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## Collagens and retinal Müller cells in healthy and diseased vitreoretinal interface

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## **Chapter 1**

### **General Introduction**

### **The Extracellular Matrix and Cells in Healthy and Diseased Vitreo-retinal Interface**

### **The Vitreoretinal Interface**

Vitreoretinal interface includes the cortical vitreous, inner limiting membrane (ILM) and the endfeet of retinal Müller cells. It is a complex extracellular matrix (ECM) structure where vitreous cortex and ILM interact with each other through various molecular mechanisms.

#### **1. Vitreous**

The vitreous body (corpus vitreum) is a transparent and highly hydrated ECM structure containing a network of heterotypic collagen fibrils (predominantly type II collagens). Three specific areas can be readily recognized within the vitreous: (1) the basal and intermediate vitreous, (2) the vitreous cortex, (3) the central vitreous. The vitreous collagen fibrils are densely packed in the basal and cortical vitreous. The density of the collagen fibrils decreases gradually towards the center. The basal vitreous is an annular structure firmly attached to both the retina and the non-pigmented ciliary epithelium at the ora serrata. The vitreous cortex is the peripheral shell of the vitreous body that borders the retina. The collagen fibrils in the cortex run parallel along the retina and blend into the anterior vitreous base. The posterior vitreous cortex is absent over the optic disc and thinned over the macula and peripapillary zone.

##### **1.1. Suprastructural organization of vitreous**

While type II collagens form the main scaffold of the vitreous body, the other ECM proteins, including type V/XI and IX collagens, proteoglycans (PGs) and glycoproteins (GPs), are responsible for stabilizing the collagen network, the hydrated state and volume of the vitreous body. The chondroitin sulphate chains of type IX collagen and opticin maintain the space between the collagen fibrils and prevent self-aggregation of the collagen fibrils. Hyaluronic acid is highly hydrated and fills the spaces between

the collagen fibrils. The vitreous body in young adults fully occupies the vitreous cavity and is strongly attached to the retina at the vitreous base, macula, optic disc and along the major retinal vessels. By its supramolecular organization, the vitreous body is able to resist and buffer tractional, compressive and impact forces, thus protecting the retina.

## **1.2. Ageing of the vitreous**

The age-related degenerative changes of the vitreous body result in a collapse of the gel structure due to its progressive liquefaction (synchysis) and aggregation of the collagen fibrils (syneresis). The macroscopic and microscopic alterations, as well as the changes in biochemical composition of the ageing vitreous have been extensively studied and reviewed by several authors.<sup>1, 2</sup> The proposed hypothesis is that age-related vitreous liquefaction results from degenerative changes of the ECM molecules coated on the surface of collagen fibrils which are involved in the spacing of the network of collagen fibrils. Bishop et al demonstrated that ageing was associated with a significant loss of type IX collagen and its chondroitin sulfate side-chain on the surface of the type II collagen fibrils predisposing these fibrils to lateral aggregation.<sup>3</sup> Age-related accumulation of advanced glycation end products (AGEs) can induce a decrease in viscosity and average molecular weight of hyaluronan, which has been considered to be caused by the free radical attack to hyaluronan.<sup>4</sup> Another theory suggested that enzymatic degradation of type II collagen could also have a role in the development of vitreous liquefaction. Los et al reported that collagen fragments were found in the vicinity of liquefied spaces in human vitreous.<sup>5</sup> Van Deemter et al identified type II collagen fragments degraded by the matrix metalloproteinases MMP-1, MMP-8 and trypsin in the vitreous cavity.<sup>6, 7</sup> Additionally, plasmin and its precursor plasminogen were found to increase with ageing in the vitreous.<sup>8</sup> The potential proteolytic activities of plasmin could play an

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important role in vitreous collagen remodelling as well. Therefore, ageing may challenge the balance of collagen synthesis and degradation resulting in a destruction of the collagen fibril networks.

### 2. Inner Limiting Membrane

The ILM of the retina is the basement membrane of retinal Müller cells. It is the histological border of the vitreous cortex and retina and an important component of the vitreoretinal interface. The vitreal side of the ILM is attached to the stromal ECM vitreous fibrils and the retinal side of the ILM to the endfeet of retinal Müller cells through tight junctions.<sup>9</sup>

#### 2.1. The structure of ILM varies topographically

ILM is a transparent ECM membrane and invisible by conventional imaging technology. Light and transmission electron microscopy (TEM) studies showed that the ILM is a thin sheet of ECM with regional variations in thickness. The ILM is thinned at the vitreous base, macula, margin of the optic disc and along the major retinal vessels and it is absent over the optic nerve head.<sup>9</sup> The application of atomic force microscopy (AFM) allowed a study of the native ILM without dehydration, which is important since dehydration will cause an important underestimation of the thickness of the ILM. The AFM study indicated that the ILM has a high water content *in vivo* by demonstrating 30 to 50% reduction in thickness after enzymatic removal of the highly hydrated GAG side chains.<sup>10</sup> The average thickness of the fully hydrated, native ILM was  $3488 \pm 460$  nm. The ILM is thinnest within a central  $400 \mu\text{m}$  zone of the fovea with an average thickness of  $138 \pm 80$  nm and gradually increases to  $3711 \pm 300$  nm at a distance of 900 to  $1050 \mu\text{m}$  from the center of the fovea.<sup>11</sup>

#### 2.2. Biochemical composition and suprastructural organization of ILM

The ILM shares a similar supramolecular organization with basement membranes elsewhere in the human body, but it also has its own specific features in terms of biochemical composition and functions. The components of the ILM are ECM proteins which come together to form a sheet-like structure by interacting with each other through specific crosslinks. The ECM proteins include type IV collagen, laminin, heparan-sulphate proteoglycans (HSPGs), nidogen, perlecan, agrin, fibronectin, and type VI and XVIII collagens.<sup>11, 12</sup> The assembly of the basement membrane is initiated by deposition of laminins on the cell surface and interactions between laminins and the cell surface receptor (such as the integrin family and dystroglycan).<sup>12, 13</sup> Type IV collagens form an independent network as the main scaffold of the basement membrane which interacts with the laminin network through specific molecular mediators, such as nidogen/entactin and type VI collagen.<sup>14-16</sup> The scaffold formed by independent laminin and type IV collagen networks thus provides interaction sites for other BM associated molecules to bind and construct a fully functional BM. In chick embryogenic eyes, a collagenase induced disruption of retinal basal lamina can be rescued by application of laminin-1 but not laminin-2 nor type IV collagen. This observation indicated that the deposition of laminin-1 on the retinal cell surface is essential for the further deposition of type IV collagens and other BM molecules and the formation of a fully functional ILM.<sup>17</sup>

### **2.3. Origin and turnover of ILM**

2.3.1. Lens and ciliary body are the main producers of ILM proteins during embryology

During embryogenesis, the majority of the ILM proteins is produced by the lens and ciliary body and not by the retina. An *in situ* hybridization study showed that the mRNA expression of ILM proteins in chick embryos, such as laminin  $\beta$ 1, the  $\alpha$ 1 chain of type IV collagen, nidogen-1, perlecan and the  $\alpha$ 1 chain of type XVIII collagen, are

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produced by the lens and ciliary body.<sup>18</sup> A similar result has been reported in humans.<sup>19</sup> The ILM proteins produced by the lens and ciliary body then are transferred through the vitreous to the retina. The vitreous thus serves as a reservoir for ILM proteins during the embryogenic phase. Western blot analysis has confirmed that the ILM proteins in vitreous are most abundant during the early embryogenic phase, decrease rapidly during the late embryogenic phase and the first 2 years of the postnatal period, and are barely detectable in adulthood.<sup>19</sup> However, not all ILM proteins follow this time course in the vitreous and not all ILM proteins originated from lens and ciliary body. Transferrin, fibronectin and macroglobulin remain present in steady concentrations in the vitreous throughout adult life. Agrin has been shown to be produced by the retina, whereas type IV collagen can be produced by the optic nerve. Additionally, retinal glial cells including astrocytes and Müller cells can produce type I-VII, IX and XI collagens some of which are involved in the vitreoretinal interface.<sup>20</sup> These findings suggest a low level of ILM protein synthesis in the adult retina which supports the theory of dynamic remodeling of the vitreoretinal collagens during ageing.

### 2.3.2. Thickening and stiffening of aged ILM is the result of a process of age-related matrix remodelling

During ageing, the human ILM displays a series of structural and biological alterations which are the result of age-related biochemical and molecular changes. The thickness and stiffness of the ILM appear to increase over time. This increase in thickness has been demonstrated both by TEM and AFM. With regard to the major components of the ILM, the relative proportion of type IV collagen in relation to the total amount of ILM proteins increases while the relative proportion of laminin decreases. An age-related accumulation of advanced glycation end products in the ILM may contribute to the stiffening of the ILM.<sup>21-23</sup> Additionally, the distribution of

ECM proteins in the ILM alters as well. By immune histology, Kohno et al showed that fibronectin and laminin in the ILM of young adults appear to be present as a thick band at the posterior pole. Only occasionally they appeared as a bi-laminar structure, while this bi-laminar distribution became common in aged ILM.<sup>24</sup> The exact molecular organization, especially at the central part of the ILM (foveal ILM), has not yet been elucidated.

### **3. Retinal Müller cells**

Retinal Müller cells, the predominant glia in the human retina, share the basic bipolar morphology of radial glial cells in the central neural system and play a number of important roles in normal and diseased retina (for a review, see references 25 and 26).<sup>25</sup> The Müller cells span the entire retinal thickness. Their somata are located in the inner nuclear layer, and two stem processes extend radially. The inner stem process approaches the retinal side of the ILM and forms endfeet that are part of the vitreoretinal interface. The outer stem process reaches the subretinal space into which it sends numerous microvilli to connect with photoreceptors.<sup>26</sup>

Müller cells have been suggested to play a central role in the formation of epiretinal membrane (ERM) at the vitreoretinal interface. Upon stress or pathological stimuli, the Müller cells are activated and undergo gliosis which is characterized by cellular hypertrophy, upregulation of the intermediate filaments vimentin and glial fibrillary acidic protein (GFAP), and a transient or long-lasting proliferation of the Müller cells. The migration of Müller cells onto the vitreal surface has been suggested to represent the initial event of epiretinal membrane formation both in idiopathic epiretinal membrane and proliferative vitreoretinopathy.<sup>27, 28</sup> Furthermore, Müller cells have been found to transdifferentiate into a myofibroblast-like phenotype which contributes to the excessive collagen production and membrane contraction observed in those ocular pathologies.<sup>29</sup> The migration, proliferation and

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transdifferentiation of Müller cells can be regulated by a large number of growth factors, such as transforming growth factor  $\beta 1$  and  $\beta 2$  (TGF- $\beta 1$  and TGF- $\beta 2$ ), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) and insulin-like growth factor (IGF) (for a review, see references 30 and 31).<sup>30, 31</sup> Recent advancements in matrix biology indicated that the collagenous components and the mechanical properties (tension, traction and rigidity) of the ECM are also involved in the regulation of cell behavior. Lindqvist et al and Wang et al both reported that mechanical traction induced cell signalling pathways involved in activation of Müller cells.<sup>32, 33</sup> Müller cells also change their gene expression profile in response to matrix remodeling. This can be observed *in vitro* when they are grown on substrates with various stiffnesses.<sup>34</sup>

### **4. Molecules that are involved in vitreoretinal adhesion**

The adhesion of the cortical vitreous to the adjacent ILM, namely, vitreoretinal adhesion, involves a series of complex molecular adhesion mechanisms. The basal vitreous is the firmest zone of attachment between the vitreous and retina. The vitreous fibrils at the vitreous base are oriented perpendicularly to and are densely interwoven with the ILM. With age, a progressive posterior extension and interdigitation of collagen fibrils with the peripheral retina and ILM occurs and results in an increase of the anteroposterior width of the vitreous base.<sup>35</sup> At the pre-equatorial and equatorial regions, areas were found where vitreous fibrils focally penetrate the ILM and where ILM-associated type IV collagen was seen to be focally interrupted at sites where it extends itself into the vitreous cortex.<sup>36</sup> Based on clinical findings and some histological studies, the presence of focal attachments is also probable at the posterior pole, and specifically in areas overlying retinal blood vessels, the macula and optic disc. Focal vitreoretinal attachments may explain why - in case of a spontaneous posterior vitreous detachment (PVD) - retinal tissue may be damaged.

Thereby, intravitreal hemorrhage, retinal tear formation, and ultimately rhegmatogenous retinal detachment may occur. Kishi et al reported that cortical vitreous remnants were found on the foveal surface of the retina in 22% (26 out of 59) of autopsy eyes that had had spontaneous PVD.<sup>37</sup> At the posterior pole, cortical vitreous fibers run parallel to the retinal surface, and there is only very limited evidence showing that the vitreous fibrils may penetrate the inner limiting membrane of the retina forming adhesions with the underlying retina. Therefore, the proposed hypothesis is that the adhesion molecules, such as fibronectin, laminin, heparan sulphate proteoglycans and opticin, are responsible for the vitreoretinal adhesion by interacting with both type II collagens in the vitreous fibrils and type IV collagens in the ILM.<sup>24, 38, 39</sup> Kohno et al reported a linear and laminar distribution of fibronectin and laminin in the equatorial and posterior ILM and suggested that fibronectin and laminin are responsible for the vitreoretinal adhesion because of their high affinity to bind with collagens, proteoglycans and hyaluronic acid.<sup>24</sup> Additionally, the newly identified collagens in the vitreoretinal interface, such as type VI, VII and XVIII collagen may also be involved in the molecular mechanism of vitreoretinal adhesion.

Age-related changes of the vitreous and ILM ultimately result in changes in vitreoretinal adhesion leading to the development of PVD with or without other associated complications. In the normal ageing process, the vitreous body gradually liquefies and collapses, which can be detected clinically as a gradually enlarging pre-macular vitreous pocket and optical clear area in the center of vitreous cavity.<sup>40</sup> When the vitreoretinal adhesion gradually weakens in parallel to the progression of vitreous liquefaction, the collapsed vitreous will be accompanied by a detachment of the cortical vitreous, which results in the development of PVD. PVD has been suggested to start as a localized shallow separation of cortical vitreous from the

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perifoveal retina, which progresses over months or years with no clinical symptoms.<sup>41</sup> The traction exerted by the collapsed vitreous can induce tissue dehiscence at the level of the cortical vitreous resulting in vitreoschisis; or at the retinal level resulting in vitreomacular traction syndrome and idiopathic macular hole.<sup>42, 43</sup> Vitreoretinal traction has also been reported to induce intra-retinal cell damage, such as the progression of diabetic macular edema and age-related macular degeneration. These evidences suggested that vitreoretinal traction may have an impact on retinal tissue. Indeed, accumulating evidences suggest that retinal cells, particularly retinal Müller cells and retinal pigment epithelial cells are mechanosensitive, and can respond to traction by altering their gene and protein expression. Therefore, it is possible that mechanical traction may have a role in the pathogenesis of vitreomacular diseases.

### **5. Epiretinal membrane at the vitreoretinal interface**

The fibrocontractive vitreoretinal diseases, including proliferative vitreo-retinopathy (PVR), proliferative diabetic retinopathy (PDR) and macular epiretinal membrane (ERM) either idiopathic or secondary to primary ocular diseases remain the major causes of irreversible visual function damage despite advancement in modern surgical techniques. The myofibroblast, a cell type that produces excessive amounts of extracellular matrix (mainly collagens) and induces contractile forces to surrounding tissues, has a crucial role in the formation and contraction of the ERM.

In normal wound healing processes, myofibroblasts are activated during the initial stage and usually undergo apoptosis when the wound is healed. In fibrotic diseases, myofibroblasts are persistently activated resulting in excessive deposition of collagens and severe tissue contraction, which impedes organ function. Myofibroblasts have heterogeneous origins and their regulation involves a series of biochemical and biophysical factors. While local fibroblasts are the prominent source

of myofibroblasts, a large panel of cells, including epithelial cells, endothelial cells and bone-marrow derived cells have been shown to contribute to the myofibroblast population. The regulatory roles of profibrogenic cytokines have long been recognized. Besides these soluble factors in the microenvironment, both the biochemical and biophysical properties of the ECM have been shown to be actively involved in the regulation of fibrosis.<sup>44, 45</sup>

Recent advances in extracellular matrix biology indicate that the ECM proteins have diverse cellular effects beyond providing structural support. The dynamic remodelling of the ECM during fibrosis results in an alteration of the biochemical and biophysical properties of the ECM. These alterations play an influential role in fibrosis. First, the newly produced ECM proteins could promote the fibrotic process. Type IV collagen can induce an epithelial to mesenchymal transition process in mammary epithelial cells.<sup>46</sup> Type VI collagen, which is upregulated in fibrotic matrix remodelling, promotes myofibroblast transdifferentiation of corneal and cardiac fibroblasts.<sup>47, 48</sup> Second, recent experimental findings showed that the mechanical tension and stiffness of the ECM regulate the formation of myofibroblasts.<sup>49-51</sup> Matrix stiffness is essential in the transforming growth factor  $\beta$  (TGF $\beta$ ) induced transdifferentiation of fibroblasts into myofibroblasts in lung, liver and conjunctiva.<sup>52-54</sup> Furthermore, a stiff substrate allows the myofibroblasts to exert contractile forces to the surrounding matrix by which they trigger an integrin-mediated activation of latent TGF $\beta$  in the ECM.<sup>55</sup>

The ERM formed at the vitreoretinal interface has been considered as an aberrant wound healing process with fibrocontractive membrane formation. The formation of myofibroblasts and fibrotic tissue are the main causes of damage to visual functioning. To prevent the detrimental effects of the ERM, one of the most promising approaches is to prevent the formation of myofibroblasts or to promote

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their apoptosis. By examining factors that induce myofibroblast formation more closely, novel treatment targets may be identified.

In summary, the vitreoretinal interface is a highly organized extracellular matrix structure with a dynamic remodelling process during an entire life time. The compositional changes due to ageing and fibrosis result in alterations in its biochemical and biophysical properties. These alterations, in turn, have a crucial role in the pathogenesis of age-related and fibrotic vitreoretinal diseases. Identifying the key elements in the vitreoretinal interface and the response of the retinal cells to alterations therein, may eventually lead to the development of effective and non-surgical approaches to treat and prevent vitreoretinal fibrotic diseases.

### **6. Aim and outline of this thesis**

The research hypothesis is that the extracellular matrix regulates ERM formation at the vitreoretinal interface by both its biochemical components (type VI collagen) and its biophysical properties (stiffness).

To provide evidences to support our hypothesis, we studied:

- 1) The ultrastructural organization of collagens at the vitreoretinal interface.
- 2) The collagens in ERMs associated with idiopathic macular hole and idiopathic epiretinal membrane and the involvement of epiretinal cells with a retinal Müller cell origin.
- 3) The regulatory role of matrix elastic modulus in the myofibroblast transdifferentiation of retinal Müller cells.

In Chapter 2, we summarized the recent advancements in epidemiological and clinical findings of idiopathic epiretinal membrane (iERM), the collagens that may participate in fibrosis and iERM formation, and the regulatory roles of the

mechanical properties of the extracellular matrix in matrix remodelling during fibrosis. The appreciation of the regulatory role of matrix remodelling related to ageing and fibrosis permits the construction of a plausible sequence of events that lead to the development of iERM.

In Chapter 3, we focused on the ultrastructural features of the vitreoretinal interface by using immuno-transmission electron microscopy. The location and organization of type VI collagen and its structural relationship with type II and IV collagens were determined.

In Chapters 4 and 5, we identified the collagenous components in the ERMs associated with idiopathic macular hole and idiopathic epiretinal membrane. One of the important findings is that type VI collagen was present in the ERM of idiopathic epiretinal membrane but absent in ERM of idiopathic macular hole. Additionally, with flat-mount and double immuno-labelling of cell phenotype specific makers, the retinal Müller cells were shown to be an important cell type participating in ERM formation by proliferation and myofibroblast transdifferentiation. Therefore, an in vitro Müller cell culture experiment was conducted and the results suggested that the expression of type I, II and VI collagens in retinal Müller cells containing  $\alpha$ -SMA positive stress fibers was affected.

In Chapter 6, we focused on the regulatory role of matrix elasticity in the transforming growth factor beta (TGF- $\beta$ ) induced myofibroblast transdifferentiation of retinal Müller cells. The results suggested that with increasing matrix stiffness and in response to TGF- $\beta$ , retinal Müller cells gain the ability of forming a contractile myofibroblast with smooth muscle actin incorporated into their cytoskeleton.

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