosion of biological knowledge about noncoding RNA has shed light on the importance of microRNAs in many diseases. Over the last several years, the involvement of microRNAs in the pathogenesis of PVR has been elucidated. In this brief review, we discussed previous studies that have shown the biological importance of microRNAs in the pathogenesis of PVR, especially focusing on EMT of RPE cells. Lastly, we shared new data based on our recent findings on the biological relationships between microRNA and inflammatory cytokines and caveolin-1.

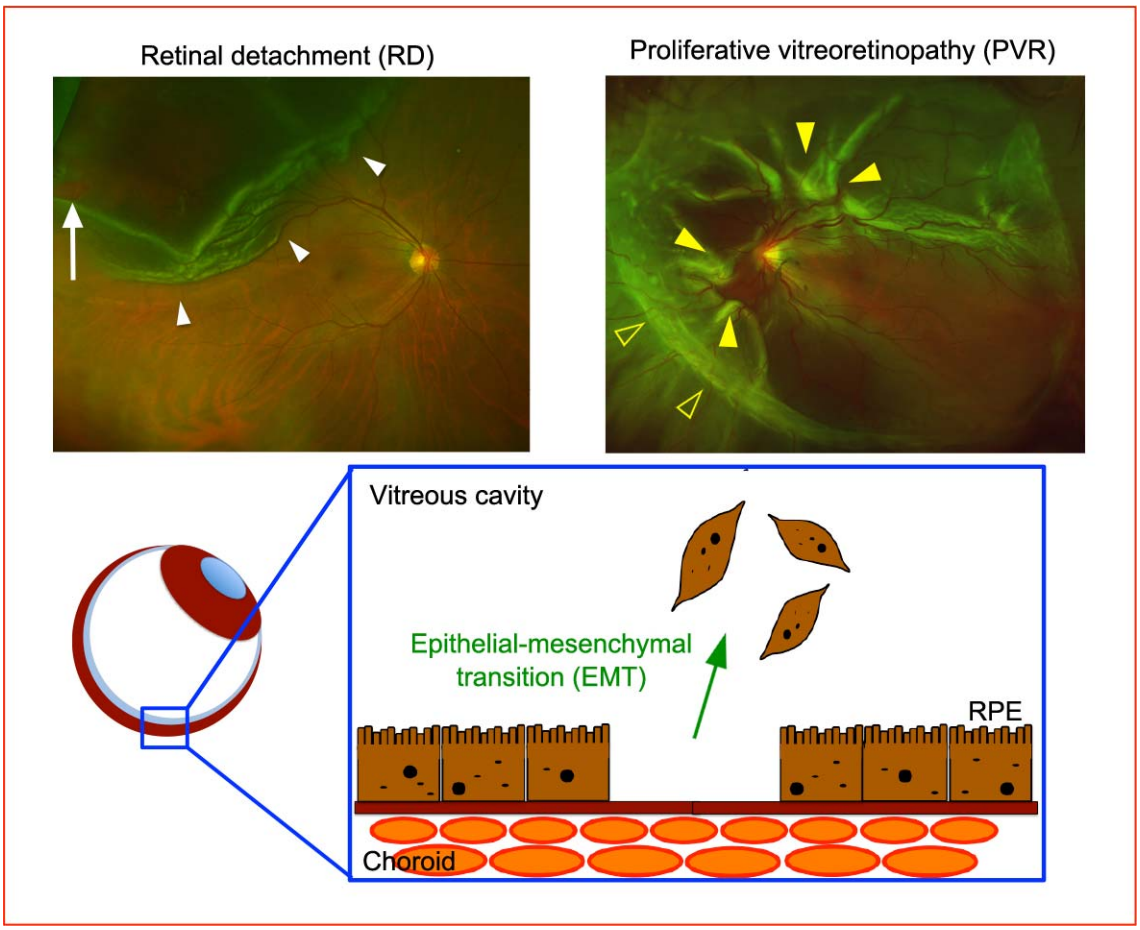

Figure 1. Illustration of pathogenesis of PVR. In eyes with RD (*left*), floating RPE cells receive certain biological signals, induce epithelial–mesenchymal transition, and RPE-derived fibrotic cells migrate on the surface of the retina. Color fundus image of PVR (*right*) showing wrinkling of the retinal surface, retinal stiffness, vessel tortuosity, and subretinal strands. The *white arrow* indicates a retinal break, and the *white arrowheads* indicate detached retina. The *yellow filled arrowheads* indicate wrinkling of the retinal surface, and *yellow open arrowheads* indicate epiretinal fibrotic membranes.

RD and PVR

The incidence of RD has been reported to be as low as 0.6 to 1.8 per 10000 people.[14] The most common type of RD is rhegmatogenous RD, which is caused mainly by retinal breaks owing to vitreous traction.[15] Although vitrectomy surgery has contributed greatly to the successful treatment of severe retinal diseases, including RD, it is difficult to recover all cases of RD completely. Patients with severe and/or long-standing RD or those with unsuccessful surgical treatment tend to suffer PVR.[16,17] PVR is diagnosed clinically by the following clinical observations:[18] existence of vitreous haze, vitreous pigment clumps, pigment wrinkling of the retinal surface, retinal stiffness, vessel tortuosity, rolled and irregular edge of retinal breaks, and subretinal strands. In an eye with RD, RPE cells float into the vitreous cavity through retinal breaks, adhere to the surface of the sensory retina, and undergo transformation from epithelial to fibrotic cells. This biological phenomenon is referred to as EMT, in which differentiated RPE-derived cell help induces PVR (Fig. 1).[19–22]

MicroRNA

Even after robust breakthroughs enabled by the whole human genome project, dozens of diseases remain of which the pathogeneses have not been elucidated perfectly. Unexpectedly, only 2% of the human genome is responsible for coding proteins.[23] Scientific approaches increasingly have begun to use transcriptome analysis. Scientists have recognized that thousands of noncoding RNAs are transcribed in the human body. One of the important but underestimated noncoding RNAs is microRNA.

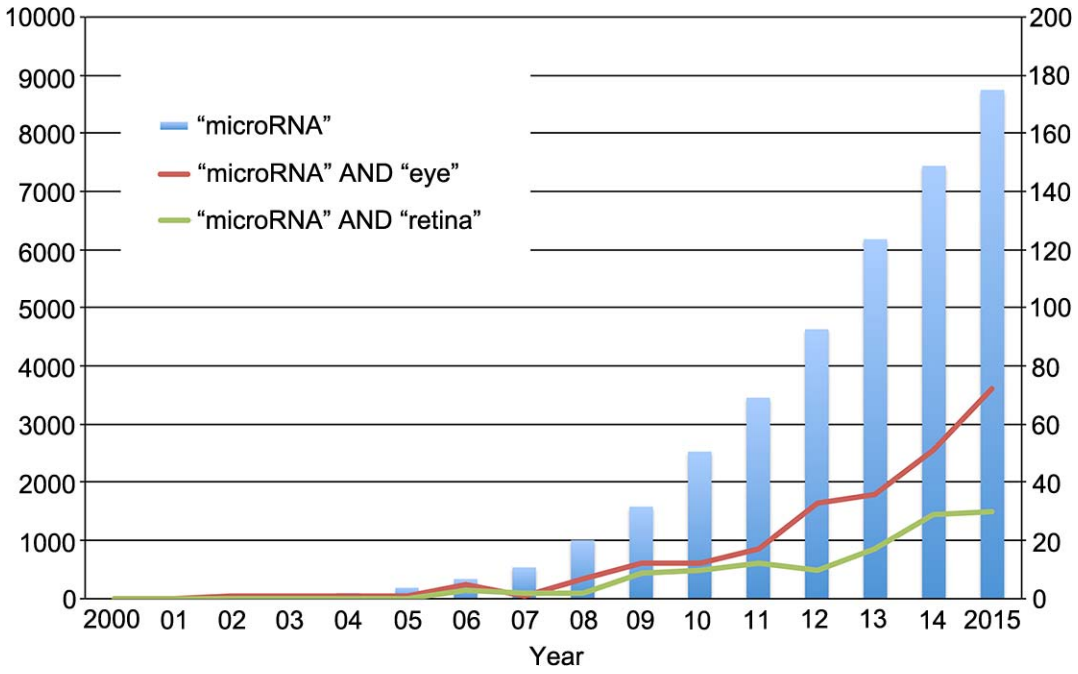

Figure 2. Growth of the number of microRNA-related publications. PubMed entries that reference the term "microRNA" are represented by the *blue bars*, those that reference "microRNA" AND "eye" are represented by the *red line*, and those that reference "microRNA" AND "retina" are represented by the *green line*.

MicroRNA is an extensive class of endogenous, noncoding, single-strand RNAs with 18 to 24 nucleotides that negatively regulate gene expression by interacting with the 3′-untranslated regions (3′UTR) of their target mRNAs.[24] By modulating the expression of their target genes, microRNAs have essential roles in homeostasis and pathogenesis.[25–27] In the human body, more than 2000 microRNAs reportedly are involved in cell proliferation, differentiation, and signaling. These microRNAs regulate cellular processes, including tumor formation, and have been linked to a number of human diseases; thus, the role of microRNA as a therapeutic target or a disease marker has been an active area of research.[28–31] In the eye, various microRNAs are thought to act on the retina or on RPE cells and to have important roles in neuroprotection and angiogenesis.[20,32–35]

Publications in MicroRNA and Vitreoretinal Diseases

The number of publications that have described microRNA experiments and ocular diseases has been increasing (Fig. 2). Soon after the first use of the term "microRNA" in the literature in 2001,[36–38] articles in PubMed referencing the keywords "microRNA" AND "eye" appeared in 2001. In 2006, articles in PubMed referencing the keywords "microRNA" AND "retina" appeared (Fig. 2). As scientific technology and knowledge of microRNA has increased, the number of microRNA-related publications in ophthalmic research also has increased each year. Table 1 lists the published studies on microRNAs in retinal diseases, including the targeted microRNAs and the cells/animals.

Detecting MicroRNAs in the Vitreous from Human Eyes

In eyes with RD or PVR, higher expressions of inflammatory cytokines have been detected in the vitreous,[39–41] presumably because a variety of factors are eluted from retinal cells, RPE cells, and vitreous cells into the vitreous. In addition, RD and PVR implicate the breakdown of blood–retinal barriers; therefore, allowing the passage of cells into the retina and vitreous cavity.[42,43] These factors have the potential to have biological effects on many intraocular cells. The vitreous occupies the cavity of the posterior segment of the eye and is in direct contact with the retina. In addition, the vitreous also is in direct contact with the RPE through the retinal breaks. Therefore, in eyes with RD and PVR, ectopic RPE cells float in the vitreous. Examining microRNA expression in the vitreous in patients with a targeted

Table 1. List of Published Studies on MicroRNA in Retinal Diseases

PMID	Authors	Sources	Species	Target MicroRNAs	Target Diseases
18034880	Loscher CJ et al. 2007	Mouse retina	Mouse	miR-376a,-691	RP
18500251	Shen J et al. 2008	Mice retina (ischemia-reperfusion model)	Mouse	miR-106a,-146,-181,-199a,-214,-424,-451	Ischemia-reperfusion injury
18834879	Loscher CJ et al. 2008	Mouse retina (P347S-Rhodopsin transgenic mouse)	Mouse	miR-1,-133, -142,-183/96/182	RP
20881307	Ishida W et al. 2011	Mouse retina	Mouse	miR-21,-142-5p,-182	Uveoretinitis
21498619	Kovacs B et al. 2011	Rat retinae	Rat	miR-146	DR
21498619	McArthur K et al. 2011	Human and rat retina	Human, rat	miR-200b	DR
21586283	Bai Y et al. 2011	Mouse retina	Mouse	miR-126	OIR
21693609	Lin H et al. 2011	RPE cells from donor eyes, ARPE-19	Human	miR-23	AMD
21816922	Conkrite K et al. 2011	Human and mouse retina	Human, mouse	miR-17∼92 cluster	RB
21885871	Feng B et al. 2011	Rat retina, Bovine RMECs	Bovine, rat	miR-146a	DR
21897745	Silva VA et al. 2011	Rat retina	Rat	miR-29b	DR
22336108	To KH et al. 2012	RB cell lines	Human	miR-24	RB
22510010	Sreenivasan S et al. 2012	RB cell lines	Human	miR-22	RB
22880027	Haque R et al. 2012	ARPE-19 cells	Human	miR-30b	AMD
22969266	Kandalam MM et al. 2012	RB tissues and RB cell lines	Human	miR-17∼92 cluster	RB
23341629	Lumayag S et al. 2013	Mouse retina	Mouse	miR-183/96/182 cluster	Retinal degeneration
23400111	Beta M et al. 2013	Human serum	Human	miR-17, -18a, -20a	RB
23404117	Murray AR et al. 2013	MIO-M1 cells, Mouse retina	Human	miR-200b	DR
23592910	Kutty RK et al. 2013	ARPE-19	Human	miR-146a, -146b-5p	AMD
23660406	Wang J et al. 2013	RB tissues, RB cell lines	Human	miR-365b-3p	RB
24018047	Ling S et al. 2013	hREC, Mouse retina	Human, mouse	miR-106a	DR
24114799	Peng Q et al. 2014	Rat retina	Rat	miR-96, -124a, -181a, -182, -183	Drug-induced retinal toxicity
24120948	Martin A et al. 2013	RB tissues and RB cell lines	Human	miR-449a, -449b	RB

Table 1. Continued

PMID	Authors	Sources	Species	Target MicroRNAs	Target Diseases
24286082	Wang J et al. 2014	RB tissues and RB cell lines	Human	miR-183	RB
24289859	Wang J et al. 2014	RB cell lines	Human	miR-183	RB
24502381	Xiong F et al. 2014	Rat retina	Rat	miR-34,-203,-212,-216,-350,-410	DR
24570140	Mortuza R.2014	hRECs	Human	miR-195	DR
24581223	Genini S et al. 2014	Dog retina	Dog	miR-9, -19a, -20, -21, -29b, -122, -129, -146a, -155, -221	RP
24607444	Ding Y et al. 2014	Human HXO-RB44 cells, RB tissue specimens	Human	miR-21	RB
24867582	Wang Q et al. 2014	HRECs	Human	miR-148	DR
24886609	Cai J et al. 2014	ARPE-19 cells	Human	miR-29s	AMD
25172656	Zhao R et al. 2015	RF/6A cells, Rat and mouse retina	Monkey, rat, mouse	miR-351	DR, ROP, RVO
25251993	Janczura J et al. 2014	hRECs	Human	miR-184	AMD
25335984	Cao Y et al. 2014	hRECs	Human	miR-200b	DR
25711632	Saxena K et al. 2015	Rat retina	Rat	miR-125-3p, -155, -207, -347, 449a ,-351, -542-3p	Light-induced retinal degeneration
25796186	Takahashi Y et al. 2015	Mouse retina	Mouse	miR-184	OIR
25802486	Haque R et al. 2015	hRECs, Rat retina	Human	miR-152	DR
25815338	Fulzele S et al. 2015	Human retina	Human	miR-146b-3p	DR
25888955	Ye EA et al. 2015	hREC	Human	miR-15b, -16	DR
26242736	Yan L et al. 2015	Mouse retina	Mouse	miR-155	OIR
26413624	Zhao R et al. 2015	Rat retina	Rat	miR-126	acidosis-induced retinopathy
26466127	Lamoke F et al. 2015	Rat retina	Rat	miR-34a	DR
26539029	Zhuang Z et al. 2015	human RMECs, Moure retina	Human, mouse	miR-155	OIR
26541434	Watanabe T et al. 2016	Rat retina	Rat	miR-223, -146a	Uveoretinitis
26595608	Garcia TY et al. 2015	RPE cells derived from hESCs and hiPSCs	Human	miR-144	AMD
26617758	Jiang Q et al. 2016	hRECs	Human	miR-200	DR
26730174	Yang Y et al. 2015	human miRNA expression profile GSE7072	Human	miR-18a, -20, -25, -181, -373, 125b, let-7b,	RB

Table 1. Continued

PMID	Authors	Sources	Species	Target MicroRNAs	Target Diseases
26818976	Hou S et al. 2016	human blood	Human	miR-23a, -146a, -301a	VKH
26822433	Lin X et al. 2016	ARPE-19	Human	miR-29	DR
26935869	Kong Y et al. 2016	Mouse retina	Mouse	miR-218	OIR
26949607	He Y et al. 2016	Rat retina	Rat	miR-181a	ischemia-reperfusion injury
26949937	Bhattacharjee S et al. 2016	Human retina	Human	miR-34-a	AMD
26960375	Hans S et al. 2016	Mouse retina, Mouse REC	Mouse	miR-218	OIR
26997512	Kadonis G et al. 2016	Human blood	Human	miR-146a	DR
26997759	Ye EA et al. 2016	hRECs	Human	miR-146a	DR
27304911	Shi L et al. 2016	Mouse retina	Mouse	miR-150	DR
27311771	Zeng K et al. 2016	Diabetic rat retinal Müller cells	Rat	miR-29b	DR
27373709	Chung SH et al. 2016	Mouse retina (transgenic mouse with Müller cell disruption)	Mouse	miR-200b	AMD, DR
27374485	Grunin M et al. 2016	Human blood with treatment-naïve neovascular AMD	Human	miR-383	AMD
27421659	Qin B et al. 2016	HRECs,rat retina tissue	Human, mouse	miR-20b	DR
27505139	Tian B et al. 2016	ARPE-19, Human RPE	Human	miR-17-3p	AMD
27527066	Palfi A et al. 2016	Mouse retina	Mouse	miR-1, -133, -142, -183/96/182	RP
27531575	Wang Q et al. 2016	human RPE	Human	miR-15a	DR
27653551	Liu CH et al. 2016	Mouse retina	Mouse	miR-129-5p, -351, -145, -150, -155, -203, -210, -375, -762	OIR
27990360	Wang LL et al. 2016	Human retina samples, RB cell lines (Y79, Weri1)	Human	miR-143	RB

RP, retinitis pimentosa; AMD, age-related macular degeneration; DR, diabetic retinopathy; ROP, retinopathy of prematurity; RB, retinoblastoma; OIR, oxygen-induced retinopathy; RMEC, retinal microvascular endothelial cell; hREC, human retinal endothelial cell; hESC, human embryonic stem cells; hiPSC, human-induced pluripotent stem cell; ROS, reactive oxygen species; VKH, Vogt-Koyanagi-Harada syndrome; PDR, proliferative diabetic retinopathy; RVO, retinal vein occlusion.

Table 2. List of Published Studies on MicroRNA in Human Vitreous Fluid

Author	Source	Target MicroRNA	Target Disease	Detection Method
Ragusa et al., 2013	Vitreous fluid	miR-146a, 26a	Uveal melanoma	Taqman array human microRNA cards followed by the confirmation by quantitative real-time PCR
Odriozola et al., 2013	Vitreous fluid (autopsy)	miR-142-5p, 541	Determine the time of death	Taqman array human microRNA cards followed by the confirmation by quantitative real-time PCR
Tuo et al., 2014	Vitreous fluid	miR-155	Uveitis	miRCURY LNA panel followed by the comfirmation by quantitative real-time PCR
Hirota et al., 2015	Vitreous fluid	miR-15a, 320a, 320b, 93, 29a, 423-5p	PDR	miRCURY LNA panel
Takayama et al. 2016	Vitreous fluid	miR-148a-3p	RD	miRCURY LNA panel followed by the comfirmation by quantitative real-time PCR
Usui-Ouchi et al., 2016	Vitreous fluid	miR-21	PVR, PDR	Taqman array human microRNA cards followed by the confirmation by quantitative real-time PCR

vitreoretinal disease is a great tool for understanding the relationship between microRNAs and vitreoretinal disease. Table 2 lists the previous publications of studies that have analyzed microRNAs from surgically collected human vitreous.[44–49] In most studies, microRNAs were detected by microRNA arrays, followed by reconfirmation by quantitative real-time polymerase chain reaction assay. Almost all of the current surgical instruments used in vitrectomy surgery are disposable and RNase-free, and collecting the vitreous from patients without any additional risks is possible during the regular surgical procedure. It is presumed that the number of studies involving collecting and analyzing microRNA in the vitreous will increase.

MicroRNA and EMT in RPE Cells

Biological evidence has been established showing that specific microRNAs induce/inhibit EMT in other cells.[50–52] Recently, aberrant expression of microRNAs in RPE cells undergoing EMT was reported.[47,49,53–59] RPE cells undergo EMT and contribute to the pathogenesis of PVR. We focused on previous studies that have examined the effect of microRNAs on human RPE cells via EMT. Table 3 shows the list of publications that have examined the biological interactions between microRNA and EMT-related factors; for example, TGFβ. Chen et al.[55] reported that 185 microRNAs were downregulated and 119 microRNAs were upregulated in TGFβ2-treated ARPE-19 cells, which suggested that microRNAs have important roles in EMT of RPE cells. Li et al.[57] revealed that miR-29b is downregulated and has a significant role in TGFβ1-induced EMT of ARPE-19 cells. Overexpression of miR-29b prevents progression of EMT by targeting AKT2, a noncanonical signaling pathway in TGFβ-induced EMT. Wang et al.[58] showed that miR-204/211 preserves the epithelial phenotype in primary human fetal RPE (hfRPE) cells, indicating a pivotal role of miR-204/211 in EMT of hfRPE cells. A large number of genes and proteins regulate EMT in RPE. Most microRNAs regulate the transcription of these EMT-related factors and indirectly regulate EMT in RPE cells.

MicroRNA and Other Factors in PVR

A large number of cytokines, such as EGF and TGF-β, induce EMT. In addition, TGF-β cooperates with other cytokines, such as FGF, and enhances EMT effectively.[60–62] For instance, TGF-β has been shown to induce isoform switching of FGF receptors, which sensitizes the cells to FGF-2 and suggests that TGF-β and FGF-2 cooperate with each other and regulate EMT.[61] Recently, we found that miR-148a

Table 3. List of Published Studies on MicroRNA and Cultured RPE Cells

Author	Cells	Target microRNA	Related Protein	Major Findings
Wang et al., 2010	Fetal human RPE	miR-204, 211	TGFβR2, SNAIL2	miR-204/211 maintained epithelial barrier function
Kutty et al., 2010	Primary human RPE	miR-155	IFNγ, TNFα, IL-1β	Upregulation of miR-155 in hRPE cells treated with IFNγ, TNFα, IL-1β
Adijanto et al., 2012	Fetal human RPE	miR-204, 211	MITF	MITF regulated miR-204/211 expression and promoted RPE differentiation
Chen et al., 2014	ARPE-19	-	TGFβ2	185 miRNAs were downregulated and 119 miRNAs were upregulated by TGFβ2 treatment
Li et al., 2014	ARPE-19	miR-29b	TGFβ1, AKT2	Downregulation of miR-29bs in ARPE-19 cells treated with TGFβ1
Jun et al., 2016	ARPE-19	miR-124	TGFβ1	Downregulation of miR-124 in ARPE-19 cells treated with TGFβ1
Wang et al., 2016	Primary human RPE	miR-182	c-Met, HGF/SF	Downregulate miR-184 in PVR induced c-Met upregulation
Hou et al., 2016	ARPE-19	miR-34	LGR4	miR-34 suppressed LGR4, contributing to an inhibition of cell migration
Takayama et al. 2016	Primary human RPE, ARPE-19	miR-148a-3p	N/A	miR-148 induced αSMA expression
Usui-Ouchi et al., 2016	ARPE-19	miR-21	N/A	miR-21 did not affect EMT in RPE cells

was expressed specifically in the vitreous from eyes with RD and that miR-148a expression correlated with the size of the retinal breaks and duration of RD, which has been reported to be the possible cause of PVR.[47,63] Based on the aforementioned reports showing that the vitreous has multiple upregulated inflammatory cytokines, we examined correlations between miR-148 expression and inflammatory cytokines from eyes with RD. Demographic and clinical characteristics, and the results of inflammatory cytokine levels of the 20 patients with RD are listed in Table 4. miR-148a expression in the vitreous was correlated significantly with FGF-2 expression levels in the vitreous of eyes with RD; however, FGF-2 expression secreted from hRPE cells transfected with miR-148a mimic did not significantly differ from that of cells transfected with control microRNA in vitro (Fig. 3). In the meantime, our recent study found that caveolin-1, an integral membrane protein, was upregulated in proliferative membranes from eyes with PVR and had a pivotal role in suppression of EMT in RPE cells.[64] Therefore, we investigated whether specific microRNAs have biological relationships with the expression of caveolin-1 in RPE cells. The microRNAs that were detected specifically in the vitreous and subretinal fluid from the eyes with RD were listed in our previous study.[47] In addition, Table 5 lists the microRNAs that were detected specifically

Table 4. Demographic and Clinical Characteristics and the Biological Parameters of the Patients with RD

Number of Sample	20
Age, y	59.4 ± 14.0
Time from onset of RRD to vitrectomy, d	9.6 ± 13.8
Range of retinal detachment, degrees	179.6 ± 89.3
Area of retinal break, pixels	20358.1 ± 27931.8
Hsa-miR-148a-3p, relative expression	24.5 ± 63.5
FGF-2, pg/mL	22.7 ± 22.7
IFN-γ, pg/mL	0 ± 0
IL-10, pg/mL	0 ± 0
IL-12p40, pg/mL	0 ± 0
IL-1β, pg/mL	0 ± 0
IL-6, pg/mL	90.1 ± 86.2
IL-8, pg/mL	48.4 ± 55.0
MCP-1, pg/mL	7556.7 ± 4707.6
TNFα, pg/mL	1.5 ± 4.7
VEGF, pg/mL	132.1 ± 126.8

in the subretinal fluid. Using miRBase and microRNA.org databases,[65,66] we hypothesized that miR-199a-5p is a possible regulator of caveolin-1. We found that caveolin-1 was suppressed by miR-199a-5p but not by miR-148a (Fig. 4). These results, which corroborated those obtained from our previous study, indicated that higher expression of miR-148a in the vitreous may contribute to the pathogenesis of PVR by an unknown mechanism and miR-199a-5p in the subretinal fluid may have a role in the pathogenesis of PVR by regulating caveolin-1 expression in RPE cells.

Discussion

Due to the developments of surgical devices and biological understanding, the success rate for structural recovery of PVR has been improved. Nevertheless, a certain number of patients with PVRs become blind even after thorough surgical and medical intervention. Achieving understanding of the pathologic mechanisms underlying the development of fibrotic membrane spreading on the surface and beneath the sensory retina is critical to completely overcome PVR. MicroRNA is a novel and powerful class of modulators that regulate gene expression, and its involvement in the pathogenesis of PVR now is becoming clear. Over the last few decades, biological tools to examine inflammatory cytokines have revolutionized research capabilities. Consequently, the involvement of inflammatory cytokines in many retinal diseases also has been elucidated. In addition to further development of scientific tools for microRNA detection, measurement, and functional analy-

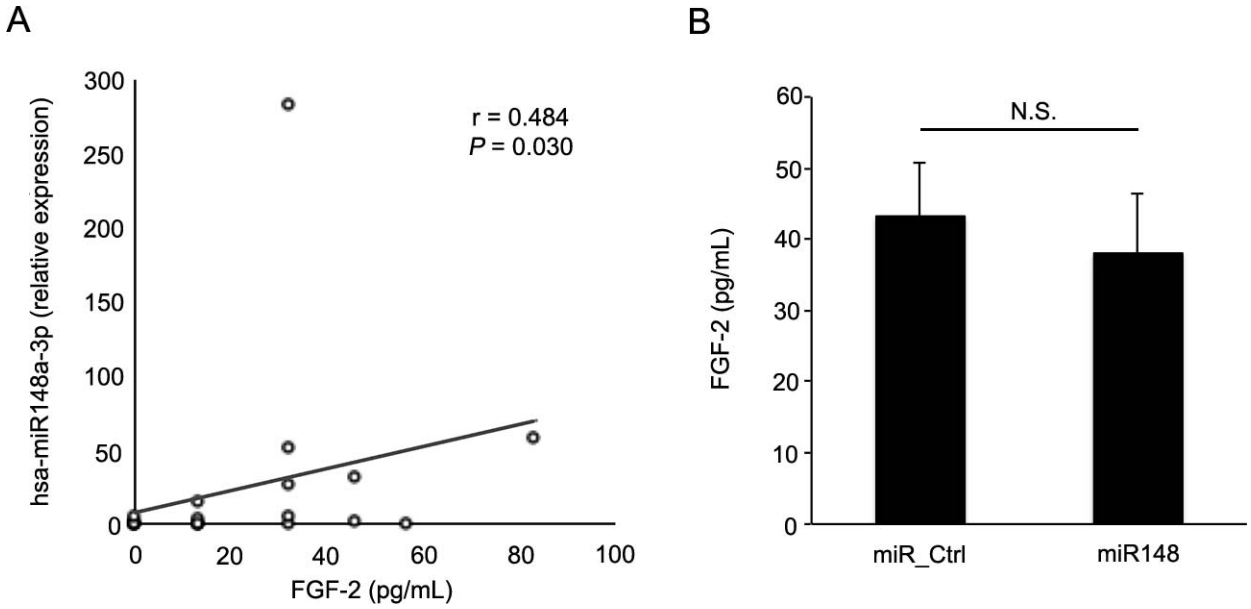

Figure 3. Correlation between microRNA expression and inflammatory cytokines. (A) miR-148a expression in eyes with RD was significantly correlated with FGF-2, but not with IFN-γ, IL-10, IL-12p40, IL-1β, IL-6, IL-8, MCP-1, TNFα, and VEGF. (B) FGF-2 levels that was secreted in the medium from hRPE cells transfected with miR148_mimic did not show significant difference from those with control microRNA ($n = 12$, $P = 0.54$).

Table 5. List of MicroRNAs Detected Specifically in the Subretinal Fluid

microRNA	Sample #1	Sample #2	Sample #3	Average
hsa-miR-584-5p	6.7700	4.1800	6.1500	5.7000
hsa-miR-376a-3p	5.8500	4.0900	3.4300	4.4567
hsa-miR-296-5p	4.0100	4.3000	3.8900	4.0667
hsa-miR-382-5p	4.4600	2.8900	4.0000	3.7833
hsa-miR-1	0.8100	3.0100	6.6500	3.4900
hsa-miR-93-3p	2.6400	3.0700	3.2600	2.9900
hsa-miR-10b-5p	2.1100	3.3400	3.0300	2.8267
hsa-miR-154-5p	3.5400	2.3200	2.5700	2.8100
hsa-miR-127-3p	3.3600	2.5800	2.1800	2.7067
hsa-miR-133a	−0.8900	1.3300	6.9600	2.4667
hsa-miR-199a-5p	1.4800	2.0900	0.9800	1.5167
hsa-miR-200a-3p	1.3700	1.9500	−0.1600	1.0533

Samples #1 to 3: Subretinal fluid from RD eye. Values are relative expression of each microRNA.

sis, the close relationship of microRNA and cytokines must be elucidated. Identifying the role of other microRNAs in EMT in RPE cells in vitro and in PVR in vivo could be helpful for understanding more precisely the involvement of EMT-related gene expression. In this brief review, we showed the lists of microRNAs that reportedly were relevant to retinal diseases, including PVR. Subsequently, based on our previous studies, we proposed two microRNAs that possibly have important roles in PVR. There might be a difficulty in constructing efficient experimental design for microRNA research: in our case, we first targeted miR-148 because it was expressed most abundantly in the vitreous of RD.[47] We found miR-148 induced EMT in RPE cells. However, we could not find a specific gene that affects EMT of RPE cells under miR148 regulation. In the meantime, we found the role of caveolin-1 in EMT, and we also found miR199 is a possible regulator of caveolin-1. Finding

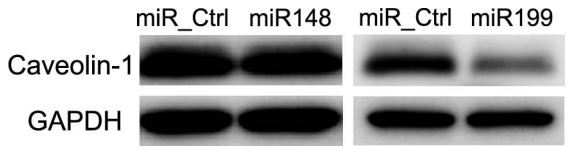

Figure 4. Caveolin-1 expression is altered by miR-199, but not by miR-148a. Shown is a comparison of caveolin-1 expressions in primary human RPE cells after being transfected with miR148-mimic versus control microRNA (miR_Ctrl) and with miR199-mimic versus control microRNA (miR_Ctrl). Caveolin-1 is suppressed by miR-199, but not by miR-148a.

a set of microRNA and its targeted gene in the same specific disease is the key to exploring microRNA-based therapeutic possibility. Further improvement of the microRNA research will accelerate the development of microRNA agonists/antagonists that can be used as a new class of drugs to regulate the progression of PVR.

Materials and Methods

Patients with Retinal Detachment and Sample Collection and Analysis

The methods for sample collection have been described in our previous studies.[47,63] Briefly, all vitreous samples were collected by dry vitrectomy and subretinal fluid was collected during scleral buckling surgery and immediately stored at −80°C. The vitreous was thawed and centrifuged for 5 minutes at 2000*g*, 4°C to remove contaminating cells only once before performing the MILLIPLEX MAP Human Cytokine/Chemokine Panel (Merck Millipore, Billerica, MA), a bead-based multiplex immunoassay that allows simultaneous quantification of the following human cytokines: FGF-2, IFN-γ, IL-10, IL-12p40, IL-1β, IL-6, IL-8, MCP-1, TNFα, and VEGF. Values under the detection limits were defined as "0" in the statistical analyses. Our adhered to the guidelines of the Declaration of Helsinki; the study was approved by the Nagoya University Hospital Ethics Review Board. Written informed consent was obtained from all patients before their enrolment. Spearman's rank correlation coefficient was used to assess the association between expression levels of hsa-miR-148a-3p and cytokines in the vitreous. $P < 0.05$ were considered a statistically significant correlation.

MicroRNA Mimic Transfection

The methods for the microRNA mimic transfection and sample collection have been described in our previous studies.[47,63] Briefly, primary hRPE cells (Lonza, Walkersville, MD) were transfected with 60 pmol of miR-148a-3p mimic and miR-199 mimic (Invitrogen, Carlsbad, CA) before use in further in vitro experiments. hRPE cells were cultured in serum-free antibiotic-free Dulbecco's Modified Eagle's Medium premixed with Ham's F-12 (1:1 ratio; Sigma-Aldrich Corp., St. Louis, MO,) before incubation with Lipofectamine RNAiMAX Transfection Reagent and miR-148a-3p mimic for 48 hours. hRPE

cells also were transfected with negative control mimic (miR_Ctrl) in the same manner and used as controls. Culture medium then was replaced with fresh medium containing 10% FBS and antibiotics and used in further experiments.

Western Blotting and ELISA

Following hsa-miR-148a-3p mimic, hsa-miR-199 mimic, or miR_Ctrl transfection and TGF-β2 stimulation, hRPE cells were washed with PBS 3 times and then lysed in RIPA buffer (Sigma-Aldrich Corp.) containing a protease inhibitor cocktail (Roche Diagnostics, Ltd, Mannheim, Germany). The lysate was centrifuged at 15,000*g* for 15 minutes at 4°C, and the supernatant was collected. The protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Protein samples (20 μg) from human tissues or culture cells were run on 5% to 20% sodium dodecyl sulfate precast gels (Wako, Tokyo, Japan) and transferred to polyvinylidine fluoride (PVDF) membranes by using an iBlot blotting system (Invitrogen). Transferred membranes were washed in 0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, pH 8.0, 0.05% Tween 20 (TBS-T; Sigma-Aldrich Corp.) and then blocked in 5% nonfat dry milk/TBS-T at room temperature (RT) for 2 hours. Membranes then were incubated with anti-caveolin-1 antibody (catalog no. 3238: 1:200; Cell Signaling Technology, Beverly, MA) at 4°C overnight. Protein loading was assessed by immunoblotting using an anti-GAPDH antibody (catalog no. 2118: 1:3000; Cell Signaling Technology). Membranes then were incubated with horseradish peroxidase-linked secondary antibody (catalog no. 7074: 1:3000; Cell Signaling Technology) for 1 hour at RT. Signals were visualized by enhanced chemiluminescence (ECL plus; GE Healthcare, Piscataway, NJ) and captured by using ImageQuant LAS-4000 (GE Healthcare). For measuring FGF-2 levels in the medium from hRPE cells, human FGF basic Quantikine ELISA Kit (catalog no. DFB-50; Minneapolis, MN) was used.

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