Polarity and epithelial-mesenchymal transition of retinal pigment epithelial cells in proliferative vitreoretinopathy

Hui Zou [1], **Chenli Shan** [1], **Linlin Ma** [1], **Jia Liu** [1], **Ning Yang** [1], **Jinsong Zhao** [Corresp. 1]

[1] Eye Center, The Second Hospital of Jilin University, Changchun, China

Corresponding Author: Jinsong Zhao
Email address: jinsongzhao2003@163.com

Under physiological conditions, retinal pigment epithelium (RPE) is a cellular monolayer composed of mitotically quiescent cells. Tight junctions and adherens junctions maintain the polarity of RPE cells, and are required for cellular functions. In proliferative vitreoretinopathy (PVR), upon retinal tear, RPE cells lose cell-cell contact, undergo epithelial-mesenchymal transition (EMT), and ultimately transform into myofibroblasts, leading to the formation of fibrocellular membranes on both surfaces of the detached retina and on the posterior hyaloids, which causes tractional retinal detachment. In PVR, RPE cells are crucial contributors, and multiple signaling pathways, including SMAD-dependent pathway, Rho pathway, MAPK pathways, Jagged/Notch pathway, and Wnt/β-catenin pathway, are activated. These pathways mediate the EMT of RPE cells, which play a key role in the pathogenesis of PVR. This review summarizes the current body of knowledge on the polarized phenotype of RPE, the role of cell-cell contact, and the molecular mechanisms underlying the RPE EMT in PVR, emphasizing key insights into potential approaches to prevent PVR.

Polarity and Epithelial-mesenchymal Transition of Retinal Pigment Epithelial Cells in Proliferative Vitreoretinopathy

Hui Zou[1], Chenli Shan[1], Linlin Ma[1], Jia Liu[1], Ning Yang[1], Jinsong Zhao[1]

[1] Eye Center of the Second Hospital, Jilin University, Changchun City, Jilin Province, China

Corresponding Author:
Jinsong Zhao;
Eye Center of the Second Hospital, Jilin University, 4026 Yatai Street, Changchun, Jilin, 130021, China
Email address: jinsongzhao2003@163.com

Abstract

Under physiological conditions, retinal pigment epithelium (RPE) is a cellular monolayer composed of mitotically quiescent cells. Tight junctions and adherens junctions maintain the polarity of RPE cells, and are required for cellular functions. In proliferative vitreoretinopathy (PVR), upon retinal tear, RPE cells lose cell-cell contact, undergo epithelial-mesenchymal transition (EMT), and ultimately transform into myofibroblasts, leading to the formation of fibrocellular membranes on both surfaces of the detached retina and on the posterior hyaloids, which causes tractional retinal detachment. In PVR, RPE cells are crucial contributors, and multiple signaling pathways, including SMAD-dependent pathway, Rho pathway, MAPK pathways, Jagged/Notch pathway, and Wnt/β-catenin pathway, are activated. These pathways mediate the EMT of RPE cells, which play a key role in the pathogenesis of PVR. This review summarizes the current body of knowledge on the polarized phenotype of RPE, the role of cell-cell contact, and the molecular mechanisms underlying the RPE EMT in PVR, emphasizing key insights into potential approaches to prevent PVR.

42

Introduction

Proliferative vitreoretinopathy (PVR) is a complex blinding disease that occurs after rhegmatogenous retinal detachment (RRD), surgical interventions, or ocular trauma. As a prolonged and exaggerated scarring process, PVR is characterized by the formation of contractile fibrocellular membranes in the vitreous cavity and on the inner and outer surfaces of the retina (Committee 1983; Mudhar 2020; Tosi et al. 2014). At present, surgical interventions, including vitrectomy, membrane peeling, pneumatic retinopexy, and scleral buckle, remain the mainstay of treatment in PVR. Although work in recent decades has led to advancements in surgical techniques and management, PVR cannot be effectively treated and is still the most common cause of failure to reattach the retina (Coffee et al. 2014; Khan et al. 2015; Mitry et al. 2012; Wickham et al. 2011). In addition, in spite of successful anatomic reattachment, the visual function of such cases cannot be improved, due to the retinal damage resulting from the mechanical contraction of fibrous membranes. Therefore, in order to improve postoperative visual function and reduce the incidence of this serious complication, it is particularly important to explore new prophylactic and therapeutic approaches based on a deeper understanding of the pathogenesis of PVR.

59   A growing body of evidence indicates that the mechanisms of PVR are orchestrated by

60   multiple elements (Idrees et al. 2019; Jin et al. 2017; Pastor et al. 2016), such as growth factors

61   (Charteris 1998; Ni et al. 2020; Pennock et al. 2014; Wubben et al. 2016), cytokines (Bastiaans

62   et al. 2018; Harada et al. 2006; Limb et al. 1991), extracellular matrix proteins (Feist et al. 2014;

63   Miller et al. 2017) and various cells (Eastlake et al. 2016; Pennock et al. 2011; Shu & Lovicu

64   2017). According to the histopathology of PVR, the fibrocellular membrane of PVR is composed

65   of excessive extracellular matrix (ECM) and multiple types of cells, and retinal pigment

66   epithelial (RPE) cells have been indicated as the most consistently present and the most abundant

67   (Amarnani et al. 2017; Ding et al. 2017; Hiscott et al. 1989; Machemer & Laqua 1975), proving

68   that the RPE cell plays a crucial role in PVR. Under physiological condition, the polarized RPE

69   cell is non-proliferative by cell-cell contact. However, when the eye suffers from a retinal break

70   or trauma, RPE cells are exposed to various growth factors and cytokines that are produced by

71   activated immune cells, leading to the disruption of junctional complexes in RPE cells.

72   Subsequently, activated RPE cells detach from Bruch's membrane, migrate through the defect of

73   the retina, proliferate, and transform into myofibroblasts, forming fibrotic membranes (Chen et

74   al. 2015; Morescalchi et al. 2013; Palma-Nicolás & López-Colomé 2013). In an analogous

75   process to exaggerated wound healing response, these membranes can attach to the retina and

76   contract, resulting in further retinal detachment and poor vision (Chiba 2014; Garweg et al.

77   2013). It is noteworthy that due to the loss of cell-cell contact, RPE cells undergo epithelial-

78   mesenchymal transition (EMT), which is pivotal in the development of PVR. During EMT, RPE

79   cells transdifferentiate into mesenchymal cells that are characterized by increased motility, and

80   enhanced ability to proliferate, resist apoptosis and produce extracellular matrix proteins, thus

81   participating in PVR (Tamiya & Kaplan 2016; Zhang et al. 2018c). These indicate that in-depth

82   knowledge of EMT may provide insight into potential approaches to prevent PVR. Therefore,

83   this review focuses on the polarized phenotype of RPE and molecular mechanisms of RPE cell

84   EMT, discussing the role of RPE cells in PVR.

Survey methodology

86   We used the PubMed database to search available literature based on keywords including

87   "proliferative vitreoretinopathy(PVR)" and "retinal pigment epithelial cell". To include more

88   information on the polarity of RPE, we also searched articles about the structure and function of

89   cell-cell junctions in RPE cells that explored the role of cell-cell contact in EMT.

90   **1. The Polarized Retinal Pigment Epithelial Cell**

91   The human RPE cell achieves terminal differentiation at four to six weeks of gestation and

92   subsequently remains mitotically quiescent (Lutty & McLeod 2018; Stern & Temple 2015). The

93   RPE, which is situated between the photoreceptors and the choroid, plays many complex roles

94 indispensable to the health of the neural retina and the choroid. These roles include recycling of
95 components of the visual cycle, absorption of light to protect from photo-oxidative stress,
96 production of essential growth factors, immunological regulation of the eye, phagocytosis of
97 photoreceptor outer segments generated during daily photoreceptor renewal, and transportation
98 across the blood retina barrier (BRB) (Ferrington et al. 2016; Fields et al. 2019; Mateos et al.
99 2014; Naylor et al. 2019; Strauss 2005; Vigneswara et al. 2015). In order to maintain these
100 multiple functions, RPE cells display a highly specialized structural and functional polarity.
101   Similar to other epithelia, the RPE displays three characteristics of the epithelial phenotype:
102 apical plasma membrane, junctional complexes, and basolateral domain. RPE cells display
103 structural polarity, with apical microvilli and melanosomes, and basal microinfolds. The
104 abundant melanin granules in RPE cells absorb stray light, a process that is essential for visual
105 function (Strauss 2005). In a polarized cell, the distributions of surface proteins on the apical and
106 basal plasma membranes are different, contributing to the performance of cellular functions
107 (Khristov et al. 2018). However, a highly polarized distribution of ion channels, transporters and
108 receptors in RPE is different from that observed in conventional extraocular epithelia (Lehmann
109 et al. 2014). For example, Na, K-ATPase(Sonoda et al. 2009) and monocarboxylate transporters
110 (MCT) 1(Deora et al. 2005) are localized to the apical aspect of RPE cells, while chloride
111 transporter CFTR(Maminishkis et al. 2006) is basally located. On the apical plasma membrane,
112 RPE cells phagocytize the photoreceptor outer segments, which are regulated by polarized
113 receptors. Bulloj et al. (2018) found that binding of Semaphorin 4D (sema4D) to RPE apical
114 receptor Plexin-B1 suppresses outer segment internalization, contributing to the maintenance of
115 photoreceptor function and longevity. The RPE also transports fluid out of the subretinal space,
116 and regulates bidirectional nutrient transport between the outer retina and the choroid, in a
117 manner dependent on the polarized distribution of membrane channels and transporters(Strauss
118 2005). The RPE basolaterally secretes extracellular matrix components and factors, which
119 participate in ECM remodeling and maintain the outer BRB (oBRB) function (Caceres &
120 Rodriguez-Boulan 2020). Therefore, the polarized phenotype of the RPE is vital to both the
121 oBRB and is the basis of the homeostasis of the outer retina (Caceres & Rodriguez-Boulan 2020;
122 Lehmann et al. 2014). The disruption of RPE polarity contributes to the development of several
123 retinal diseases, such as PVR and age-related macular degeneration (AMD). A comprehensive
124 understanding of the way in which this polarity is achieved may provide insights into the
125 pathogenesis of PVR.
126   However, most available data on RPE polarity is contributed by studies performed on RPE-
127 immortalized cell lines that show partial preservation of the RPE phenotype, and were
128 extrapolated from data obtained from the prototype Madin-Darby Canine Kidney (MDCK) cell
129 line (Lehmann et al. 2014). The detailed mechanisms that determine RPE polarization remain

130 unclear. Some scholars believe that junctional complexes, including adherens junctions (AJs)
131 and tight junctions (TJs), are essential for building epithelial cell polarity and maintaining the
132 integrity of epithelial layers such as RPE (Niessen 2007; Pei et al. 2019; Tamiya & Kaplan
133 2016).

134   Tight junctions are complex cell-cell junctions formed by transmembrane proteins
135 interactions with peripheral cytoplasmic proteins (Fig 1). Transmembrane proteins include
136 occludin, members of the claudin family, and junctional adhesion molecules (JAMs). Peripheral
137 cytoplasmic proteins, such as zonula occludens (ZOs), form bridges between transmembrane
138 proteins and the actin filament cytoskeleton and play a key role in the assembly and organization
139 of TJs (Bazzoni & Dejana 2004; Bazzoni et al. 2000; Naylor et al. 2019).

140   The RPE tight junctions regulate the paracellular movement of solutes via size and charge
141 selectivity (Benedicto et al. 2017; Caceres et al. 2017; Naylor et al. 2019).Occludin and claudins
142 determine the permeability and semi-selectivity of the TJs, and as such play critical roles in the
143 oBRB (Balda et al. 2000; Fields et al. 2019; Furuse et al. 1998; Günzel & Yu 2013; Rosenthal et
144 al. 2017). JAMs regulate TJ assembly and function by recruiting other proteins to the TJ and play
145 an important role in the barrier property of TJs (Balda & Matter 2016; Orlova et al. 2006; Shin et
146 al. 2006). In patients with RRD, damage to TJs elicits the breakdown of oBRB and promotes the
147 penetration of growth factors and cytokines, aggravating PVR. As well as having a barrier
148 function, TJs define the physical separation between apical and basal domains of the plasma
149 membrane, to maintain RPE cell polarity (Campbell et al. 2017; González-Mariscal et al. 2014;
150 Sluysmans et al. 2017). The two extracellular loops of occludin mediate adhesion of adjacent
151 cells and block the movement of plasma components. The C-terminal domain combines directly
152 with ZOs, subsequently interacting with the actin cytoskeleton, which is essential to organizing
153 and maintaining cell polarization (Balda & Matter 2016; Furuse et al. 1994; Shin et al. 2006;
154 Tarau et al. 2019). Feng et al. (2019) demonstrated that during EMT, the breakdown of TJs
155 resulting from loss of claudin-1 causes ARPE-19 cells to lose their epithelial phenotype and
156 transform into fibroblasts, promoting the development of PVR. TJs are involved in the regulation
157 of signaling pathways that govern various cellular functions such as proliferation, migration, and
158 differentiation (Bhat et al. 2018; Shi et al. 2018; Sluysmans et al. 2017). Vietor et al. (2001)
159 found that decreased amounts of occludin can cause up-regulation and translocation of the
160 adhesion junction protein β-catenin, which interacts with the transcription factor lymphoid
161 enhancer-binding factor (LEF)/T cell factor (TCF) in the nucleus, leading to a loss of the
162 polarized epithelial phenotype in EpH4 cells. ZOs, adaptor proteins within the TJ complex,
163 exhibit dual localization at TJs and in the nucleus. Under injury or stress, the disruption of TJs
164 increases ZO-2 nuclear accumulation, driving its interaction with transcription factors, and
165 inducing MDCK epithelial cell proliferation (Islas et al. 2002; Shi et al. 2018; Traweger et al.

166   2003). In differentiated RPE cells, the interaction between ZO-1 with ZO-1-associated nucleic
167   acid-binding protein (ZONAB) maintains cell-cell contact by sequestering ZONAB at the TJ or
168   in the cytoplasm, maintaining cells dormancy. However, when damage to TJs decreases ZO-1
169   levels, ZONAB is translocated into the nucleus, leading to the up-regulation of cyclin D1 (CD1)
170   and subsequent cell proliferation (Balda et al. 2003; González-Mariscal et al. 2014). Therefore,
171   TJs provide a structural foundation for the maintenance of cell-cell contact. Georgiadis et al.
172   (2010) demonstrated that the overexpression of ZONAB or knockdown of ZO-1 could result in
173   increased RPE proliferation and the development of EMT. Recent research has confirmed that
174   during EMT, ZO-1 is decreased in ARPE-19 cells, and the knockdown of either ZO-1 or AJ
175   protein E-cadherin leads to the downregulation of the other protein, indicating the existence of an
176   interaction between the two junctional complexes (Bao et al. 2019). Due to the importance of TJs
177   in the maintenance of integrity and functionality of epithelial cells, several researchers have
178   focused on novel factors that stimulate the formation of TJs, such as nicotinamide (Hazim et al.
179   2019) and lysophosphatidic acid (Lidgerwood et al. 2018). Studies into these factors may
180   produce well-differentiated RPE cell lines and a platform to enable the rapid expansion of our
181   understanding of many RPE functions and retinal pathologies. This approach could be conducive
182   to finding novel therapeutic interventions for PVR.
183   Besides the TJ complex described above, another type of junctional complex called AJs
184   plays a key role in the maintenance of the integrity of epithelial cells and cell-cell contact (Fig 1).
185   Cadherins, the major proteins of AJs, belong to the glycoprotein superfamily, of which there are
186   more than 20 members. The cytoplasmic domain of cadherins regulates interactions between
187   cadherins and catenins, including β-catenin, α-catenin, and p120-catenin, and other scaffolding
188   proteins such as ZO-1, to maintain cell shape and modulate cell proliferation (Aberle et al. 1994;
189   Nelson & Nusse 2004; Wheelock & Johnson 2003). In quiescent adult RPE cells, epithelial
190   cadherins (E- and/or P-cadherin) sequester β-catenin at the AJs to maintain cell-cell contact.
191   Reduction of cadherin levels or dissociation of AJs allows β-catenin to translocate into the
192   nucleus, where it interacts with the transcription factor LEF, and activates the transcription of
193   various genes, including Snail and cyclin D1, which participate in RPE cell EMT via the
194   canonical Wnt/β-catenin signaling pathway (Gonzalez & Medici 2014; Lamouille et al. 2014;
195   Nelson & Nusse 2004; Yang et al. 2018) . Tamiya et al. (2010) suggested that the loss of P-
196   cadherin causes the loss of cell-cell contact and initiates RPE cell migration and EMT. These
197   events coincide with a switch in cadherin isoform expression from P- to N-cadherin. In addition,
198   hepatocyte growth factor (HGF) and its receptor c-Met can destabilize cell-cell adhesion and
199   elicit nuclear translocation of β-catenin, resulting in RPE cell migration (Lilien & Balsamo 2005;
200   Liou et al. 2002). Jin et al found that HGF induces loss or redistribution of junctional proteins
201   ZO-1, occludin, and β-catenin in RPE explants, potentially damaging barrier function and

202 increasing the migration of RPE cells, resulting in retinal detachment(RD) and PVR (Jin et al.
203 2002; Jin et al. 2004). Given the importance of HGF in the interruption of RPE junction, HGF
204 may be a potential target for the prevention and treatment of PVR. However, this possibility
205 needs further study.

206   Under physiological conditions in the eye, TJs and AJs maintain the specialized structural
207 and functional polarity of RPE cells and play a pivotal role in the maintenance of cell-cell
208 contact; they sequester EMT signaling effectors ZONAB and β-catenin at the junction or
209 cytoplasm to prevent cells from responding to mitotic factors, causing cells to leave the cell-
210 cycle (Fig 1). Thus, normally, RPE cells form a cobblestone-like monolayer of immotile,
211 polarized, and mitotically quiescent cells. However, once junctional complexes break down, RPE
212 cells undergo EMT, which is an important contributor to proliferative vitreoretinopathy. In this
213 pathological process, RPE cells lose their structural and functional polarity and transdifferentiate
214 into mesenchymal cells, which proliferate, resist apoptosis, possess migratory ability, and
215 produce abundant ECM, leading to the formation of an aberrant scar-like fibrocellular
216 membrane.

217 **2. De-differentiated RPE and Fibrocellular Membrane**

218   Proliferative vitreoretinopathy is characterized by the formation of fibrocellular membranes
219 composed of proliferative and migratory cells and excessive, aberrant ECM. Histopathological
220 analysis of PVR has demonstrated that PVR membranes have contractile activity and strain the
221 retina, leading to tractional retinal detachment (TRD), which is responsible for blurring vision.

222   Several studies (Feist et al. 2014; Takahashi et al. 2010) have found that the cellular
223 components of PVR membranes include RPE cells, myofibroblasts, fibroblasts, glial cells and
224 macrophages, and that myofibroblasts are critical for the formation and contractile activity of
225 fibrocellular membranes. Based on the indirect immunofluorescence evaluation of human PVR
226 membranes, Feist et al. (2014) showed that myofibroblasts originate principally from RPE cells
227 through EMT. Myofibroblasts are characterized by increased expression of alpha-smooth muscle
228 actin (α-SMA) and incorporation of α-SMA into newly formed actin stress fibers, which
229 enhances their contractile properties. Myofibroblasts also secrete excessive matrix and pro-
230 fibrogenic factors, promoting the contraction of PVR membranes that ultimately cause
231 irreversible loss of vision (Gamulescu et al. 2006; Hinz et al. 2001; Shu & Lovicu 2017; Tamiya
232 & Kaplan 2016; Tomasek et al. 2002).

233   In addition to myofibroblasts, abnormally increased ECM reinforces the continuous
234 contractile tension of PVR membranes, and this mechanical tension, together with specialized
235 ECM proteins, regulates myofibroblast differentiation and its function, contributing to PVR. In
236 PVR membranes, the primary components of ECM are collagen and fibronectin. The majority of

237 collagen fibrils are type Ⅰ collagen, which is synthesized by RPE cells and Müller cells.

238 Collagen fibrils provide tensile strength to the ECM, and activate Rho, resulting in the

239 translocation of myocardin-related transcription factor (MRTF) into the nucleus and promoting

240 RPE cell EMT (Guettler et al. 2008; Miralles et al. 2003). Fibronectin may also play a significant

241 role in PVR. During pathological ECM remodeling, fibronectin is one of the earliest ECM

242 components recruited, serving as a scaffold for other ECM proteins (Kadler et al. 2008; Miller et

243 al. 2017; Miller et al. 2014). Extra domain (ED)-A fibronectin, a splice variant of fibronectin, is

244 increased in TGF-β2-induced RPE cells and induces myofibroblast differentiation, participating

245 in PVR (Khankan et al. 2011).

246   Under normal conditions, ECM breakdown by proteases such as matrix-metalloproteases

247 (MMPs) plays a crucial role in ECM remodeling and the release of growth factors, maintaining

248 tissue homeostasis in cooperation with ECM synthesis, reassembly, and chemical modification

249 (Bonnans et al. 2014; Craig et al. 2015; Lindsey et al. 2016). As mentioned above, the polarized

250 RPE is able to basolaterally secrete the extracellular matrix components fibronectin and

251 collagens, MMP and tissue inhibitors of MMPs (TIMPs), which participate in ECM remodeling.

252 However, under pathological conditions such as inflammation and retinal injury, RPE cells lose

253 their apical-basal polarity, undergo EMT and abnormally secrete MMPs, TIMPs and ECM

254 proteins, leading to dysregulated ECM remodeling (Greene et al. 2017). Such ECM has aberrant

255 composition and organization and mechanical properties, and enhances matrix stiffness and

256 strain, which disrupts the normal structure and function of the retina, exacerbating the

257 progression of PVR.

258 **3. RPE and Epithelial-mesenchymal Transition**

259 3.1 EMT of RPE Cell

260   Epithelial-mesenchymal transition is an important biological process, in which epithelial

261 cells transdifferentiate into mesenchymal cells. Although EMT can occur in normal embryonic

262 development and wound healing, it also participates in pathological processes such as fibrosis,

263 cancer progression, and PVR. There are three distinct subtypes of EMT: type 1 occurs during

264 tissue and embryo development, type 2 is involved in wound healing and organ fibrosis, and type

265 3 is associated with cancer progression and metastasis (Dongre & Weinberg 2019; Kalluri &

266 Weinberg 2009). This review focuses on type 2 EMT, which is crucial to PVR. During EMT,

267 due to junctional complexes damage, RPE cells relinquish their apical-basal polarity, reorganize

268 their cytoskeletal architecture, and convert into spindle-shaped cells (Fig 1). These cells

269 downregulate the expression of epithelial proteins such as E-cadherin and ZO-1, and increase

270 expression of mesenchymal drivers including N-cadherin, vimentin, α-SMA and fibronectin (Li

271 et al. 2020). This mesenchymal transdifferentiation of RPE cells can increase the directional

272   motility of individual cells, confer resistance to apoptosis, and facilitate cell proliferation and
273   dysregulated ECM remodeling, eventually leading to the formation of PVR membranes.
274   3.2 Transcription Factors of EMT
275   The details of the molecular mechanisms that drive RPE cell EMT and lead to PVR remain to
276   be clarified. Emerging evidence suggests that diverse extracellular inductive signals, including
277   soluble cytokines and growth factors, and ECM components, can modulate the expression and

278   activity of EMT‐ associated transcription factors and act together to control the initiation and

279   progression of EMT in responding epithelial cells (Yang et al. 2020). Among the various
280   transcription factors involved in the induction of EMT, core transcription factors including Snail
281   1, Snail 2(also known as Slug), Twist 1 and zinc-finger E-box-binding (Zeb) 1 have been
282   identified as important regulators of RPE cell EMT. These factors impact the expression of genes
283   that control repression of the epithelial phenotype and activation of the mesenchymal phenotype
284   (Boles et al. 2020; Feng et al. 2019; Li et al. 2019; Li et al. 2014; Liu et al. 2009; Palma-Nicolás
285   & López-Colomé 2013). For example, thrombin can repress the expression of E-cadherin by
286   stimulating Snail 2 expression and promote the expression of N-cadherin by phosphoinositide 3-
287   kinase (PI3K)/PKC-ζ/mTOR signaling in Rat RPE cells (Palma-Nicolás & López-Colomé 2013).
288   During RPE dedifferentiation in primary culture, Zeb1 is overexpressed and binds to the MITF A
289   promoter to repress the cyclin dependent kinase inhibitor, p21CDKN1a, resulting in RPE cell
290   proliferation and EMT (Liu et al. 2009). These EMT transcription factors often act in concert,
291   functionally cooperating at target genes by the convergence of signaling pathways. However, the
292   molecular details of how these transcription factors contribute to EMT are still elusive
293   (Lamouille et al. 2014; Stone et al. 2016).
294   3.3 Epigenetic Factors of EMT
295   Due to the importance of epigenetic regulation of EMT, epigenetic modifiers have attracted
296   increasing attention. Unlike transcription factors, epigenetic modifications are more stable/long-
297   term. However, evidence has shown that epigenetic modifiers work in concert with transcription
298   factors at different molecular layers to regulate the EMT process (Skrypek et al. 2017). Several
299   epigenetic factors have been described including DNA methylation, histone modification and
300   non-coding RNA. Because of the specific machinery utilized for EMT activation, these
301   modifications are characterized by cell type specificity. In RPE cells, Methyl-CpG-binding
302   protein 2 (MeCP2), a DNA methylation reader, plays a crucial role in the induction of EMT, and
303   DNA methylation may participate in the pathogenesis of PVR (He et al. 2015; Li et al. 2020). He
304   et al. (2015) found high levels of expression of MeCP2 in all human PVR membranes, and
305   concluded that MeCP2 mediates α-SMA expression through Ras GTPase activating protein
306   (RASAL1). Furthermore, DNA methylation inhibitor 5-Aza-2' deoxycytidine (5-AZA-dC)

307 reportedly inhibits the expression of TGF-β-induced α-SMA and FN in human fetal RPE cells. It
308 appears that 5-AZA-dC may have therapeutic value in the treatment of PVR. However, the
309 mechanisms underlying the blockade of α-SMA and FN expression are complex, and further
310 investigation is warranted.
311 Recently, the role of histone modifications associated with EMT has been assessed in RPE
312 cells. However, there has been little research into the regulation of RPE cell EMT by histone
313 modification. Boles et al. (2020) reported that TGF-β1 and TNF-a co-treatment (TNT) induces
314 an EMT program in adult human RPE stem cell (RPESC)-RPE cells, involving an apparent
315 reorganization of H3K27ac and H3K4me1 patterns at distal enhancers. The regions that gain
316 H3K27ac tend to have a high H3K4me1/H3K4me3 ratio, indicating that they have enhancer
317 activity and are associated with upregulated genes. Xiao et al. (2014) found that the expression
318 of histone deacetylases (HDACs) in TGF-β-induced EMT of RPE cells was increased, and that
319 Trichostatin A (TSA), a class I and II HDAC inhibitor, attenuated TGF-β2-induced EMT by
320 inhibiting the canonical SMAD pathway and the non-canonical signaling pathways, including
321 Akt, p38MAPK, ERK1/2 pathways and Notch pathway. Therefore, histone modifications may
322 participate in the regulation of RPE cell EMT, and HDAC inhibitors may have potential as drugs
323 for the prevention and treatment of PVR.
324 The study of EMT mechanisms at the RNA level has provided new perspectives on the
325 treatment of PVR (Kaneko & Terasaki 2017; Wang et al. 2016). MicroRNAs (miRNAs) are
326 small noncoding RNAs that contribute to cellular processes by regulating gene expression. In
327 differentiated RPE cells, microRNA-204 is highly expressed, and represses the expression of
328 type Ⅱ TGF-β receptors and Snail 2, maintaining epithelial structure and function. In contrast,
329 low expression levels of miR-204 and anti-miR-204 promote RPE cells proliferation,
330 participating in EMT (Wang et al. 2010). MicroRNA-194 overexpression can also suppress RPE
331 cell EMT by attenuating the expression of Zeb1 (Cui et al. 2019). In addition to miRNAs, long
332 non-coding RNAs (lncRNAs) contribute to the regulation of RPE EMT (Zhang et al. 2019). In
333 RPE cells treated with PVR vitreous or TGF-β1, MALAT1 expression is increased, and
334 knockdown of MALAT1 attenuates the phosphorylation of SMAD2/3 and the expression of
335 Snail, Slug, and Zeb1, preventing cell migration and proliferation (Yang et al. 2016). In patients
336 with PVR, MALAT1 is increased in the blood, and is reduced after surgery. Thus, MALAT1
337 may be a potential prognostic and diagnostic indicator for PVR (Zhou et al. 2015).
338 3.4 Signaling Pathways of EMT
339 During RPE cell EMT, extracellular signals change the expression of genes encoding
340 epithelial and mesenchymal proteins and mediate cellular behavior such as cell migration,
341 proliferation, and apoptosis through a network of interacting signaling pathways that contribute

342 to the development of PVR (Chen et al. 2014a; Chen et al. 2014b; Lee-Rivera et al. 2015).

343 Among these, transforming growth factor-β (TGF-β) and its intracellular cascades play a key

344 role in the EMT of RPE cells.

345   TGF-β induces EMT of RPE cells via two pathways: the classical SMAD-dependent

346 pathway and the SMAD-independent pathway (Fig 2) (Cai et al. 2018; He et al. 2017; Heffer et

347 al. 2019; Ishikawa et al. 2015; Takahashi et al. 2015; Yao et al. 2019; Zhang et al. 2017; Zhang

348 et al. 2018b; Zhou et al. 2017). In the SMAD dependent pathway, TGF-β binds to cell surface

349 receptor complexes, and activates type Ⅰ TGF-β receptors, which phosphorylate SMAD2 and

350 SMAD3. The activated SMADs combine with SMAD4 to form a SMAD complex, which then

351 enters the nucleus and combines with regulatory elements to regulate the expression of key genes

352 associated with EMT. In addition to SMAD-dependent signaling, TGFβ induces EMT through

353 SMAD independent signaling pathways including Rho GTPase-dependent pathways (Lee et al.

354 2008), PI3K/Akt pathway (Huang et al. 2017; Yokoyama et al. 2012), mitogen-activated kinase

355 (MAPK) pathways (Chen et al. 2017; Lee et al. 2020; Matoba et al. 2017; Schiff et al. 2019) and

356 Jagged/Notch signaling pathway (Zhang et al. 2017). The MAPK signaling pathways include

357 extracellular signal-regulated kinase(ERK) MAPK pathway, p38 MAPK pathway, and JUN N-

358 terminal kinase (JNK) pathway (Parrales et al. 2013; Schiff et al. 2019; Xiao et al. 2014; Zhang

359 et al. 2018a).

360   The Rho pathway has been reported to regulate the assembly and organization of the actin

361 cytoskeleton and associated gene expression, and may be essential for the fibrotic response of

362 RPE cells in PVR. In TGF-β1-treated ARPE-19 cells, activated RhoA or its downstream effector

363 Rho kinase (ROCK) increase the kinase activity of LIM kinase (LIMK) which then

364 phosphorylates cofilin. This phosphorylation attenuates the activity of cofilin, which promotes

365 actin polymerization and reorganizes the actin cytoskeleton, leading to stress fiber formation

366 (Lee et al. 2008). TGF-β-induced RhoA activation also facilitates cell migration and increases α-

367 SMA expression in primary RPE cells (Tsapara et al. 2010). Itoh et al. (2007) demonstrated that

368 ROCK inhibitor Y27632 and RhoA inhibitor, simvastatin, suppress TGF-β2-induced type Ⅰ

369 collagen expression in ARPE-19 cells, and confirmed the existence of crosstalk between the

370 SMAD pathway and the Rho pathway. Some studies have suggested that activated SMAD3

371 induces NET1 gene expression to regulate RhoA activation in RPE cells (Lee et al. 2010).

372 Moreover, thrombin can activate Rho and ROCK, leading to myosin light chain (MLC)

373 phosphorylation and actin stress fiber formation in EMT of RPE cells (Fig 3)(Ruiz-Loredo et al.

374 2011). Therefore, ROCK inhibitor and RhoA inhibitor may be new potential therapeutic target

375 drugs for PVR.

376   The PI3K/Akt pathway mediates a broad range of cellular functions, such as cell

377   transformation, migration, proliferation, apoptosis, and gene expression (Aguilar-Solis et al.

378   2017; Liu et al. 2019). During PVR, binding of TGF-β to its receptor activates PI3K, resulting in

379   the phosphorylation of Akt; activated Akt inhibits glycogen synthase kinase 3β (GSK-3β),

380   promoting EMT in RPE cells (Shukal et al. 2020; Zhang et al. 2018a). Researchers have found

381   that inhibition or knockdown of GSK-3β promotes cell migration and collagen contraction in

382   ARPE-19 cells, while GSK-3β overexpression and PI3K/Akt inhibitor reverse these cellular

383   responses (Huang et al. 2017). Some studies have shown that thrombin can activate PI3K,

384   resulting in increased cyclin D1 expression and RPE cell proliferation, processes that are

385   involved in the development of PVR through PDK1/Akt and PKCζ/mTORC signaling (Fig 3)

386   (Lee-Rivera et al. 2015; Palma-Nicolás & López-Colomé 2013; Parrales et al. 2013).

387   In addition to the PI3K-AKT pathway, other kinase pathways contribute to EMT in

388   cooperation with the SMAD-dependent signaling pathways. In human RPE cells, TGF-β

389   activates TGF-β-activated kinase 1 (TAK1), which subsequently transduces signals to several

390   downstream effectors, including p38 (Heffer et al. 2019), JNK (Kimura et al. 2015) and nuclear

391   factor-κB (NF-κB) (Chen et al. 2016b), which participate in EMT. Dvashi et al. (2015) found

392   that TAK1 inhibitor caused a reduction in both p38 and SMAD2/3 activity, attenuating cell

393   migration, cell contractility and α-SMA expression in TGF-β1-induced RPE cells. Moreover, the

394   ERK MAPK pathway plays a role in TGF-β-induced EMT and cooperates with other signaling

395   pathways in the regulation of EMT in RPE cells. Recent studies (Chen et al. 2014b; Tan et al.

396   2017; Xiao et al. 2014) have shown that blocking the ERK1/2 pathway inhibits the

397   phosphorylation of SMAD2 and the Jagged/Notch pathway. Inhibition of the Jagged/Notch

398   signaling pathway can alleviate TGF-β2-induced EMT by regulating the expression of Snail,

399   Slug and Zeb1 (Fig 3); this also suppresses the ERK1/2 signaling (Chen et al. 2014b).

400   The contribution of growth factors other than TGF-β, such as HGF, fibroblast growth factor

401   (FGF), epidermal growth factor (EGF) and platelet derived growth factor (PDGF) should also be

402   factored in with regard to the induction of RPE EMT. These factors bind to and stimulate the

403   autophosphorylation of transmembrane receptors on Tyr, subsequently participating in RPE cell

404   EMT via PI3K/Akt pathway, ERK MAPK pathway, p38 MAPK pathway (Fig 3) (Chen et al.

405   2016a; Ozal et al. 2020).Chen et al. (2012) explored the role of Wnt/β-catenin signaling in PVR,

406   and found that when EGTA disrupted contact inhibition in RPE cells, EGF+FGF2 could activate

407   Wnt signaling and increase nuclear levels of β-catenin, which interacts with TCF and/or LEF,

408   leading to cell proliferation (Fig 3); and EGF+FGF2 cooperated with TGF-β1 to induce EMT

409   through SMAD/Zeb1/2 signaling. Acting together, various inductive signals received by RPE

410   cells from their niche can trigger the activation of EMT programs by individual intracellular

411   cascades or the crosstalk of multiple intracellular signaling pathways.

412   3.5 Interventions of RPE EMT

413   Therapeutic interventions against RPE EMT have largely been explored in mechanistic
414   experiments using in vitro cell culture and in vivo animal models. To date, some promising drug
415   candidates have been trialed in preclinical studies of PVR, including TGF- β receptor inhibitors,
416   peroxisome proliferator-activated receptor (PPAR)-γ agonists, retinoic acid receptor-γ (RAR-γ)
417   agonists and methotrexate (Shu et al. 2020; Zhou et al. 2020).Nassar et al. (2014) found that
418   TGF-β receptor 1 inhibitor LY-364947 (LY) attenuates RPE cell transdifferentiation *in vitro*, and
419   that intravitreal injection of LY completely prevents PVR and TRD *in vivo*. Evidence is
420   emerging to show that the up-regulation of PPAR-γ expression may be beneficial for the
421   treatment of fibrosis in several organs(Wang et al. 2019). Hatanaka et al. (2012) reported that
422   PPAR-γ agonist pioglitazone could prevent TGF-β-induced morphological changes and the up-
423   regulation of EMT-related markers in primary monkey RPE cells, through inhibition of the
424   SMAD pathway. Some drugs, including dichloroacetate (DCA)(Shukal et al. 2020), salinomycin
425   (SNC)(Heffer et al. 2019), resveratrol(Ishikawa et al. 2015), protein kinase A inhibitor H89 (Lyu
426   et al. 2020) and heavy chain-hyaluronan/pentraxin3(He et al. 2017), reportedly inhibit EMT in an
427   *in vitro* EMT cell model and prevent PVR development by blocking the activation of theTGF-β
428   pathway. Thus, inhibition of EMT by pharmacological agents may be an effective strategy to
429   prevent PVR development.

Conclusion

431   Clinical and experimental studies have shown that RPE cells play an important role in PVR.
432   Junctional complexes are crucial for the maintenance of RPE polarity. Under the influence of
433   growth factors and cytokines, RPE cells lose cell-cell contact and apical-basal polarity, and
434   undergo EMT via multiple signaling pathways, which promote cell proliferation, migration, and
435   ECM production. RPE cells further transform into myofibroblasts and form fibrocellular
436   membranes that have contractile activity and strain the retina, leading to tractional retinal
437   detachment in PVR. As a complex refractory blinding disorder, PVR involves multiple signaling
438   pathways and factors. In addition, the specialized polarity of RPE cells is fundamental for retinal
439   homeostasis, and RPE EMT plays a key role in the development of PVR. Nevertheless, further
440   research into the mechanisms underlying RPE polarity and EMT is needed to prevent this
441   devastating complication. A deeper understanding of RPE polarization is fundamental for
442   elucidating the mechanism of EMT initiation and progression, and is essential to exploring the
443   potential pharmacologic prophylactic and therapeutic approaches to PVR. Various factors, such
444   as microenvironmental signals, transcription factors, and epigenetic factors, participate in the
445   regulation of EMT at different molecular levels. Further studies about the detailed molecular
446   mechanisms of EMT are needed to facilitate the development of therapeutic strategies for PVR.

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Figure 1

Role of cell-cell contact in health and PVR

(A) Tight junctions and adherens junctions maintain cell-cell contact and cell polarity in RPE cells. Mature RPE cells with cell-cell contact remain dormant by sequestering EMT effectors to prevent nuclear localization. ZO-1 sequesters nucleic acid-binding protein (ZONAB) at tight junctions/cytoplasm, and adherens junctions sequester β-catenin by binding to epithelial cadherins. Tight junctions have a barrier function that control the passage of solutes. **(B)**Loss of cell-cell contact initiates EMT. Deconstruction of junctional complexes or reduction of epithelial cadherins/ZO-1 elicits nuclear localization of ZONAB/β-catenin and activation of their target genes, and disrupts the outer blood retinal barrier, facilitating the release of growth factors and cytokines, which further aggravate PVR.
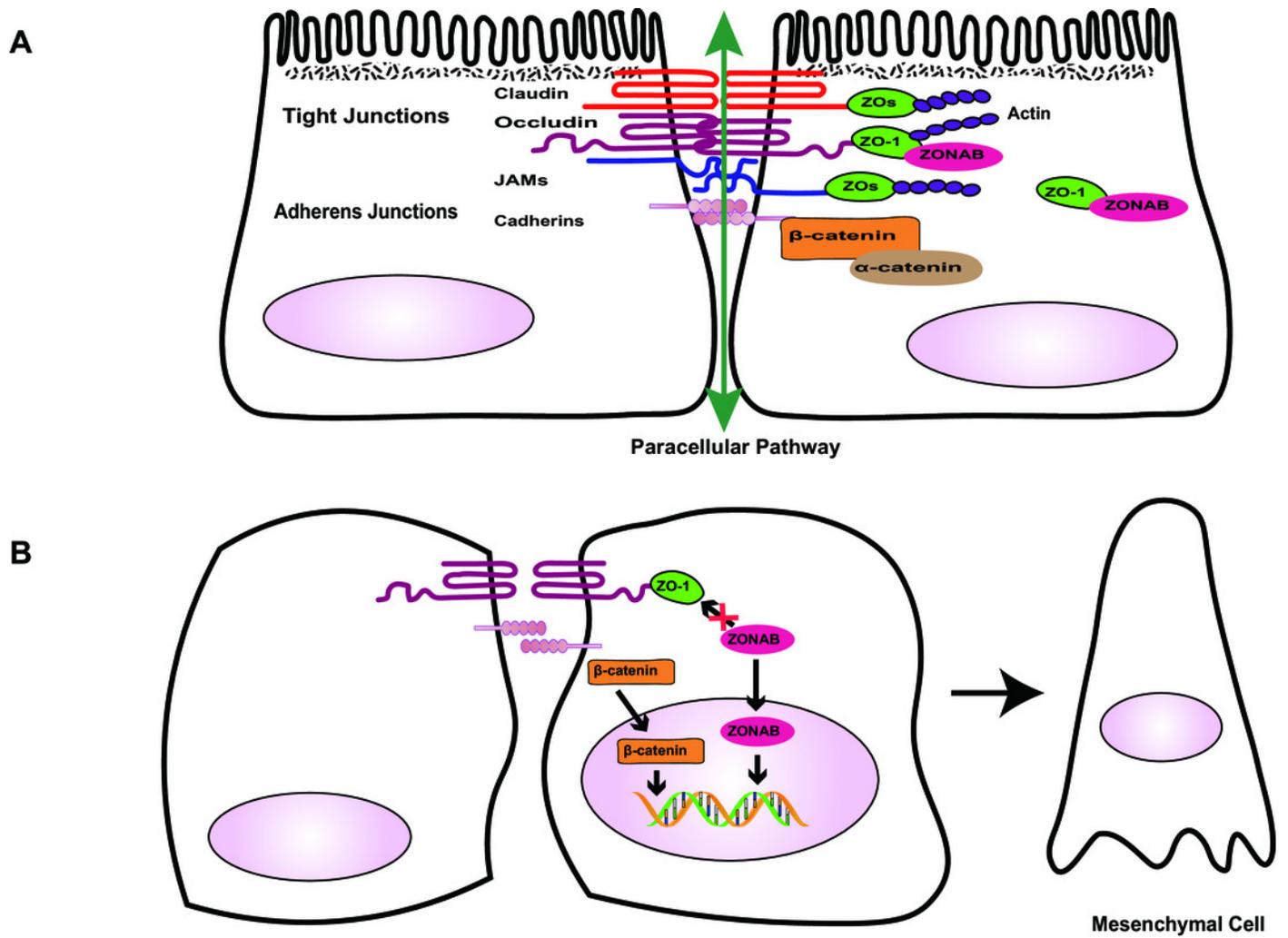
Figure 2

Signaling pathways of TGF-β-induced RPE cells EMT

Transforming growth factor-β (TGF-β) activates various signaling pathways that cooperate to cause EMT. Besides canonical SMAD-dependent signaling, TGF-β can activate the Rho, PI3K/AKT, ERK MAPK, p38 MAPK, JUN N-terminal kinase (JNK) and nuclear factor-κB (NF-κB) pathways.
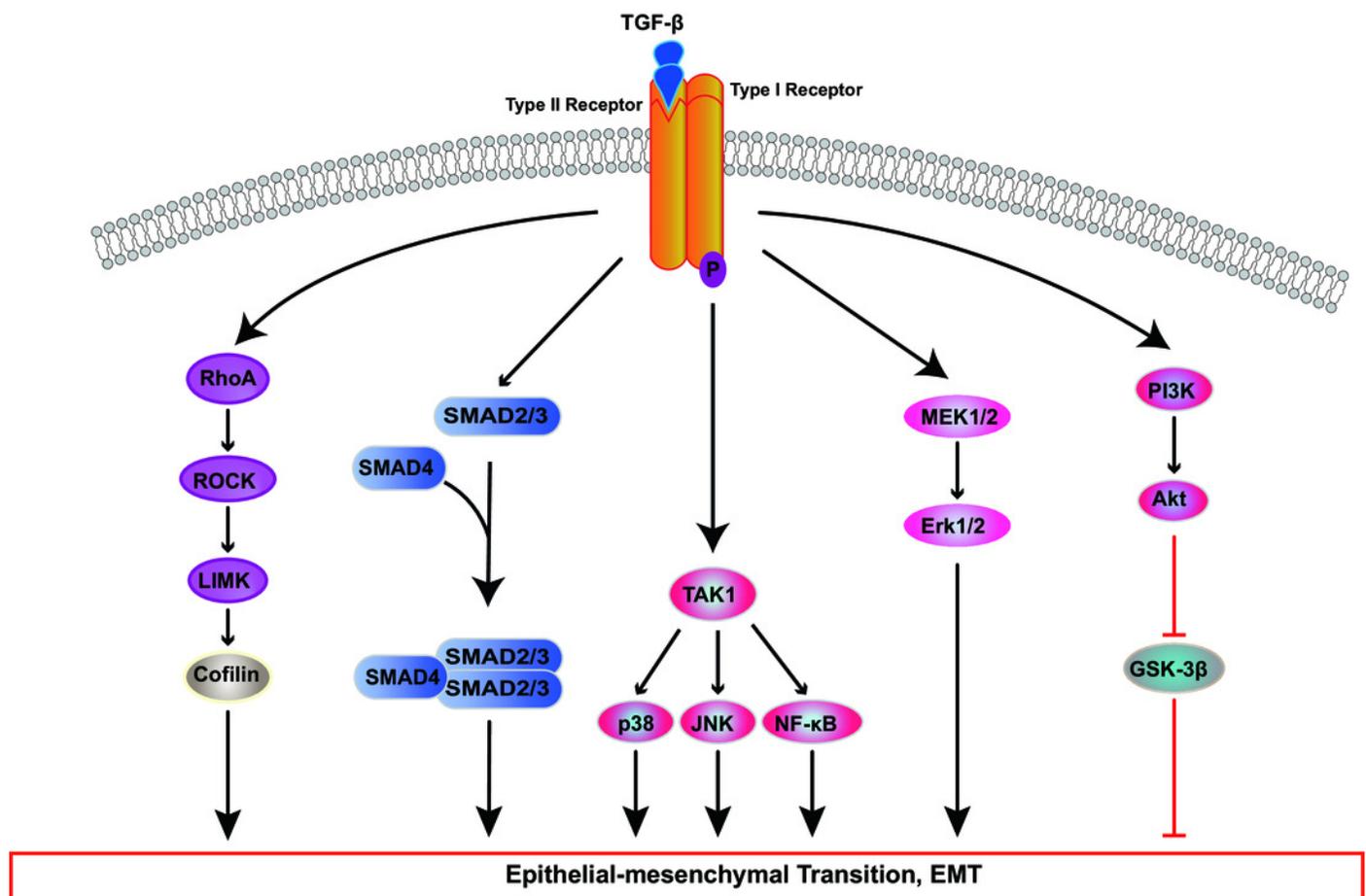
Figure 3

RTK, Wnt, Notch, and Thrombin signaling in RPE cells EMT

Growth factors (GFs) stimulate receptor tyrosine kinases (RTKs) and induce EMT through PI3K-AKT and ERK MAPK signaling pathways. Thrombin activates PI3K and Rho signaling. PI3K promote EMT through Akt and mTOR pathways. WNT signaling promotes EMT by inhibiting glycogen synthase kinase-3β (GSK-3β) to result in nuclear localization of β-catenin, which interact with the transcription factors lymphoid enhancer factor (LEF) /T cell factor (TCF) and change genes expression. The intercellular interaction of Jagged ligands with Notch receptors induces EMT through the cleavage and release of the Notch ICD, which then activate target genes.