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Self-assembling nanofiber hydrogels to attenuate epithelial mesenchymal transition in lens epithelial cells

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CHAPTER 1

Introduction

1.1.   Epithelial Mesenchymal Transition and its regulators

EMT classification

Elizabeth Hay, in 1968 using a chick streak model, mentioned for the first time a process of transformation of epithelial cells to mesenchymal cells during the embryogenic stage [1] and characterized several markers to identify this process [2, 3]. Since that time the use of "transformation" changed to transition. There are now more markers to identify epithelial and mesenchymal stages and also the intermediate stage between epithelial and mesenchymal cells. The epithelial mesenchymal transition is nowadays associated with more processes than embryogenesis alone. Epithelial mesenchymal transition (EMT) is the process of movement or migration of polarized epithelial cells with changes in their phenotype and genotype to mesenchymal-like cells. This process starts with degradation of the basal membrane, reorganization and alteration of cell markers and finish with a complete change in cell phenotype in a location that is different from the original.

EMT classification was been discussed since 2007 when Radhu Kalluri [4] introduced types 1, 2 and 3. Type 1 EMT is associated with embryogenesis [5, 6]. After the fertilization of the egg, there is the formation of the primitive streak that will define the site of granulation and initiate the three germ layers (ectoderm, mesoderm and endoderm) that will create all tissues in the body. The primitive streak is composed of epithelial-like cells of the epiblast. These cells express proteins that dictate the cell differentiation towards mesenchymal and associated cell migration [5, 7]. Most of the transformation and migration of these primitive layers of cells are regulated by EMT and depend of Wnt signals [8], Snail, Eomes and Mesps transcription factors [9-12]. Also in the embryogenesis, EMT has a crucial role during neural crest formation [13]. Epithelial cells from neuroectoderm have the ability to migrate and generate the neural crest by EMT processes using EMT genes as Snail, Sox and Slug [14, 15]. It is important to mention that in this enormous phenomenon of embryogenesis the polarity of cells that allow cell migration is fundamental. The migration is not only involving EMT but also endothelial-mesenchymal transition (EndMT) [16, 17].

Type 2 EMT is associated with tissue regeneration and fibrosis. In an organ, when the inflammatory response is taking too long the fibrotic process starts. EMT is associated with fibrosis in the liver, kidneys, lungs, heart and lens of the eye [18-21]. This process is an unsuccessful attempt to repair the damaged tissues; its failure will cause loss of epithelial homeostasis and deposition of fibrotic tissue in the extracellular matrix (ECM). Epithelial cells are located on the basal membrane and have a well-defined apical-basal polarity which, during EMT, is gradually changed to a front-rear polarity. This phenomenon is essential for cell migration and it is associated with the increase of degradation of the extracellular matrix proteins. The epithelial cytoskeleton is reorganized with formation of actin stress fibers, the shape of the cells starts to elongate and new cell-cells junctions are formed [22]. From this stage, cells start to express mesenchymal markers or intermediated stages (e.g. myofibroblasts) markers and acquire resistance to apoptosis [23]. Early

studies about kidney fibrosis identified EMT as the main source of fibroblasts arising from resident epithelial cells [24]. The inflammation process results in the release of important growth factors as, TGF-β, PDGF, EGF, and FGF [25, 26] that recruit several cells from the immune system, such as macrophages, and active resident fibroblasts that accumulate/gather in the site of the injury. This also starts the production of chemokines and MMPs. The epithelial cells under the effect of these growth factors degrade the basal membrane and lose focal adhesion, hemi-desmosome and desmosome proteins, leading to a decrease in the levels of laminin, collagen type IV and E-cadherin. Studies in kidney, liver, lung and heart fibrosis using BMP-7, an antagonist of TGF-β, showed the importance of TGF-β as an inducer of EMT [27]. After using this antagonist, E-cadherins were restored to their original levels, FSP1 and α-SMA were decreased, damaged epithelial cells were repaired and the organs improved their function, being a signal of reversal of EMT, also referred to as mesenchymal epithelial transition (MET) [28]. More recently, investigations with BMP-7 in mice fibrotic lungs and skin suggested that BMP-7 alone is not enough to avoid EMT or to restore the functionality of the organ [29].

Several investigators pointed to fibroblasts-specific protein 1 (FSP1), α-SMA and collagen *type I* as valid markers to characterize mesenchymal products from EMT in fibrotic tissues [26]. In chronic inflammation it was found that epithelial cells can maintain theirs morphology and some epithelial markers as E-cadherins, but that they also can express fibrotic markers as α-SMA and FSP1. These cells therewith were in the early stage of fibrosis, expressing intermediate markers of EMT, meaning that they express markers for both epithelial cells and fibrosis. Over the course of the inflammatory process these cells will leave the epithelial layer due to the basal membrane degradation and will be accumulated in other parts of the organ where they will express all EMT markers and show a pronounced fibrotic tissue type [30, 31] (Fig.1).

Type 3 EMT is associated with cancer and metastasis. The abnormal accretion of epithelial cells together with an increase of angiogenesis [32], hypoxia [33] and high levels of TGF-βs (mainly TGF-β_1) [34] create the first cancer cells. In this first stage of the disease, the survival rates are larger than when the cancer cells are disseminated in the body by metastasis. This capacity of the cells to migrate through the basal lamina and invade tissues and organs at other locations is strongly associated with EMT [35, 36]. The capacity of invasion is associated with malignant phenotypes and therefore malignancy and EMT are also connected. However, some debates about this topic are arising because the migratory cancer cells do not express mesenchymal phenotypes or genotypes. Fabulously, Ramón y Cajal [37] draw in 1890 the first mesenchymal phenotype in breast cancer but even nowadays it is difficult to correlate EMT and cancer due the lack of acceptable markers for histological samples. Nowadays, facts indicate that after the formation of cancer cells in situ, there is the presence of epithelial markers. However these markers will slowly change to a cancer stem cells (CSCs) phenotype and genotype with the capacity to migrate. These subpopulation of self-renewing tumor cells have the ability to generate new tumors and express EMT markers. In the beginning of this process a dual phenotype is present; a cancer epithelial cells population show the epithelial phenotype and CSCs populations express EMT markers with an invasive/malignant phenotype. This

last subpopulation enters in the blood circulation and tumor propagation starts. The affinity of some cancer cells for a particular organ is explained by the quality of the microenvironment that the new organ can give to the cancer cells, where a high level of vasculature is favorable for cancer seeding and proliferation. This could be the reason why lungs, liver and bone marrow are pivotal organs for cancer dissemination [38]. Finally when CSCs arrive at other organs or tissues they will be coupled to the healthy cells and some epithelial markers will be expressed again due the ability of CSCs revert to cancer cells. This last processed is MET and happen in all cancer metastasis [39, 40]. The turnover between cancer cells and CSCs by EMT and MET are responsible for oncogenic mutations in the secondary tumors.

One of the most important steps in cancer metastasis is the propagation of the tumor cells. This is just possible due the degradation of the basal lamina of the in situ cancer, mainly caused by the downregulation of the cell adhesion molecules as desmosomes, tight junctions and gap junctions. The reprogramming between EMT and MET is led by changes in the miRNA, that has a fundamental role in the second tumor [41]. Other observations that support the mesenchymal phenotype in metastasis is that normal epithelial cells not in contact with the basal lamina start an apoptotic process [42], but mesenchymal cells do not need to be in contact with the basal lamina in order to survive, so the changes of phenotype from epithelial to mesenchymal cells are fundamental for metastasis of cancer cells.
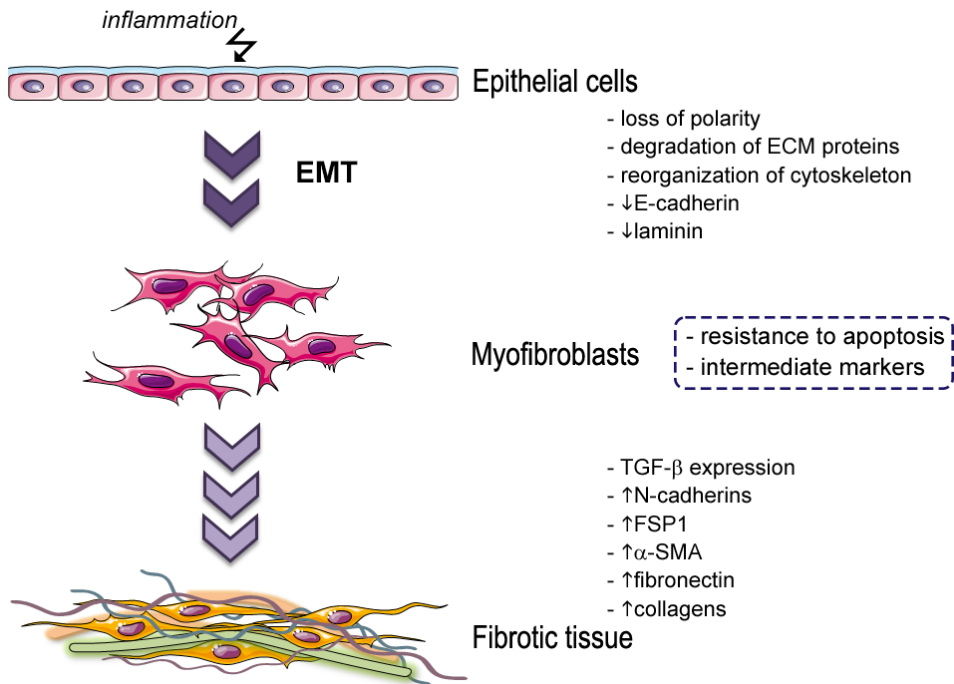
Figure 1: Changes in epithelial cells toward Epithelial-Mesenchymal Transition (EMT) into a fibrotic tissue. The epithelial cells, under inflammation, change their characteristics to become fiber-like cells. Their typical polarity is lost, the ECM proteins are degraded and the cytoskeleton move to a more elongate cell membrane, These "new" cells, myofibroblasts, acquire capacity to migrate, resistance to apoptosis and express intermediate markers of epithelial and mesenchymal cells during EMT. The maturation of the cellular differentiation into fibers takes place together with the production of a fibrotic ECM. The fibrotic cells are expressing, among others, high levels of TGF-βs, N-cadherins, fibroblasts-specific proteins-1 (FSP1) and α-SMA. In parallel, the deposition of fibronectin and collagens are enlarged and the fibrotic tissue is created.

Signaling in EMT

In all the different types of EMT there are key events that are characteristic: Loss of cell-cell junctions, changes of apical-basal polarity to front-rear polarity, changes in cell cytoskeleton, downregulation in epithelial markers and upregulation in mesenchymal markers, increase of cell mobility, changes in ECM proteins and resistance to apoptosis. All these changes have common markers and common pathways as well as molecules that stimulate the EMT process or the reverse MET process. There is still debate about to what level EMT can be reversed. Knowledge of these pathways can lead to new treatments to avoid EMT.

In the next paragraphs, some of the markers associated with the changes in the phenotype of cells during EMT both at the mRNA and protein level are described. A list of markers was selected to further understand the scientific work of this thesis.

Cell-surface markers

The cadherin switches are one of the most important signals of transition of cells from epithelial to mesenchymal phenotype [30, 43]. E-cadherin repression or cleavage is associated with increase of mesenchymal neural cadherin (N-cadherin) and OB-cadherins. In fact, N-cadherins connections are weaker than E-cadherins, which facilitate cell migration [44]. The cleavage of E-cadherins (in the membrane) is regulated by MMP-3 and through cytoplasmic ROS production Snail-1 is activated by phosphorylation of GSK-3β [45]. E-cadherin inhibition by Snail-1 or other inhibitors induce β-catenin/Tcf-Lef1 that result in increased expression of vimentin and other mesenchymal proteins. The activation of β-catenin is dependent on phosphorilisation unless GSK-3β is silenced by Wnt signaling. The Wnt pathway can be activated by TGF-β that switch on β-catenin and consequently stimulate mesenchymal genes. TGF- β can also directly activate Tcf-Lef1 thought SMAD-2 [46].

DDR2 (discoidin domain receptor tyrosine kinase 2), a collagen receptor, is also an important inducer of mesenchymal genes. DDR2 upregulates MMP-1 and cell mobility when linked to collagen *types I* or *X* in the ECM. Recently it was discovered that TGF-β_1 promotes collagen *type I* synthesis and DDR2 expression. An inhibition of DDR2 downregulates EMT markers and cell migration induced by TGF-β_1 [47]. It is also demonstrated that DDR2 activation increases the Snail 1 presence in the nucleus which is associated with cleavage or absence of E-cadherins [48].

Cytoskeletal markers

Vimentin

Vimentin, an intermediate filament, is essential for strength and movement of mesenchymal cells [49]. It is highly expressed in mesenchymal cells during embryogenesis and therefore is an important marker for EMT type 1. It is also related with the aggressiveness of cancer due to its importance for cell mobility [50]. But in adults, epithelial and endothelial cells also express vimentin which makes it difficult to use this protein as a single marker for fibrosis or EMT type 2. However the augmentation of this protein during the transition of epithelial cells to mesenchymal cells is clearly established.

Alpha-smooth actin (α-SMA)

The well-known actin isoform alpha-smooth actin can be seen as a hallmark marker in the detection of EMT [51, 52]. Epithelial, endothelial or mesenchymal cells can be activated to become myofibroblasts and can be detected by the enhancement of α-SMA.

In EMT, these cells are considered an intermediated of fibroblasts, essential to the creation of fibrotic tissue.

Kidney fibrosis is associated with the accumulation of α-SMA-positive myofibroblasts. Small GTPase Rho has an influential role in TGF-β_1 -induced renal epithelial cells going into EMT. Together with Rho kinases (ROK) it can modulate cell contacts and reorganize the cell cytoskeleton. It is also known that the pathway Rho/ROK is essential for the expression of SMA in smooth muscle cells [53]. Thus the TGF- β family has a primordial effect on α-SMA expression.

Actin filaments are responsible for the mechanical support of the cells and together with myosine play a fundamental role in traction and mobility of the cells. The actin filaments in normal epithelial cells are arranged in a thin group of fibers contrary to the thick fibers present in the differentiated mesenchymal cells [54]. Genes responsible for the actin cytoskeleton assembly are constantly activated during EMT induced by TGF-β. This emphasises the importance of α-SMA and actin in the development of EMT.

β-catenin

This protein is used as a marker in all three types of EMT. It regulates the linkage of E-cadherins to the cytoskeleton and controls gene expression associated with EMT via the Snail family [55, 56]. In normal epithelial cells it is located at the cell membrane and during EMT this protein is detected mainly in the nucleus where it regulates EMT gene expression together with T cell factor complex (TCF/LEF) [57].

TGF-β is connected with the augmentation of β -catenin. It is known that the transcription factors SNAIL 1 and 2 regulate E-cadherin transcription and that these can induce the β-catenin-TCF complex that up-regulates TGF-β signals and consequently EMT [58].

Extracellular matrix proteins

Fibronectin

This extracellular glycoprotein recognized by integrins has an essential role in cell proliferation, migration, adhesion and survival. Alterations in its localization and shape can be related to fibrosis or cancer [59, 60]. However, fibronectin is also associated with embryonic development and wound healing and it is highly expressed during cell division in the majority of cellular types, as for example, fibroblasts, mesenchymal stem cells, endothelial and epithelial cells [61, 62]. Due to its importance in all these cellular processes this protein is considered to be a non-specific marker for EMT.

Laminin

Laminin is one of the main constituents of the basement membrane. This membrane is disrupted in the beginning of the EMT process due to the transformation of epithelial cells to myofibroblasts. The glycoproteins, laminin 1 ($\alpha_1\beta_1\gamma_1$) and laminin 5 ($\alpha_3\beta_3\gamma_2$) are mostly associated with EMT types 2 and 3. Laminin 1 is downregulated in vitro and is lost in vivo during fibrosis [63]. Laminin 5 is upregulated in the disruption of the basement membrane related to cancer metastases [64].

Collagen type I and type III

Collagen *type I* and *III* are deposited in matrix in large quantities in fibrosis. Deposition of collagen *type I* promotes EMT in lung cancer cells by upregulate the autocrine TGF-β_3 signal [65]. But there is also evidence that collagen *type I* acts as an activator of the Snail family and lymphoid enhancer-binding factor 1 (LEF-1). These transcriptional factors are associated with the suppression of E-cadherins which means that collagen *type I* can act by altering the protein expression in epithelial cells to initiate migration and EMT [66].

The origin of these collagens *in vitro* and *in vivo* is not totally clear. Studies in mice with interstitial renal fibrosis show that α-SMA positive cells generate collagen *type I* and *III*, similar with findings in patients with kidney disease where deposits of collagen *type III* were found associated with α-SMA positive cells [67]. However there are also studies reporting that the deposition of collagen *type I* and *III* with fibrosis in lungs and kidneys seems to be caused by myofibroblasts derived from fibroblasts rather than from transformed epithelial cells. Although, the significant larger quantities of epithelial cells compared with fibroblasts in kidneys may be crucial for the deposition of collagens in this organ [68].

Collagen type IV

Like laminin, collagen *type IV* is an essential constituent of the basal membrane. Collagen *type IV* includes 6 different alpha isoforms. Three of these alpha isoforms curl together on their non-collagenous sites to create the triple collagen helix [69]. The NC1 domain in each N-terminal of the collagen triple helix is indicated as promotor of the collagen aggregation. Collagen *type IV* can modulate cell behaviour by linkage to integrins [70].

Collagen *type IV* being a fundamental constituent of the basal membrane is not highly related with the development of EMT in fibrosis, but its quantity can rise during the maturation of fibrosis. For example in kidney fibrosis the fibrotic matrix is filled with high quantities of collagens *type I* and *III* and residual quantities of collagen *type IV* from the basal membrane [71]. In the other hand, the degradation of collagen *type IV* is an essential step during the EMT type 1 of the heart development (endocardial cushions) in an embryo [72]. All these characteristics make it difficult to assign a defined role of collagen *type IV* in EMT and associated fibrosis.

Collagen type VI

Collagen *type VI* plays a major role in ECM due to its structural support to the cells. Its essential role in creating networks of microfilaments between ECM proteins it is reflected in its presence in most of the tissues. Collagen *type VI* is mostly constituted by three polypeptides which encode three different genes, COL6A1, COL6A2, and COL6A3. It is referred to as an activator and modulator of several signal pathways related with fibrosis, inflammation, angiogenesis, apoptosis and proliferation [73, 74]. Several studies also identified an upregulation of collagen *type VI* in progression and metastasis of cancer cells [75]. Collagen *type VI* has been reported as an antiapoptotic molecule for some cells such as fibroblasts [76] and endothelial cells [77] and at the same time, it is a stimulator of cell proliferation and angiogenesis. These factors can create favourable environments for cancer cell survival. Another association of collagen *type VI* and metastasis is related to the augmentation of TGF- β signalling which is directly related with the advance of EMT in the cancer cells [73].

Levels of collagen *type VI* are also high in fibrotic cells and this collagen has been used as a marker of mesenchymal activation towards the fibrotic process [78]. Due to its role in cell-matrix and matrix-matrix interaction, this protein is essential for fibrogenesis.

Transcription factors

Fibroblast transcription site-1 (FTS-1)

Fibroblast-specific protein- 1 (FSP1) is a Ca^{2+}-binding S100 proteins involved in various cellular and extracellular processes as Ca^{2+} signal transduction, cell growth, differentiation, motility and angiogenesis [79]. FSP1 are found in kidney fibrosis due the high accumulation of fibroblasts in this disease [80]. FSP1 is considered an important marker for EMT in cancer and fibrosis since the early transition from epithelial to fibroblast cells [24, 81, 82]. FTS-1 can be coupled and regulate several EMT genes including, E-cadherins, vimentin, α-SMA, ZO-1 and β-catenin. CBF-A and KAP-1 can form a complex with FTS-1 and determine EMT genes activity. CBF-A/KAP-1/FTS-1 complex is associated with EMT type 2 in kidney epithelial cells. But studies have shown that the complex CBF-A/KAP-1/FTS-1 is a proximal activator of EMT-associated genes [83]. Since its formation is sufficient to activate the expression of genes encoding fibroblasts-specific protein-1 (FSP-1), a key protein expressed on fibroblasts derived from EMT.

Snail transcription factor

Snail family are frequently activated or inactivated in the process of EMT or MET, mainly SNAIL1 (or SNAIL) and SNAIL2 (most known as SLUG). SNAILs linkages by carboxy-terminal zinc-finger to E-box of DNA sequences and they are the main repressor of E-cadherins genes [15]. After binding to the E-box, SNAIL coordinates methylation and acetylation to the histone. Normally, methylations are attributed to repressive chromatin and acetylation to active chromatin [84]. Both process can occur during and after the

transition process of the cells and can be defined as "bivalent control" [85] [86]. As a result of this process, gene expression for E-cadherins can be stimulated during MET and repressed during EMT. SNAL1 expression is also dependent of signals from Notch, TGF-βs and WNT family.

Growth factors - TGF-β family proteins

TGF-β proteins act by kinases receptors which are included, TGF-βs, bone morphogenetic proteins (BMPs) and activins. The signal transduction of these proteins is made by Smad dependent or independent pathways [87]. TGF-β expression is connected with in vivo EMT events as kidney fibrosis, hepatic fibrosis and lung fibrosis.

TGF- β_1 is highly used, for in vitro and in vivo studies, to activate epithelial or fibroblasts cells into myofibroblasts and consequently fibrosis [88]. TGF- β_1 works by phosphorylation / activation of receptors for Smads. This complex is accumulated in the nucleus where is directly responsible for gene transcription [89]. Although has been proved that *in vitro* an increase of TGF- β_1 is associated with an overexpression of pre-fibrotic and fibrotic genes and proteins [90, 91], in *in vivo* new facts are emerging. In a mice model for renal fibrosis, was detected that the renal tubular epithelial cells that overexpress TGF- β_1 significantly enlarge tubulointerstitial fibrosis [92]. However recently, *in vivo* studies also showed that the inhibition of TGF- β is not enough to stop renal fibrosis after an injury [93].

Despite the new insights for the role of TGF- βs in vivo, there are no doubts about the importance of these growth factors during EMT and fibrosis. And mechanisms to annul the effects of TGF- β are being study as well. The bone morphogenetic proteins (BMPs) family is the antagonist of TGF- βs. During the embryo development, TGF- βs and BMPs are counterbalancing the cellular process, but during EMT this balance is disrupted by the increase of TGF- βs. Both share *type I* (e.g. ALK-2, 3 and 6) and *type II* receptors but they can differ on its localization and on binding interfaces [94]. Although has been found over 20 BMPs, the BMP-2, 4 and 7 are the most related EMT proteins [95].

BMP-2 has suppressed the effects of EMT in in vivo model for renal fibrosis by reversing the TGB- β_1 reduction of Snail expression [96]. BMP-4 can inhibit EMT induced by TGF- β via Smad pathway on retinal epithelium [97]. BMP-7 reverses the TGF- β_1 effect by Smad-5, which is reflected as an increase of E-cadherins and revival of the epithelial phenotype [27].

EMT in eye lens

Lens epithelial cells (LEC) are the cells present in the interior of the lens of the eye. They are typically in a hexagonal like shape, lined near the basal membrane as a monolayer [98]. These peculiar characteristics are signaling a well-defined polarity and straight epithelial connections between cell membranes. During an inflammatory process, cells receive the stimulus to de-differentiate. Then the connection with basal membrane and the cell-cell connections start to become weak and the epithelial phenotype gradually

changes to the mesenchymal-like phenotype. In parallel, the epithelial phenotype is modified to detached and elongated cells, with characteristics increasingly similar with (myo)fibroblasts. These fibers-like cells also gain the capacity to migrate all around the interior of the lens. The deposition of the cells on the posterior side of the lens leads to a definite myofibroblast appearance with related ECM composition. This process results in a fibrotic tissue. The entire process is called posterior capsule opacification (PCO) [99].

Cataract, one of the most common eye disease in the world, is an opacification of the nucleus of the lens. The surgical intervention to remove the catarogenic nucleus and replace it with an intra-ocular lens is relatively simple and fast. It restores clear vision with a low risk of complications. However, during the removal of the lens material, the monolayer of the lens epithelial cells is damaged. The residual cells respond to the inflicted damage and the inflammatory process by displaying EMT behavior leading to (posterior) capsular opacification (Fig.2). This process slowly leads to a secondary loss of vision and is treated with a neodymium:YAG capsulotomy surgery, again restoring clear vision.
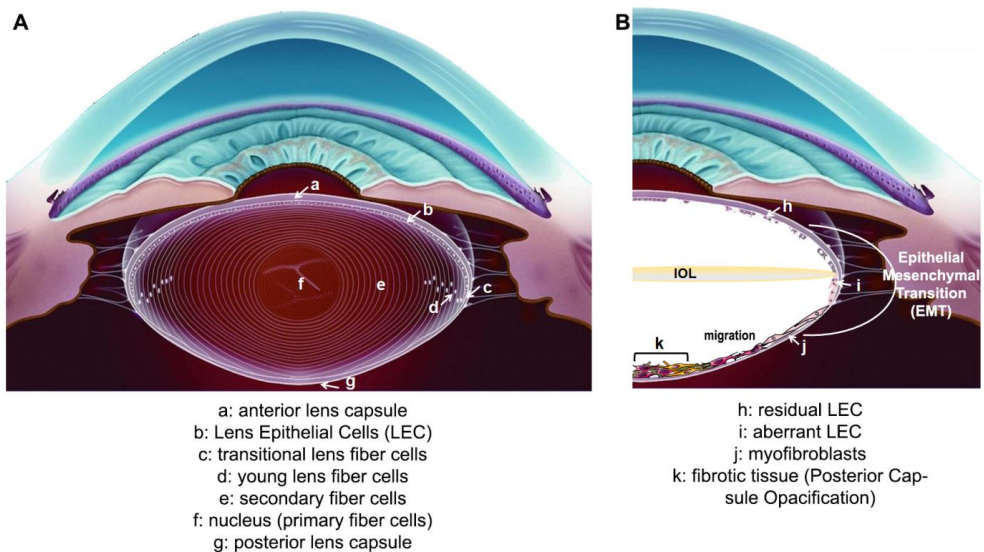

a: anterior lens capsule
b: Lens Epithelial Cells (LEC)
c: transitional lens fiber cells
d: young lens fiber cells
e: secondary fiber cells
f: nucleus (primary fiber cells)
g: posterior lens capsule

h: residual LEC
i: aberrant LEC
j: myofibroblasts
k: fibrotic tissue (Posterior Capsule Opacification)

Figure 2: A) Healthy eye lens and its morphology. B) Eye lens after an insertion of an intra-ocular lens (IOL). Described is the Epithelial-Mesenchymal Transition (EMT), where the residual LEC starts to migrate to the posterior lens capsule, as myofibroblasts, and together with collagens and fibronectin create a fibrotic tissue, denominated posterior capsule opacification (PCO).

Several techniques to optimize the extraction of the nuclei and to optimize the design of the IOL have been done without success [100]. Also the materials of the IOLs have been subject of studies. Frequently used materials for IOLs are silicone, poly(methyl)methacrylate (PMMA) and acrylates [101]. Hydrophobic coatings are preferably used instead of the hydrophilic [102] and sharp-edge shapes are associated with less PCO than the round-edged design of the IOL [103, 104]. Despite these findings no materials or material coatings have fully prevented the formation and deposition of fibrotic tissue. Considering the tools available to the ophthalmologists and the success rates of cataract surgery one could argue that fibrosis in the lens capsular bag is not really an issue. But the hallmark of lens replacement would be the application of lenses that have the ability to accommodate, therewith restoring clear vision and accommodation. Such a mechanism relies on an intact, flexible capsular bag – zonula fiber system. As fibrosis tends to stiffen tissues, this has to be avoided. This implies that strategies to prevent EMT and subsequent fibrosis are still highly relevant and subject of world-wide research.

1.2.   Aims of the thesis

In search of biomaterials that can be used to control the EMT response in lens epithelial cells the research described in this thesis deals with the use of low molecular weight gelators (LMWG) consisting of self-assembling nanofibers that can be used as a surface coating and as an injectable gel.

The aim of this thesis was to assess the potential of different nanofiber compositions in controlling EMT in lens epithelial cells. This potential should be assessed using different *in vitro* models of lens epithelial cell interactions with nanofiber–based materials.

The following sub-aims were defined:

1) To select suitable nanofiber-based materials with potential to interfere with EMT using a 2D model coating;
2) To establish a 3D model to further assess the potential of selected nanofiber-based materials in avoiding EMT, including the incorporation of relevant adhesion peptides integrated in the nanofibers;
3) To develop an *in vitro* / *ex vivo* porcine lens capsule model in which the bioactive potential of nanofiber-based materials can be assessed.

Furthermore, the use of fluorescent stains was applied onto the cornea epithelium in order to assess cornea damage as a robust method to study interactions of lubricants and other agents with the cornea epithelium.

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