Shaping vessels and microenvironment: adipose stromal cells in retinal-related diseases

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Chapter 7

Conclusions and future perspectives

General discussion

Shaping vessels and microenvironment: adipose stromal cells in retina related disease

The studies proposed in this thesis aimed at evaluating the adipose stromal/stem cells (ASC) plasticity to support and maintain the microcirculation in the context of diabetic retinopathy. Considering the multitude of biochemical changes during the onset and progression of diabetic retinopathy, cell therapy warrants deeper understanding of the molecular mechanisms underlaying cell-to-cell communication and the ability to shape the surrounding microenvironment. The ultimate aim is to engineer cells to effectively reestablish homeostasis in the pathological retinal microenvironment.

This thesis contributed new knowledge that moves the field closer to this ultimate goal:

The first two experimental chapters in this thesis (chapter 3 and 4), focused on the role of notch signaling in guiding ASC-driven vessel network formation by endothelial cells. Notch signaling is indeed involved in this process by promoting migration and assembly of endothelial cells.

Endothelial cells morphogenesis was analyzed in a confined three-dimensional microenvironment (chapter 5). This chapter illustrated ASC exert pericytic-like function (acting as supporting cells to the microvessels) by secreting ECM components. This process allows endothelial cells positioning and subsequent assembly in vessels-like networks.

In chapter 6, fibulin1 was investigated for its possible role in the pathogenesis of diabetic retinopathy and ECM organization by ASC.

The initial features that led several scientists to focus on the ASC regenerative characteristics encompass their multipotency, pro-angiogenic milieu and the phenotypical acquisition of perivascular cells/pericytes feature[1-4]. Pericytes have the fundamental role of supporting the retinal microcirculation through direct contact with endothelial cells[5]. The secretion of a basement membrane and communication with other cell populations of the retina (i.e. glia cells), establish a tight control and protection from the signals of the systemic circulation through the blood-retina barrier[6]. The dysregulated catabolism of glucose exerts an important metabolic stress on the microvascular endothelial cells constituting the capillaries in the eyes. Consequently, endothelial cell apoptosis and pericyte migration leaves portions of the capillary bed unguarded and subjected to the action of cytokines and growth factors secreted by the cells in the microenvironment[7]. Therefore, the hypothesis that ASC could replace retinal pericytes and confer stability to the aberrant retinal microcirculation is appealing in the field of regenerative medicine[8]. However, several factors and limitations need to be considered to successfully induce physiological angiogenesis in the retinas affected by diabetes. First of all, a single animal model that recapitulates the non-proliferative and the proliferative stages of diabetic retinopathy is not available. Without an appropriate model to study the long-term effects of cell therapy during the progression of the disease it becomes complex to monitor the fate of the implanted cells. Moreover, the pathological retinal microenvironment switches from ischemic areas which require angiogenesis to reestablish the capillary bed, to regions with pathological proliferative angiogenesis where, ideally, the implanted cells could modulate the identity of the endothelial cells and stabilize the microcirculation towards homeostasis. To date, numerous studies reported the ASC secretion of growth factors involved in regulation of angiogenesis, proliferation, inflammation and maintenance[9]. In **chapter 2**, we reviewed the current knowledge on the

application of mesenchymal stromal/stem cells for cell therapy in the eyes. Trophic mediators secreted by ASC and relevant to the diabetic retinal microenvironment are various. For example, FGF, VEGF, NGF, BDNF, GDNF and CNTF, are all involved in maintenance of processes such as survival and proliferation of several types of cells[1]. How these factors modulate the retinal microenvironment and, in reverse, the response to these factors shapes the activity of implanted cells needs further elucidation. However, evidence in animal models suggested several benefits upon ASC injection in the eyes. ASC can improve neuronal function (measured through ERG), alleviated vascular leakage, reduced apoptosis, increased intraocular levels of neurotrophic factors and, importantly, no pro-vasculogenic microenvironment changes were observed[9-12]. Furthermore, ASC have been shown to withstand the oxidative stress initiated by glucose. This is very important since resident pericytes migrate away for the same reason. Finally, a low-grade response of pro-inflammatory mediators also developed due to the progression of DR. In fact, a challenge for implanted ASC is to maintain macrophages as a M2 immunosuppressive phenotype, regulate T cells responses and limit the induction of an adaptive immune response. The latter is speculative since studies on the regulation of the immune/inflammatory response are lacking.

Recent studies focusing on the role of pericytes depletion in the retina begin to shed light on the molecular pathways which induce irreversible damage to the microcirculation in diabetic retinopathy. Under physiological conditions pericyte-derived angiopoietin-1 activates the tie2 receptor which phosphorylates and inactivates FOXO1. This pathway promotes intracellular junctions of the blood-retinal barrier and attenuates inflammation. Pericyte-free endothelial cells enact a positive feedback loop with activation of endothelial transcription factor NFAT, increased Tie-2 phosphorylation and

upregulation of Angpt2 and VEGFR2. The alterations of these signal molecules sustain pathological neo-angiogenesis. Concomitantly, NFAT increases the secretion of pro-inflammatory chemokines by endothelial cells. Consequently, disruption of intracellular junctions favors monocyte infiltration, which then differentiate into macrophages that produce pro-angiogenic factors (i.e. VEGF, PIGF) and pro-inflammatory TNF-α[13]. In this scenario, endothelial cells respond to adverse stimuli in an attempt to reestablish the physiological balance between anti- and pro-angiogenic factors.

Another class of proteins involved in development and maintenance of the blood-retinal barriers is notch signaling. Notch receptors and ligands on the surface of endothelial cells and pericytes, dictate the molecular events that discriminate between tip leading endothelial cells and the following stalk-cells[14]. VEGF is also involved in stimulating the nascent vessels; notch signaling, however, attenuates the response of stalk cells to VEGF by reducing its receptors[15]. In this way, neo-angiogenesis can proceed in only one direction. During the initiation of the vasoproliferative stage of retinopathy, angiogenesis control of endothelial cells is dysregulated, and the response of sensitized endothelial cells to pro-angiogenic stimuli in addition to dysregulated notch signaling, leads to excessive vasoproliferation[16]. In **chapter 3**, we hypothesized that the ASC pericytic function exerts angiogenic control on endothelial cells through notch signaling. In this study, investigation of the distribution of notch receptors and ligands on ASC and endothelial cells revealed a predominance of NOTCH2 expression and overall equal expression of the notch components respectively. NOTCH2 has affinity for both Delta and Serrate ligands[17] and positive feedback loops between nuclear complex NICD, ERG and β-catenin have been proposed to explain the regulation of angiogenesis and homeostasis in the vascular plexus[18]. In order to investigate the role of NOTCH2 expressed on the ASC in the communication with

endothelial cells, ASC NOTCH2 expression was reduced by short hairpin RNA. Downregulation of ASC NOTCH2 inhibited the capillary-like tube formation of endothelial cells, as examined in both 2D and 3D coculture. Notably, endothelial cells cultured alone in a three-dimensional setting, failed to spontaneously assemble into a tube-like structure. These first results suggested that the process of vasculogenesis initiated by ASC requires cell-to-cell contact with the endothelial cells in order to promote endothelial cells morphogenesis. Whether NOTCH2 controls the ASC pericytic features with regard to sensing endothelial cells' signals for attraction and migration, was hypothesized and tested with the chemoattractant PDGF-BB. This growth factor, secreted by endothelial cells, recruits pericytes during vascular growth and acts as mitogen in activated cells, allowing proliferation and survival for vascular development[19]. Migration of ASC SH-NOTCH2 (NOTCH2 down regulation in short hairpin treated ASC) to recombinant PDGF-BB was reduced compared to the wild type ASC. Furthermore, PDGFRB produced by ASC was downregulated in ASC SH-NOTCH2, confirming the relationship between endothelial chemoattractant and the ASC capacity to respond. Recent findings by Park and co-workers confirmed the crucial role of PDGF/PDGFRB signaling for the formation and maturation of the blood-retinal barrier in postnatal stages. Interestingly, PDGFB/PDGFRB was not involved in the maintenance of blood-retinal barrier by endothelial cells and pericytes[20]. We next asked the question whether conditioned medium collected from ASC SH-NOTCH2 could have deleterious effects on endothelial cells proliferation. In this experiment, endothelial cells equally proliferated in all the experimental conditions ruling out any adverse effect from ASC SH-NOTCH2 secretome. In the last steps of this study, ASC SH-NOTCH2 were used to quantify the regenerative capacity on the retinal capillary-bed regression obtained through an oxygen induced retinopathy mouse model (OIR). The retinas capillary-beds treated

with ASC SH-NOTCH2 and controls (ASC WT and ASC SH-SCR) resulted in reconstitution of the avascular areas of the retinas. By measuring the amplitude of ON-bipolar cells by means of electroretinogram (ERG), we showed that both ASC WT and ASC SH-NOTCH2 had a positive improved amplitude of these layers of the retina compared to untreated animals. In contrast, ASC SH-NOTCH2 did not distribute evenly and deeply in the retinas' layers as observed for ASC WT. Recapitulating, ASC injected into the vitreous of the eyes to repair the diabetic retinas need to respond to the microenvironment and, PDGFRB might function as mediator for a subsequent ASC-endothelial cells communication. ASC expressing NOTCH2 is indispensable to induce angiogenesis in endothelial cells. This process should allow endothelial cells to effectively respond to VEGF whereas ASC protect endothelial stalk cells from pro-angiogenic growth factors and ensure an angiogenic process toward homeostasis[15].

In **chapter 4**, we further investigate another possible mediator, namely JAGGED1, known to be expressed by endothelial cells and to bind to NOTCH2[17]. Gene expression profiles in in vitro cultured ASC, endothelial cells and their coculture showed expression of JAGGED1 which was not altered by hyperglycemia. In vitro cocultured ASC and microvascular endothelial cells expressed VEGFR1, receptors found on tip cells during angiogenesis, resulted in higher expression compared to VEGFR2 whose expression is induced in stalk cells to suppress the tip-cell phenotype[21]. These measurements were consistent in hyperglycemia condition. We previously showed that ASC NOTCH2 downregulation inhibited the interaction and subsequent capillary-like tube formation by endothelial cells. By targeting γ-secretase, one of the enzymes involved in notch intracellular domain (NICD) release upon notch ligand-receptor binding[22], we confirmed the importance of this interaction to promote vessel-like network formation in a coculture of ASC and endothelial cells. In the same

setting, a peptide targeting JAGGED1 and therefore inhibiting its binding to notch receptors, did not interfere with the network formation between ASC and endothelial cells. This result suggested that other notch receptors might compensate for JAGGED1 inhibition and that ASC can still exert their pericytic-supporting properties. As proof of concept, we used an in vivo model of capillaries formation namely Choriolliallantoid membrane (CAM) from the chicken embryo. Firstly, during the development of CAM, JAGGED1 inhibitor was added and regression of the developmental vascular network was observed. In accordance with previous finding, we showed that ASC partially rescued the formation of CAM in the presence of JAGGED1 inhibitor. Conclusively, we demonstrated that the juxtacrine (cell-to-cell communication) interaction between ASC and endothelial cells are influenced by notch signaling. On the one hand, notch receptors on ASC are mandatory to dock and promote capillary-like network formation by endothelial cells. On the other hand, JAGGED1 is a ligand that rather influence endothelial cells network whereas ASC can still function as supportive cells suggesting compensation of other notch ligands expressed by endothelial cells.

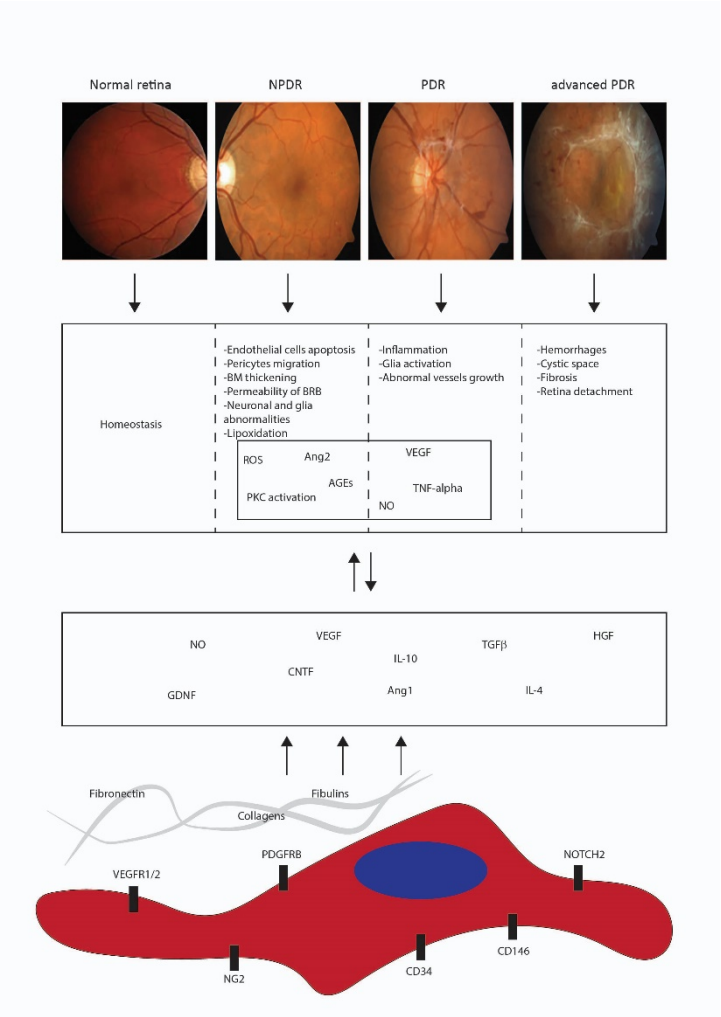

Figure 1. Molecular relationship between the progression of DR and cell therapy. NPDR (non-proliferative DR), PDR (proliferative DR). Retinas images[44] and[45].

Part II: Extracellular matrix remodeling

Cellular assembly of capillary-like tubes in vitro driven by adipose stromal cells

Tissue regeneration studies that leverage multipotent stem cells must take into account several aspects: cellular etiology, source, differentiation, response to the host microenvironment in pathological conditions, and finally, the acquired phenotype has to comply safely in the target organ[8,23]. As shown in figure 1, the progression of diabetic retinopathy involves a series of shifts in chemokine, growth factors and cellular responses that need to be thoroughly dissected in order to effectively use ASC for cell therapy[24-26]. Before animal experimentation, the complexity of organs can be partially recreated in vitro using three-dimensional microenvironments. Through this approach, detailed information about cellular morphogenesis and activity can be moreefficiently translated in vivo. To this end, we developed a three-dimensional microenvironment that allowed close observation of ASC and endothelial cells interactions. In **chapter 5**, we examined the temporal events that culminate in an interconnected capillary-like tube formation by ASC driven endothelial cells. Several studies investigated this interaction, showing ASC acquiring perivascular position and expressing some pericytic markers such as SM22α, NG2 and PDGFR[27,28]. However, cellular morphogenesis, in time, had not previously been investigated. Moreover, our constructions of 3D-printed scaffolds conferred a controlled and limited microenvironment where cellular events could be conveniently monitored by tracking cells for a span of time that easily exceed thirty days. In addition, some of the extracellular matrix (ECM) components involved in the multicellular assembly were analyzed. For example, fibronectin is a glycoprotein that interacts with collagen, heparin and fibrin. Simultaneously, fibronectin serves as receptor for various integrins, therefore offering both structural and signal functions[29]. In

our model, fibronectin was ubiquitously secreted by ASC in about ten days after cellular assembly took place. In fact, embedding ASC and endothelial cells in matrigel resulted in ASC remodeling of the matrigel for subsequent endothelial cells sprouting. At the same time, endothelial cells could migrate toward one another to assemble in numerous aggregates, establishing the first stage of vasculogenesis. Importantly, we showed that endothelial cells alone, in the same setting, could not assemble spontaneously, conferring to ASC the trigger for vasculogenesis[30]. ASC could be found in two positions, one being structural, the other being perivascular. The latter showed ASC to be in intimate contact with endothelial cells' aggregates. Time-lapse microscopy showed that ASC were moving in an oscillatory fashion, suggesting mechanical pressure may be required by endothelial cells for subsequent sprouting. Endothelial cells sprouted and connected aggregates in a capillary-like tube formation which was observed after ten days of coculture. ASC fibronectin secretion could be detected after four days and, at ten days fibronectin occupied most of the extracellular space between cells. Importantly, a small portion of endothelial cells did not participate in the aggregate formation. In fact, these cells were observed to remain in between aggregates and subsequently elongated toward them. Putatively, these "solitary" endothelial cells functioned as intermediates for the aggregates sprouting endothelial cells to effectively form interconnected capillary-like tubes. After twenty-three days of coculture aggregates maintained a dynamic phenotype in terms of cells movements as well as bridges of cells connecting aggregates between one another. The majority of cells could cover a distance spanning between two to ten microns per thirty minutes. The fastest cells were covering twenty microns per thirty minutes.

Perturbation of the ECM composition caused by diabetes comprises diverse pathophysiological factors that include reactive oxygen species (ROS), advance glycation-end products (AGEs) and overall metabolic

stress caused by hyperglycemia[31]. Proteins of the basement membranes constituting the microcirculation of several organs such as kidneys and the retina, become increasingly up-regulated whereas proteins that are not normally present in physiological conditions contribute to the thickening and pathological changes of the basement membranes composition[32]. For instance, collagen IV and agrin are up-regulated in diabetic conditions, while fibronectin and tenascin, which are usually moderately present in basement membranes, also contribute to the thickening of the ECM surrounding microvessels[33]. We measured ASC fibronectin deposition during the vasculogenic process in vitro by endothelial cells. We used human vein endothelial cells (HUVEC) and porcine retina microvascular endothelial cells (PREC) in combination with ASC. The highest fibronectin deposition was observed in the coculture between ASC and HUVEC. The immunoglobulin PECAM-1 was used to visualize the capillary-like tubes formed by endothelial cells. In the latter setting, PECAM-1 was also deposited more abundantly compared with the combination of ASC and PREC. These results agreed with the abovementioned studies, in fact, ASC adapted to the source of endothelial cells by depositing more or less fibronectin depending on the diameter of the putative vessels that could be formed. However, the mechanism behind ASC fibronectin deposition and how endothelial cells modulate it is currently unknown. Fibronectin is indispensable for rebuilding tissues conferring routes for cells migration, proliferation and contraction. Moreover, growth factors (i.e. PDGF, VEGF, and bone morphogenic protein (BMP)) bind to fibronectin and regulate wound healing process[29]. The relation between ASC matrix deposition and the growth factors gradient during capillaries repair in diabetic retinopathy could offer new therapeutic targets. In the last experimental setting, ASC derived from diabetic patients were used. Diabetic ASC failed to induce capillary-like tubes by endothelial cells which remained constrained in to aggregates with little signs of sprouting. Fibronectin was moderately deposited

compared to wild type ASC. In contrast, as shown in **chapter 6**, diabetic ASC cultured in monolayer exhibited a net higher fibronectin deposition when compared to wild type ASC. In addition, the contribute of hyperglycemia to ASC fibronectin deposition appear to be negligible, suggesting that diabetic ASC perturbation derives from molecular mechanisms independent from hyperglycemia-induced changes. Alternatively, it is possible that perturbation of metabolic pathways that control oxidative phosphorylation and electron transport chains overcame the physiological state of diabetic ASC, because in vitro, hyperglycemia does not induce any additional perturbation in terms of ECM deposition. In this scenario, we hypothesized that supramolecular organization of the ECM might significantly contribute to the ASC angiogenic capacity in the retina and therefore, understanding this modulation could open new routes for intervention, as well as implementation in the current knowledge. We focused on fibulin-1, a constituent of basement membranes that interacts with collagens and fibronectin[34-36]. Little is known about localization and fibulin-1 function in the retina. However, fibulin-1 was found to be involved in axonal outgrowth in mouse retina through an in-silico pipeline for complex genetic networks[37]. In the vitreous of patients with proliferative diabetic retinopathy, mass spectrometry-based proteomics identified upregulation of fibulin-1 together with several isoform of collagens, fibronectin and proteoglycans[38]. Importantly, fibulin-1 was detected as constituents of the arterial ECM and, arteria thickenings were associated with an increase deposition of fibulin-1 in patients with type 2 diabetes[39]. In diabetic mice (ins2akita) we found that fibulin-1 was down-regulated compared to the distribution in the inner plexiform layer and inner nuclear layer of the wild type mice retinas. Diabetic ASC exhibited higher deposition of fibulin-1 and fibronectin compared to wild type ASC. Lastly, ASC in coculture with endothelial cells showed fibulin-1 deposition on previous pattern left from migrating cells during vasculogenesis.

Conclusions and future perspective

The main aim of this thesis was to investigate the molecular mechanisms and the microenvironment morphogenesis involved in the pericytic function of adipose stromal cells (ASC). First, notch signaling is required for the communication between ASC and endothelial cells. ASC chemoattraction and migration depended on the expression of NOTCH2 which in turn, induced endothelial cells to form capillary-like tubes in vitro. Second, the ASC capacity to shape the vasculature was analyzed in a three-dimensional system. We dissected the temporal dynamic of ASC and endothelial cells during the process of vasculogenesis. Moreover, spatial organization, morphology and ECM deposition highlighted important features in healthy and diabetic ASC. Finally, ECM deposition in ASC and during the progression of diabetic retinopathy were analyzed in parallel to elaborate on new strategies for cell therapy.

An important link was forged between the mitochondrial biogenesis, fibrosis and notch signaling in recent publications[40,41]. These relationships maydisplay similar patterns in ASC driving tissue regeneration. Therefore, modulation of notch signaling in ASC could offer a therapeutic intervention for cells therapy in the eyes. However, novel approaches that combine modulation of a large selection of genes and automated profiling of cells phenotypes are needed to precisely tackle cells heterogeneity and to identify new targets[42]. The latter approach could be integrated with functional studies aimed at understanding cells movements in three-dimensional microenvironments[43]**.** In conclusion, we identified key molecular regulators involved in ASC supportive functions on the vasculature. Approaching complexity by recreating in vitro microenvironments is of great importance for basic understanding of ASC in shaping the microenvironment and to safely translate findings into patients.

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Nederlandse samenvatting

Microvasculaire complicatie van diabetes beïnvloedt de haarvaten in het netvlies. Microvasculaire endotheelcellen vormen de haarvaten die nauw de uitwisseling tussen het bloed en de extracellulaire ruimte van het netvlies regelen. Grenzend aan capillairen komen pericyten in contact met de microvasculaire endotheelcellen om structurele en biochemische ondersteuning te verlenen. Bij diabetische patiënten komt de normale glucoseopname in het gedrang, wat leidt tot biochemische verstoring in de microcirculatie. Endotheelcellen zijn een van de eerste gevoelige cellulaire bestanddelen die in het netvlies apoptose ondergaan en de extracellulaire ruimte blootstellen aan de bestanddelen van het bloed. Vervolgens worden pericyten ofwel apoptotisch ofwel migreren onder de invloed van een ongunstige micro-omgeving. Het moleculaire scenario naar een gebrek aan juiste perfusie sinds district van endotheelcellen het capillaire bed onderbreken. Gebrek aan zuurstof en activering van angiogene stimuli in endotheelcellen activeert proangiogene mechanismen in het netvlies die een fysiologisch capillair bed proberen te herstellen. In de meest ernstige gevallen van patiënten die worden getroffen door diabetische retinopathie, groeit de microcirculatie ongecontroleerd om hemorragieën en extracellulaire ophoping van vloeistoffen te veroorzaken die een normaal gezichtsvermogen in het gedrang brengen en uiteindelijk tot blindheid leiden.

Van de medische strategieën die worden gebruikt om de microcirculatie in het netvlies te voorkomen, te behouden en te genezen, wordt in dit proefschrift rekening gehouden met cellulaire therapie. Adipose stromale cellen (ASC) geoogst uit vetweefsels vormen een aantrekkelijke bron gezien hun beschikbaarheid en, belangrijker nog, ASC fungeren als ondersteunende cellen voor de microvaatjes. Het hoofddoel van dit proefschrift was het onderzoeken

van de moleculaire, morfologische en pathologische impact van adipose stromale cellen tijdens de regeneratie van bloedvaten in de context van microvasculaire complicatie van diabetes, d.w.z. diabetische retinopathie. Dit werk is in twee delen uit een. Eerst worden de huidige strategieën van implantatie en de daaropvolgende resultaten van celtherapie besproken. Daarna volgt het onderzoek naar notch-signalering op ASC als moleculaire route betrokken bij de communicatie van ASC en endotheliale cellen evenals ASC-implantatie in de ogen om een gebrek aan functie van notch op ASC te evalueren. Vervolgens wordt een meer diepgaande evaluatie van ASC-vorming van de morfogenese van endotheelcellen in vitro onderzocht door middel van driedimensionale micro-omgevingen.

In hoofdstuk twee werd celtherapie in de context van diabetische microvasculaire complicatie in de ogen besproken. De belangrijkste resultaten met betrekking tot ASC-pericytische functie toonden hun haalbaarheid aan om de microcirculatie van het netvlies te integreren en te ondersteunen bij diabetisch geïnduceerde dieren zoals ratten en muizen. Voorlopige resultaten toonden verbetering van proangiogene stimuli na implantatie en verminderde ontsteking. Belangrijk is dat vroege schade aan de microcirculatie ofwel werd voorkomen of verminderd. Deze review was vooral gericht op ontstekingen veroorzaakt door uitgebreide schade aan het netvlies. ASC moet ontstekingsstimuli kunnen weerstaan, mogelijk verminderen en, daar naast, stabiliteit verlenen aan de haarvaten in het netvlies. In zoverre kon ASC ook differentiëren in de richting van ontstekingsremmende fenotypen zoals M2-macrofagen en Treg, samen met een repertoire van geschikte secretie van ontstekingsremmende en antiangiogene cytokines, groeifactoren en metabolische toestanden naar homeostase. Er is echter weinig bekend over inflammatoire toestanden van het netvlies tijdens diabetes na cellulaire implantatie. Daarom hebben we de immunomodulatiestimuli en ASC-resultaten die in in vitro

experimenten zijn gegenereerd, overwogen en gesuggereerd dat geschikte diermodellen en meer kennis van de fundamentele fenotypische veranderingen van geïmplanteerde ASC nodig zijn.

ASC functioneren als ondersteunende cellen die lijken op pericyten in het netvlies. Pericyten zitten naar om endotheelcellen heen, scheiden een basaalmembraan af en helpen de moleculaire trafficking tussen de bloedstroom en de extracellulaire ruimte van organen te beheersen. Om celtherapie te verbeteren, is meer kennis van de ondersteunende functie van ASC nodig op moleculair niveau. In hoofdstuk 3 en 4 werd de hypothese gesteld dat notch-signalering migratie, contact en differentiatie van ASC naar pericytisch-achtige functie regelt bij contact met endotheelcellen. Notch-signalering is een alomtegenwoordige moleculaire route, met verschillende functies in verschillende districten van het lichaam. Over het algemeen regelt notch-signalering segmentatie en cellulaire differentiatie. In ASC vonden we dat NOTCH2 voornamelijk aanwezig was tussen zijn receptoren, terwijl JAGGED1 voornamelijk aanwezig was tussen de liganden. Vervolgens hebben we onderzocht wat de bijdrage is van ASC die NOTCH2 tot expressie brengt om endotheelcellen in vitro te induceren. Wanneer ASC NOTCH2-expressie was verminderd (ASC SH-NOTCH2), konden endotheelcelnetwerken niet formeren in vergelijking met wildtype. Deze functie is ook getest in een driedimensionaal cocultuursysteem en heeft de tweedimensionaal verkregen resultaten bevestigd. Eerdere studies toonden aan dat endotheelcellen pericyten aantrekken door de uitscheiding van groeifactoren. PDGFRB is een receptor op ASC (of pericytes) die een rol speelt bij respons op endotheliaal uitgescheiden PDGF-BB. In normale omstandigheden migreerde ASC naar medium verzameld uit endotheelcellen (geconditioneerd medium), evenals naar medium met de toevoeging PDGF-BB.ASC met NOTCH2 verminderde expressie kon darrentegen niet collectief migreren naar de eerder genoemde

stimuli. Gezien deze eerste resultaten, vroegen we of implantatie in de ogen ASC SH-NOTCH2-interactie met de microcirculatie zou verhinderen en bijgevolg hun werkzaamheid zou verminderen. Het diermodel dat voor dit experiment werd gebruikt lijkt op de proangiogene staat van endotheelcellen in het netvlies, maar het is geen omgeving die door diabetes wordt beschadigd. ASC SH-NOTCH2 is niet geïntegreerd in de microcirculatie in vergelijking met de wildtype. Bovendien toonde het elektroretinogram geen significante verschillen in de reactie van de cellen op lichtstimuli en daarop volgend herstel. Samengenomen bevestigden deze gegevens ASC-wildtype-integratie in de microcirculatie van het netvlies en mislukte integratie van ASC SH-NOTCH2. Dit laatste zou het gebrek aan respons van ASC op migrerende stimuli en / of het vermogen van ASC om als ondersteunende cellen te functioneren kunnen verklaren. Meer in vivo experimenten zijn nodig om de fenotypische toestand van ASC in deze context te evalueren.

Een ander belangrijk onderdeel van de notch-signalering is JAGGED1. Experimenter naar gebrek aan functie vroege vasculaire beschadiging en dodelijkheid. JAGGED1 overexpressie, leidt daarentegen tot ongecontroleerde angiogenese met een daaruit voortvloeiende vaatafwijking zoals vasoproliferatie. JAGGED1 is actief op endotheelcellen en verschillende onderzoeken toonden interactie aan van JAGGED1-ligand op endotheelcellen die onmisbaar zijn voor pericytische differentiatie op pericyten. We stelden daarom dat ook JAGGED1 op ASC betrokken is bij de communicatie en inductie van vorming van bloedvaten door endotheelcellen. Interessant genoeg werd gevonden dat glucose de JAGGED1-expressie op ASC of microvasculaire endotheelcellen in vitro niet beïnvloedt. Om de bijdrage van notch aan de communicatie tussen ASC en endotheelcellen te onderzoeken, werden twee types remmers gebruikt. Ten eerste is γ-secretase vereist om een deel van de notch-receptoren

op het oppervlak van de cellen enzymatisch bloot te stellen. γ-secretase-remmer was voldoende om de vorming van het bloedvatennetwerk te belemmeren. Daarentegen had JAGGED1 geen waarneembaar verschil in de vorming van netwerken tussen ASC en endotheelcellen, wat suggereert dat andere notchiganden JAGGED1 zouden kunnen compenseren. Vervolgens werd het effect van JAGGED1-remming getest op een in vivo model van net haarvatennetwerk. Hier resulteerden de haarvaten in een verminderde dichtheid en groei in vergelijking met de controle group. Wanneer ASC op het haarvatennetwerk werd geplaatst, werd een verhoogde dichtheidsgroei gedetecteerd. Voor dit laatste experiment zijn echter meer experimentele replicaten nodig om deze resultaten te bevestigen.

Dit tweede deel van het proefschrift heeft tot doel de communicatie tussen cellen (ASC en endotheliale cellen) in tijd en ruimte te evalueren. Voor dit doel werden biologisch afbreekbare scaffolds gebruikt om cellen in extracellulaire matrix te kweken en om de morfologische ontwikkeling in een langere tijdsperiode te volgen in vergelijking met tweedimensionale kweeksystemen. Deze benadering toonde cellenmorfologie en extracellulaire matrix (ECM) uitscheiding vanaf tijd 0 (het zaaien van cellen) tot ongeveer één maand. Bovendien werden ASC van diabetische patiënten en retinale microvasculaire endotheelcellen in dit systeem gekweekt om de morfologische ontwikkeling te evalueren. Endotheelcellen vormden aggregaten onder invloed van ASC, endotheelcellen alleen waren daartoe niet in staat. Grote ECM-componenten zoals fibronectine werden ongeveer vier dagen na het zaaien uitgescheiden. De wanden van de steiger scaffold door de cellen gebruikt als ankerpunten en bepaalden de limiet van het kweeksysteem. Endotheelcellen en ASC werden gelabeld door de mitochondriën en werder gevolgd in real time. ASC had een dubbele functie als structurele cellen, gedeponeerd fibronectine en ingepakte endotheelcellen in een pericytisch-achtige positie. In ongeveer 10

dagen werd een onderling verbonden netwerk gevormd met endotheelcellen die adhesie-eiwitten tot expressie brengen (PECAM-1) en ASC die functioneren als ondersteunende en structurele cellen. Het cellulaire netwerk werd gekenmerkt door tunnels met een diameter van ongeveer 20 μm. Daarentegen kon diabetische ASC de vorming van endotheelcellen niet ondersteunen. Fibronectine en PECAM-1 afzetting was ook afgenomen. De laatste afname werd ook waargenomen in een co-cultuurexperiment van ASC en retinale microvasculaire endotheelcellen. Vervolgens werd de snelheid en richting van cellen in de co-cultuur bepaald. Cellen die de aggregaten omvatten waren niet statisch en konden 15 μm / uur heen en weer transponeren. Endotheelcellen die uit de aggregaten staken, bewogen zich naar het centrum met een hogere snelheid, berekend als 21 μm / 30 minuten. Tezamen genomen voormen driedimensionale micro-omgeving een krachtige aanpak om de morfologische ontwikkeling van een co-kweeksysteem te beoordelen, zoals deze in dit proefschrift wordt gepresenteerd. ASC kon hun structurele en ondersteunende fenotype uitoefenen terwijl depositie van doeleiwit werd gevolgd in de tijd. Uit deze studie resulteerde dat ASC endotheelcellen induceren in aggregaten die de neiging hebben om met elkaar te verbinden om compacte en continue structuren te vormen. Interessant is dat diabetische ASC deze organisatorische capaciteit had verloren. Samengevat, deze methode is fundamenteel voor het vergelijken en verrijken van moleculaire mechanismen tot morfologische gebeurtenissen bij cellen tot celcommunicatie in fysiologische en pathologische omstandigheden.

In het laatste experimentele hoofdstuk (hoofdstuk 6) werden de mogelijke rollen van fibulin1 in de context van diabetische retinopathie en ASC geëvalueerd. Fibulin1 is een structureel extracellulair eiwit waarvan wordt aangenomen dat het ankerpunten aan andere belangrijke ECM-eiwitten verleent. Deze bijkomende eiwitten zouden

ECM-toegankelijkheid en cellulaire morfogenese kunnen reguleren. Daarom werd eerst een diabetisch diermodel gebruikt om de depositie en lokalisatie van fibulin1 te bepalen. Fibronectine werd ook geëvalueerd als onderdeel van de eiwitten met verbindingspunten voor fibuline1. In het netvlies van diabetische dieren bleek de fibulinl-expressie verminderd te zijn vergeleken met controledieren. Fibulin1 was echter gelokaliseerd in de binnenste plexiforme laag en de buitenste nucleaire laag van het netvlies. Het projecteren van het gebruik van ASC als celtherapie in de ogen, fibuline en fibronectine expressie werden geëvalueerd in wild type ASC en diabetische ASC. Interessant is dat zowel fibronectine als fibuline1 opgereguleerd waren in dibetische toestand vergeleken met wild type ASC. Ten slotte werd de bijdrage van ASC-fibuline-1-expressie in co-cultuur met endotheelcellen geëvalueerd in een driedimensionaal kweeksysteem. Hier werd fibuline1 tussen cellen uitgescheiden en interessant genoeg werd het aangetroffen in tunnels die eerder waren gevormd door cellenactiviteit. Kortom, fibuline1 is inderdaad een bestanddeel van de ECM die in het netvlies wordt afgezet, evenals door ASC. Hyperglycemie verhoogt waarschijnlijk de depositie door ASC, vooral ASC van diabetische patiënten. Deze resultaten wijzen op een beter begrip van de rol van fibuline1 in het netvlies onder pathologische omstandigheden en op voorzichtigheid bij het gebruik van ASC bij patiënten met diabetes, aangezien ECM-depositie lijkt te zijn aangetast. Daarom zijn gebrek aan functie-experimenten nodig om de biologische rol en de gevolgen van fibulinl-uitputting in de retina en ASC te begrijpen.

In dit proefschrift werd de potentiële rol van ASC als celtherapie voor implantatie in de ogen onderzocht. ASC functioneren als ondersteunende cellen die op de pericytische functie lijken door angiogene en vasculogene kiemen in vivo en in vitro te induceren en te beheersen. Een beperkte driedimensionale micro-omgeving toonde aan

dat ASC niet alleen bijdraagt aan het contact met endotheelcellen, maar ook aan de structuur, en dus de micro-omgeving voorbereidt op de morfogenese van endotheelcellen. Belangrijk is dat ECM-eiwitten verhoogde secretie vertoonden door gecultiveerde ASC en endotheliale cellen in pathologische toestand zoals diabetes en hyperglycemie. Verder is notchsignalering fundamenteel voor het initiëren en handhaven van vasculaire netwerkvorming door endotheelcellen. Om te zorgen voor veilige translationele aanwijzingen voor de implantatie van cellen in de ogen, kan deze aanpak leiden tot in een efficiënte studie van langetermijnkweek en modificatie van ASC en endotheelcellen met een vooruitzicht om de complexiteit te vergroten en uit afzonderlijke experimenten. Voor toekomstige experimenten moeten het morfologische potentieel van ASC, de bio-energetische toestand en de interactie met cellen van het doelorgaan in vitro zorgvuldig worden geëvalueerd om het beste cellulaire fenotype voor regeneratieve doeleinden te waarborgen.

List of abbreviations

AGE Advanced glycation end product
ALDH Aldehyde dehydrogenase
AMD Age-related macular degeneration
Ang-2 Angiopoietin 2
ASC Adipose tissue-derived stromal/stem cells
α-SMA alpha-smooth muscle actin
BDNF Brain-derived neurotrophic factor
BM-MSC Bone marrow mesenchymal stem cells
BRB Blood retinal barrier
CB-MSC Cord blood mesenchymal stem cells
CO Carbon monoxide
CNTF Ciliary neurotrophic factor
DR Diabetic retinopathy
EPO Erythropoietin EC Endothelial cells
ECM Extracellular matrix
GLO-1 methylglyoxal-metabolizing enzyme glyoxalase-1
GPx1-3 glutathione peroxidases
HGF Hepatocyte growth factor
HO-1 Heme oxygenase 1
hRPCs human retinal pericytes
IGF Insulin-like growth factor
IDO Indole amine 2,3-dioxygenase
IL interleukin
INF-γ Interferon-gamma
ISCT I International Society for Cellular Therapy
imPSCsc mouse induced pluripotent stem cells
iPSCs induced pluripotent stem cells
EAU Experimental autoimmune uveitis
ECM Extracellular matrix
ET-1 Endothelin-1
FGFb Fibroblast growth factor b
MSC Mesenchymal stromal/stem cells
MCP-1 Monocyte chemotactic protein 1
MIF Macrophages inhibitory factor
CI Mitochondrial complex one
MMP Matrix metalloproteases
MoA Mode of action
NG2 Neural/glia antigen 2
NGF Nerve growth factor

NO Nitric oxide
NOX NADPH oxidase
NT3 neurotrophin-3
NOTCH Neurogenic locus notch homolog protein
OIR Oxygen-induced retinopathy
PDGF-B Platelet-derived growth factor B
PDR Proliferative diabetic retinopathy
PEDF Epithelial derived growth factor
PGE2 prostaglandin E2
PKC Protein kinase C
RAGE Receptor for advanced glycation end products
RGS5 regulator of G-protein signaling 5
ROS Reactive oxygen species
SDF-1 stromal cell derived factor 1
SOD superoxide dismutases
SVF Stromal vascular fraction
STZ Streptozotocin
T1D Type 1 Diabetes
T2D Type 2 Diabetes
TGF-β Transforming growth factor beta
Th T helper
TNF-α Tumor necrosis factor alpha
Treg Regulatory T cell
TRX thioredoxin reductases
VEGF Vascular endothelial growth factor