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

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2019

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Terlizzi, V. (2019). *Shaping vessels and microenvironment: adipose stromal cells in retinal-related diseases*. University of Groningen.

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Chapter 1

Introduction and aims

General introduction

Complications of diabetes

The incidence of diabetes increased exponentially from 1980 to 2014; in the next decade it is estimated to affect over 300 million people worldwide. According to the World Health Organization, by 2030 diabetes will be the 7th leading cause of death in the world ¹. Type 1 diabetes refers to an autoimmune disease that affects the pancreas. β -cells production of insulin becomes impaired, hyperglycemia occurs when the immune system irreversibly damages about the 90% of β -cells. Genetic predisposition and environmental factors are at the basis of the insurgence of Type 1 diabetes. However, the exact biological mechanisms that causes insulin deficiency is currently unknown. In contrast, type 2 diabetes impairs the intake of glucose by skeletal muscles and adipose tissue which is an indirect consequence of loss of sensitivity to insulin. Both forms of diabetes lead to dysregulated levels of glucose in the body. Complications of diabetes act systemically and include organs such as kidneys, heart, nerves, eyes and the vasculature ^{2,3}.

Diabetic retinopathy

In diabetic patients, inefficient glucose metabolism leads to hypo- and hyperglycemia. If not prevented or controlled, particularly hyperglycemia primes damage to the macro- and microcirculation. However the pathological outcome in an individual is influenced by the genetic background, sex, and presence or absence of hypertension and oxidative stress ^{4,5}. Diabetic retinopathy affects approximately 35% of diabetic patients in the world ⁶. The endothelial cells and neural unit are the first retinal cellular components that undergo biochemical changes and consequently suffer damage. The microvasculature in the

eyes is maintained via an intimate communication between microvascular endothelial cells, pericytes and microglial. The endothelium forms a continuous and interconnected network (blood retinal barrier (BRB)) that ensures protection, isolation and exchange from metabolites in the circulation ⁷.

Hyperglycemia induces considerable pressure on metabolic pathways that catabolize glucose. As a consequence, the formation of reactive metabolites i.e. reactive oxygen species (ROS) and advanced glycation end-products (AGEs), compromise the structure and folding of proteins in cells ^{8,9}. Microvascular endothelial cells and pericytes in the retina are particularly exposed to these biochemical changes, inducing apoptosis as well as pericytic migration during the first phases of retinopathy progression ^{10,11}. The cellular apoptosis and migration drive vasoregression, which results in a lack of perfusion in the affected portion of retinal tissue ¹². The lack of oxygen perfusion within the endothelial cells' surroundings is sensed as a stimulus to promote angiogenesis. This process is under the control of juxtacrine (cell-to-cell contact) and paracrine (i.e. growth factors and cytokines) signaling. Moreover, vasoregression is accompanied by enhanced production of inflammatory cytokines and microglia activation ¹³. Vasoregression, microglia activation and neurodegeneration, progressively induce the last disease stage: vasoproliferation ⁶. Importantly, early diagnosis and lifestyle can drastically reduce the burden of retinopathy. In fact, through diet modulation and the use of anti-angiogenic drugs much can be achieved in delaying the onset of the disease. Finally, diabetic retinopathy remains a preventable disease, however more studies are needed to understand the causes of irreversible metabolic changes in the various retinal cellular constituents. Further expansion of this topic is covered in Chapter 2 of this thesis.

Notch signaling in shaping retinal capillaries

During development and disease, notch signaling regulates cell fate, differentiation, migration, and apoptosis^{14,15}. Notch proteins are single transmembrane glycoproteins of ~350kDa. These proteins function as receptors on the cell's surface. In mammals, four isoforms exist (Notch1-4). Five ligands Delta and Serrate (Jagged in mammals) on the cells' surface activate receptors on neighboring cells in a process often called lateral inhibition. This process is important for instructing cells not to follow the same fate as the precursor cells. For example, initial studies on *drosophila melanogaster* identified unspecified epithelial cells in the eyes which became photoreceptors¹⁶. Upon ligands binding, notch receptors are cleaved at three defined points in the ectodomain portion: extracellularly, at the membrane by metalloproteases (disintegrin and metalloproteinase domain/containing protein 10 (ADAM10) and tumor necrosis factor- α (TNF- α)-converting enzyme (TACE) or ADAM17) and in the intracellular space by the enzyme γ -secretase^{17,18}. The latter event releases the portion of protein called intracellular domain (NICD) which shuttles to the nucleus to interact with and initiate transcription of target genes. Interestingly, the intracellular portion of notch receptor contains ankyrin repeat protein (NRARP) which orchestrates vessel density during angiogenesis by linking with the wingless-type protein and catenin beta 1 (WNT/Ctnnb1) pathway¹⁹. In addition, NICD undergoes several levels of regulation through ubiquitylation which regulates notch pathway activity²⁰. In the nucleus, NICD interacts with a complex of target proteins CLS (human CBF1, fly Suppressor of Hairless Su(H), worm LAG-1) and Mastermind (Mam) to activate transcription of genes implicated in cell-fate decision²¹. Among other target genes, a basic helix loop helix (HES class) is well known, their function however, depends on context and cell types²².

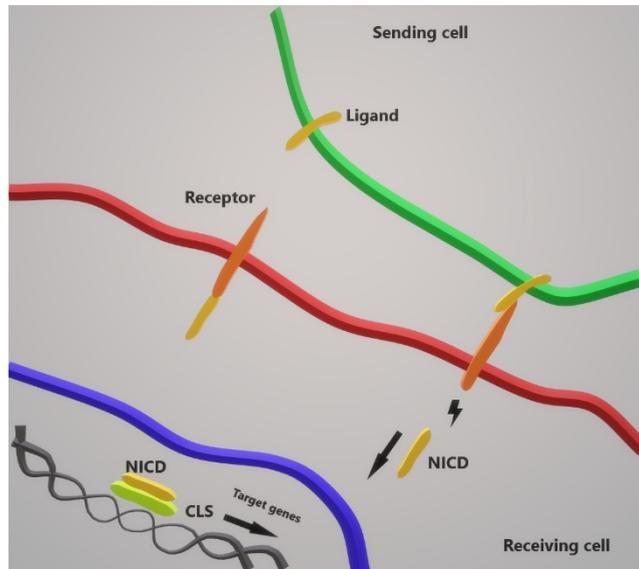


Figure1. Schematic Notch pathway. Receptor/ligand interaction and translocation of NICD to the DNA.

In the eyes, notch signaling is one of the master regulators that orchestrates sprouting angiogenesis, pericytes recruitment and vessel maturation²³. The action of notch signaling is extended to providing capacity to respond to stress stimuli (i.e. ischemic sites) and to mobilizing progenitor cells from the bone marrow to the site of injury²⁴. In the retina, gain- and loss-of-function experiments evaluated notch receptors and ligands during vascular development²⁵. Endothelial cells that lead the nascent vessels (tip cells) expressed Delta-like 4 (Dll4). Following endothelial cells, “stalk cells”, expressed Jagged1 (Jag1). Jag1 was activated by Notch1 released from Dll4 expressing tip cells. This mechanism is indispensable for counteracting Dll4 expression in stalk cells and inducing vessel maturation²⁶. Dll4-Notch1 signaling also has an important function of maintaining a balance between tip- and stalk-cells differentiation in response to potent proangiogenic factors such as vascular endothelial growth factor (VEGF)²⁷. Downstream targets of notch signaling i.e. transcription factor

recombination signal-binding protein Jkappa (RBP-J) and basic helix-loop-helix transcription factor were investigated. Gene deletion and overexpression led to spontaneous angiogenesis and inhibition of tubular structure formation in retinal endothelial cells respectively, demonstrating the role of notch signaling in maintaining vascular homeostasis^{28,29}. Microvascular complications caused by diabetes dysregulates key mechanisms which maintain vascular homeostasis in the retina. Given its capacity to modulate cell specification and spatial distribution, Notch signaling represents an interesting target³⁰.

Adipose stromal cells and the vasculature

Mesenchymal stromal cells (MSC) reside in the stroma of multiple locations throughout the body (including bone marrow (BM), placenta, and adipose tissue). All MSC have in common the capacity of differentiating to other cell types; the acquisition of phenotypes is dependent on the source and stimuli³¹. Given of their abundance, multipotency, and the structural role these cells play in supporting blood vessels (among other functions), adipose stromal cells (ASC) are of intense interest in the field of regenerative medicine^{32,33}. However, heterogeneity in ASC populations exists and, either biomarker sorting (CD34 positive cells)³⁴ or differential culture methods³⁵ have been employed to identify and isolate ASC with properties which resemble pericytes. The role of pericytes in regulating vasculogenesis is both physiological and mechanical, as permeability and structural control is required, to maintain blood vessels' homeostasis^{36,37}.

An important multifunctional cell-surface receptor found on cells of mesenchymal origin is platelet-derived growth factor receptor beta (PDGFRB). This tyrosine-protein kinase is activated by several isoforms of PDGFRB ligands (i.e. PDGF-AA, PDGF-AB, and PDGF-

BB) ³⁸. Ligand-receptor interaction favors receptor dimerization and promotes tyrosine phosphorylation which activates, in turn, GTPase activation protein of Ras (GAP) ³⁹, serine/threonine-protein kinases (AKT), mitogen-activated protein kinase (Erk1/2 MAPK) ⁴⁰, proto-oncogene tyrosine-protein kinase (SRC) and phosphatidylinositol 3-kinases (PI3K) ⁴¹. These pathways are involved in several processes such as pericyte recruitment, proliferation, vessels morphogenesis and cellular differentiation. Moreover, chondroitin sulfate proteoglycan (NG2) regulates endothelial cells motility during vascular morphogenesis. These mechanisms are achieved extracellularly by binding and modulating collagens, growth factors and matrix proteases ⁴². NG2 may also function as a signal transducer, regulating and activating integrins and focal adhesion kinases which integrate signal transducers from the extracellular space ⁴³. Described to be expressed by pericytes and not endothelial cells, NG2 represents a valuable protein for identifying perivascular cells ⁴⁴. The list of markers to identify cells with pericytic characteristics is long, and includes smooth muscle alpha-actin (ACTA2) ⁴⁵, NESTIN ⁴⁶, regulator of G-protein signaling 5 (RGS5) ⁴⁷, receptor for CXC chemokine ligand or stromal cells-derived factor (CXCL) 12 ⁴⁸. It is intuitive that a single marker to recognize ASC as pericytes is not sufficient since the expression of surface antigens is shared by MSC from different sources. However, the combination of proteome profiles, cells localization and interaction assays are helping to clarify this endeavor ^{31,49,50}. For instance, ASC express ACTA2 and NG2 on the cells' surface in a model of microvascular remodeling in vivo; ASC were found in perivascular positions and the vessel density was increased, suggesting a role for ASC in promoting angiogenesis ⁵¹. In addition, ASC can migrate toward PDGF-BB, a chemoattractant secreted by endothelial cells,

further demonstrating the features of ASC that illustrate they resemble and act like pericytes.

As described earlier, oxidative stress is one of the main causes of cellular degeneration in the diabetic retina⁵². Dysfunctional pericytes can be found during the progression of diabetic retinopathy, contributing to vasoregression and the thickening of the basement membrane⁵³. ASC exposed to ROS and reactive nitrogen species (hydrogen peroxide and S-nitroso-N-acetylpenicillamine), show high resilience to oxidative stress, implicating glutathione peroxidase and superoxide dismutase as scavenger enzymes⁵⁴. In contrast to the homeostatic secretome and perivascular positioning, ASC also secrete proangiogenic factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and transforming growth factor beta (TGF β)⁵⁵. The above defined characteristics need to be carefully evaluated when considering the potential of ASC for employment for transplantation into the diabetic retinal microenvironment. Beyond pericyte replacement, ASC face a microenvironment in which inflammatory processes are initiated and different types of cells undergo progressive damage. Therefore, the effect and the adaptation of the ASC pericytic phenotype depends on the stimuli of the whole retinal microenvironment, bearing in mind that ameliorating the blood-retinal barrier is only one of the challenges. In table 1, ASC key molecular components and their interactions and roles, are summarized

Table 1. ASC gene expression with related pericytic function

Gene	Interaction	Role
CXCL12 C-X-C Chemokine Ligand 12	Receptor CXCR4 ACKR3 Related to AKT pathway	Regulates intracellular calcium ions and chemotaxis. Positive regulator of monocyte adhesion.
CD34	Lectins Glycans	Adhesion molecule that mediates stem cells extracellular matrix attachment.
IGF1 Insulin Growth Factor 1	Receptors IGFRs Binds to integrins Activates MAPK/ERK and AKT1	Enhances glucose uptake. Induces tyrosine kinase activity.
VCAM1 Vascular Cell Adhesion Molecule 1	Interacts with integrin alpha- 4/beta-1	Mediates leukocyte-endothelial cells adhesion. Signal transduction.
ACTA2 Actin, Alpha 2, Smooth Muscle, Aorta	Protein kinase binding	Motility, structure and integrity.
CSPG4 Chondroitin Sulfate Proteoglycan 4	FAK and ERK1/ERK2, Rho GTPase activation	Regulates endothelial cells motility during microvascular morphogenesis. Regulates extracellular matrix protease activity.

MCAM Melanoma Cell Adhesion Molecule	FYN PTK2/FAK1	and	Controls adhesion of endothelial cells and intracellular junctions.
DLL1 Delta Like Canonical Notch Ligand 1	Receptors NOTCH1 NOTCH2	and	Plays a role in cell fate decision and cell-to-cell communication. Controls patterning, morphogenesis and maintenance of adult stem cells.
NGFR Nerve Growth Factor Receptor	Trk receptors which are coupled with MAPK, PI3K and Ras.		Regulates insulin dependent glucose uptake. Mediates both cell survival and death.

The ‘matrix’ of life: the perks of culturing in three dimensions

The extracellular matrix (ECM) is a mixture of proteins, glycoproteins and proteoglycans that frame and interact with different specialized cells to form a functional organ⁵⁶. Collagens are found in 28 different forms, fibronectin connects the ECM to integrins on the cells’ surface and proteoglycans regulate biochemical exchange throughout the ECM⁵⁷. The enormous variations in composition of the major ECM components confer unique specializations and functions to organs. Moreover, ECM is a dynamic system undergoing perpetual rearrangement where growth factors, cytokines and the ECM itself cross-talk with cells and profoundly influence their function. In pathological conditions, prolonged biochemical perturbation due to i.e. ischemia and inflammation, stress cells’ organelles involved in proteins maturation. Misfolded proteins affect cell-ECM interactions and consequently ECM architecture and stability⁵⁸.

The retina has unique structure and function. Photons first have to travel through the intricate organization of ganglion cells, astrocytes, bipolar cells, horizontal cells, Müller cells, pericytes and capillaries, to finally reach the rod and cones that transduce the light stimulus into a biochemical signal. At this point the response travels all the way back to the ganglion cells which transport the information to remote areas of the brain. ECM is fundamental for maintaining this complex architecture, as well as to respond to damage and to guide cells during wound healing⁵⁹. It is not yet clear which cells contribute significantly to the ECM production in the retina. However, pericytes, microglia, Müller cells and astrocytes all have the potential to contribute to the production and maintenance of the ECM in this environment^{60–62}. The main components of the retinal ECM include collagen type IV, fibronectin, laminin and heparan sulfate proteoglycans. Fibronectin for

example, is a “molecular glue” secreted by cells under form of dimers linked by disulfide bonds which interact with laminin and collagen type IV⁶³. Fibronectin not only has a structural role, but also functional, it binds to $\alpha 5\beta 1$ integrin regulating processes such as cell adhesion and migration⁶⁴. Among the vast variety of accessory extracellular proteins, fibulins establish connection with several other ECM proteins and, might have an important role in the retina during angiogenic control⁶⁵. During the progression of diabetic retinopathy, deposition of ECM components significantly increases, leading to pathological structural changes⁶⁶. Thickening of vascular basement membranes strongly influences the normal capillary architecture, which results in the occurrence of vascular permeability thereby contributing to the progression of retinopathy⁶⁷.

Through exploiting the self-organizing capacity of mammalian cells, much can be achieved in mimicking structural details of organs in culture. The combination of specific cell populations from a given organ on soft substrates, allow the following of developmental and disease stages in vitro. A more recent approach, employs undifferentiated embryonic stem cells which, with appropriate stimulatory conditions, spontaneously assemble in a surrogate micro-organ. The term organoid refers to the combination of three-dimensional culture with the propagation of stem cells. The main advantage of such an approach denotes improvements in drug testing and cells replacements prior animal or human experimentation^{68,69}.

Three-dimensional microenvironments may also function to represent portions of the organ of interest. A precise analysis of how ASC interact and shape the vasculature can provide significant advances for understanding the regenerative capacities of these cells. In fact, vascular regeneration is a growing theme in tissue engineering⁷⁰.

Although ASC are adult stem cells with a limited capacity to differentiate to other cell populations, they possess an interesting self-organizing ability that if exploited in a three dimensional microenvironment, can give important clues of the tissue of interest that is being created ⁷¹. More studies support the notion of self-assembly to enhance stemness and therapeutic potential ⁷². For example, ASC cultured in spheroids exhibited an enhanced neuroprotective potential compared to the monolayer-cultured ASC, when transplanted in the brain of a rat model for Parkinson's disease ⁷³. Similar findings showed neural cells differentiated from ASC had increased expression of growth factors believed to play a role in tissue regeneration ^{74,75}. Heterotypic cell-cell interactions between ASC and endothelial cells drives spatiotemporal organization and, in combination with molecular analysis, a better identification of ASC that support the vasculature ⁷⁶.

Aims of this thesis

During the progression of diabetic retinopathy, several cells in the retina undergo pathological changes. The tight interplay between endothelial cells and pericytes maintains the blood retinal barrier. Consequently, the entire retinal microenvironment requires restoration. Strategies to re-induce a homeostatic microenvironment exist but are not sufficiently robust to date to account for all the structural, biochemical and morphological changes provoked by diabetes. Cell therapy might offer both structural and biochemical endurance to the hyperglycemic stress. The aim of this thesis is to contribute to understanding of the molecular mechanisms involved in the ASC pericytic function and how ASC shape the surrounding microenvironment, with the ultimate aim of improving cells implantation in the eyes.

In order to employ ASC as therapeutic cells in the retina, they need to be considered from several pathological scenarios during the progression of diabetic retinopathy. In **chapter 2**, oxidative stress, inflammation and pathological proliferative angiogenesis are described in the context of the state of the art of current knowledge about cell therapy in the eyes. This review focuses on the mesenchymal stem cells' capacity for positively influencing the retinal microenvironment upon transplantation and their impact on the retinal cellular constituent affected by diabetes. Moreover, the latest strategies, biases and clinical trials are discussed to address safety and efficacy of cell therapy.

Evolutionarily conserved molecular mechanisms are at the basis of cell's communication, differentiation and morphogenesis. When considering cell therapy, the surrounding microenvironment is fundamental for instructing implanted cells to adapt to the new

microenvironment. In **chapter 3 and 4**, notch signaling is investigated as a molecular intermediate indispensable for ASC to exert their pericytic phenotype and efficiently promote angiogenesis in endothelial cells.

We asked which isoform of ASC expressed notch modulates migration and vessels network formation in vitro, and translated findings to in vivo models. The regenerative capacity of ASC were evaluated based on the integration and maintenance of capillary networks.

The second half of this thesis focuses on the ASC ability to transform the microenvironment with the aim of understanding morphological changes upon transplantation. In **chapter 5**, three-dimensional scaffolds are used to investigate the ASC interaction with endothelial cells and importantly, alterations in communication with endothelial cells when ASC are isolated from diabetic patients. In this study, multi-cellular assembly of endothelial cells and ECM deposition are followed over time under the ASC guidance in a three-dimensional microenvironment.

The hypothesis that ASC are fundamental to guide endothelial cells to form interconnected structures was tested by following ECM deposition and cellular morphogenesis. Moreover, whether ECM and structural changes occur in ASC/retinal microvascular endothelial cells coculture was investigated. **Chapter 6** describes fibulin1 as extracellular protein believed to be one of the key proteins regulating the basement membranes organization during physiological and pathological conditions. Fibulin1 is upregulated in diabetic retinopathy in response to vascular damage. Whether fibulin1 is important to establish ASC pericytic function on endothelial cells is matter of the current investigation.

Finally, chapter 7 discusses the ASC therapeutic capacity as supportive cells. In this chapter, molecular interplays between ASC and ECM and the influence of the pathological microenvironment during the progression of diabetic retinopathy are discussed. Specifically, NOTCH2 regulates the ASC pericytic phenotype in vitro. Moreover, ASC guide endothelial cells organization and vasculogenic activity in confined three-dimensional microenvironment by ECM deposition and cell-to-cell contact. Finally, accessory protein fibulin1 was targeted for its possible role in the pathogenesis of diabetic retinopathy and ASC regenerative potential. Convergence of ASC molecular pathways, ECM deposition and three-dimensional confined microenvironment are merged to discuss future perspectives.

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