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

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Chapter 2

Mesenchymal stromal/stem cells as potential therapy in diabetic retinopathy

Mesenchymal stromal/stem cells as potential therapy in diabetic retinopathy

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Summary

Diabetic retinopathy (DR) is a multifactorial microvascular disease induced by hyperglycemia and subsequent metabolic abnormalities. The resulting cell stress causes a sequela of events that ultimately can lead to severe vision impairment and blindness. The early stages are characterized by activation of glia and loss of pericytes, endothelial cells (EC) and neuronal cells. The integrity of the retinal microvasculature becomes affected, and, as a possible late response, macular edema may develop as a common reason for vision loss in patients with non-proliferative DR. Moreover, the local ischemia can trigger vasoproliferation leading to vision-threating proliferative DR (PDR) in humans. Available treatment options include control of metabolic and hemodynamic factors. Timely intervention of advanced DR stages with laser photocoagulation, intraocular anti–vascular endothelial growth factor (VEGF) or glucocorticoid drugs can reduce vision loss.

As the pathology involves cell loss of both the vascular and neuroglial compartments, cell replacement strategies by stem and progenitor cells have gained considerable interest in the past years. Compared to other disease entities, so far little is known about the efficacy and potential mode of action of cell therapy in treatment of DR. In preclinical models of DR different cell types have been applied ranging from embryonic or induced pluripotent stem cells, hematopoietic stem cells, and endothelial progenitor cells to mesenchymal stromal cells (MSC). The latter cell population can combine various modes of action (MoA), thus they are among the most intensely tested cell types in cell therapy. The aim of this review is to discuss the rationale for using MSC as potential cell therapy to treat DR. Accordingly, we will revise identified MoA of

MSCs and speculate how these may support the repair of the damaged retina.

Diabetic retinopathy

DR is a complex and multifactorial diabetic complication. Hyperglycemia, inflammation and neuronal dysfunction are the major factors in the pathophysiology of DR but also systemic factors such as hypertension may be involved. A variety of biochemical pathways are affected as discussed in detail previously (Hammes, et al. 2002; Hammes, et al. 2011b; Stitt, et al. 2016). Especially chronic hyperglycemia appears as the initiator of a vicious cycle of events by inducing biochemical abnormalities in target tissues of diabetic complications through mitochondrial overproduction of reactive oxygen species (ROS) (Brownlee 2005). Mechanisms, which lead to ROS production, are at least: increased polyol pathway flux, increased formation of advanced glycation end products (AGEs), activation of protein kinase C and increased hexosamine pathway flux. The resulting oxidative stress induces injury to all cell types in the retina. Pericytes appear to be the first cell type affected, then endothelial cells vanish leaving nonperfused acellular capillaries (Hammes, et al. 2011a). This vasoregression is accompanied by neuronal damage affecting the neurovascular unit and the blood-retina barrier. The proliferative stage is caused by severe retinal ischemia, uncontrolled expression of proangiogenic factors and endothelial cell proliferation leading to severely hyperpermeable and rupture-prone vessels. Inflammatory processes play an important additional role. Inflammatory cytokines are produced by a variety of cell types under hyperglycemic and hypoxic conditions including glial and microglial cells (Vujosevic and Simo 2017).

The disease can progress from a mild form of non-proliferative disease, marked by microaneurysms in the retina to severe stages, denoted by intraretinal hemorrhages, venous beading and intraretinal microvascular abnormalities (Stitt, et al. 2016) (Fig. 1). Tight glycemic control can significantly reduce the risk and progression of DR in early stages (Aiello and DCCT/EDIC Research Group 2014). In patients with type 1 diabetes (T1D), renin–angiotensin system inhibitors are now standard therapy if incipient diabetic nephropathy coincides. Evaluating the progression of DR in patients requires non-invasive methods. The first and most important method is funduscopy with and without permanent documentation by photography. Funduscopy focusses mainly on the detection of the main diabetes-related vascular pathologies, i.e. progressive vasoregression and increased vascular permeability (Williams, et al. 2004). This is complemented by optical coherence tomography, which enables the identification of retinal degeneration, of macular edema and of inflammatory cell invasion (Virgili, et al. 2015). Not widely used techniques like ultra-wide field imaging can assess important morphological changes in the peripheral retina and give clues on the severity of DR (Soliman, et al. 2012). Finally, sensitivity of cells constituting the neuroretina layer is measured by multifocal electroretinogram (ERG) (Harrison, et al. 2011). Abnormality in cells' electrical signals may precede the development of retinal lesions and microaneurysms in some, but not in all cases (Santos, et al. 2017). Furthermore, regular screening for clinical signs of DR helps to control disease progression and can reduce the risk of vision loss by enabling timely intervention with laser photocoagulation or intraocular drug injection (anti-VEGF or glucocorticoids) (DCCT/EDIC Research Group, et al. 2017). Beneficial effects of these therapies do not affect to all patients, may

be only transitory, or can have adverse side effects. Therefore, the clinical use is limited and new treatment strategies are needed.

The majority of established therapies target quite advanced states of DR. It is desirable to develop strategies which target early phases in DR to delay or even prevent DR. Cell-based therapies may offer direct tissue replacement and/or endogenous regeneration via trophic paracrine factors. Besides MSC, endothelial progenitor cells (EPC)/endothelial colony forming cells and pluripotent embryonic or induced pluripotent stem cells (iPSC) have been tested (Park 2016).

Due to their broad mechanisms of action, involving secretion of trophic factors, similarity to pericytes, extracellular matrix (ECM) modulation, ROS scavenging potential and finally immune modulatory capacities, MSC may affect DR disease progression at different disease stages from vasoregression to vasoproliferation (Fig. 2).

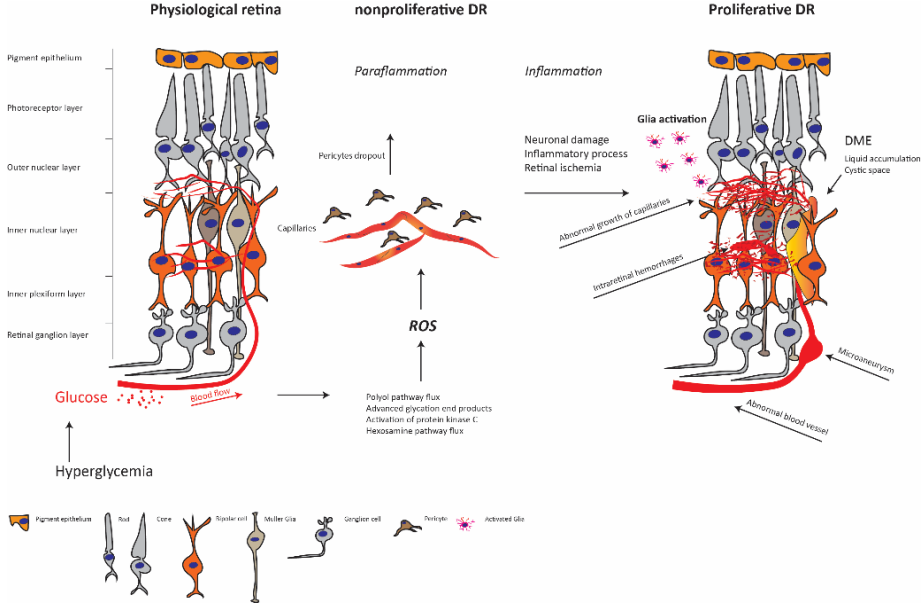

Fig. 1. Diabetic retinopathy (DR) is a multifactorial microvascular disease that is caused by chronic hyperglycemia and subsequent adverse metabolic sequelae. The resulting cell stress causes a series of events which may lead to severe vision impairment and blindness ultimately. The early stage of DR is characterized by activation of glia and loss of pericytes, endothelial cells (EC) and neuronal cells. The integrity of the retinal microvasculature is negatively affected while a late response may comprise the development of macular edema in nonproliferative DR. Alternatively, DR may proceed towards so-called proliferative DR. This is induced by the local ischemia which is a strong pro-angiogenic trigger that causes abnormal capillary growth, intraretinal hemorrhages and the formation of cysts among others. Together, this is the leading cause of deteriorated vision and blindness in diabetics.

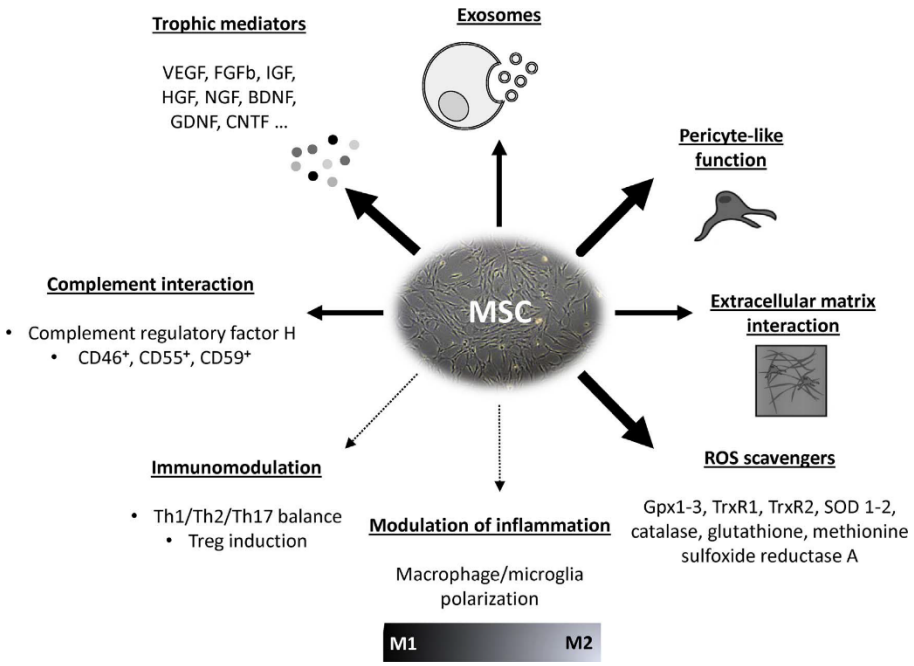

Fig. 2. Proposed mechanism of action (MoA) of MSC in DR. Thick arrows indicate MoA based on the use of MSCs in preclinical models of DR (see also Table 2). Dotted arrows indicate known MoA based on diabetes studies at a systemic level, but not explicitly in DR (requires confirmation in (pre)clinical models). Regular arrows indicate hypothesized MoA, based on the use of MSCs in other than diabetic diseases, which however have not been described in DR until now.

Animal models of DR

Firstly, rodents represent a valuable animal model to investigate the impact of cell therapy in DR because of their short life cycle and responsiveness to genetic manipulation. Secondly, the use of streptozotocin (STZ), specifically toxic to β cells in the pancreas, induces reproducible severely hyperglycemic conditions mimicking essentials features of the human disease. STZ causes destruction of the pancreatic islet and, subsequent hypoinsulinemia and hyperglycemia (Rossini, et al. 1977). In the retina, early signs of DR corresponding to preclinical human disease stages are evident, i.e. loss of pericytes, vasoregression, neurodegeneration and glial activation (Hammes 2018). There are several other animal models with different characteristics and dynamics, such as the Ins2Akita (Akita) mouse model which holds a mutation in the gene encoding insulin-2 causing spontaneous diabetes (Barber, et al. 2005).

It has to be noted that the current animal models can only serve as models for incipient DR as none of the animals develops PDR. In order to obtain a mouse strain with PDR, transgenic mice, in which the bovine rhodopsin promoter is coupled to the gene for human VEGF, have been crossed with Akita mice (Okamoto, et al. 1997). The hybrid, named Akimba, shows several signs of PDR such as microaneurysms, leaky capillaries, and capillary dropout (Rakoczy, et al. 2010). Finally, the angiogenic aspects of PDR can be simulated by exposing mice to hyperoxia resulting in retinal ischaemia followed by proliferative vascular damage in the retina layers (oxygen-induced retinopathy (OIR) (Smith, et al. 1994). These are the main animal models which resemble a general frame of diabetic driven complication in the eyes.

Mesenchymal stromal cells: a brief overview

MSC were first described by Friedenstein in 1970 as fibroblast colony-forming cells, isolated from bone marrow, with osteogenic differentiation potential. It is noteworthy to emphasize that Friedenstein himself, already at their first description, introduced them as new "therapeutic" component to be considered in bone marrow transplants (Friedenstein, et al. 1970).

After their first description MSC were widely studied, bringing new insights regarding their biology and functions. Moreover, different sources of MSC were found beyond bone marrow (BM-MSC) such as adipose tissue (adipose tissue-derived stem/stromal cells - ASC) (Bajek, et al. 2016; Kern, et al. 2006), dental pulp (Perry, et al. 2008), cord blood (CB-MSC) (Bieback, et al. 2004) and Wharton´s jelly (WJ-MSC) (Joerger-Messerli, et al. 2016). However, at the same time, misconceptions about MSC were arising, limiting their applicability in therapeutic approaches (Phinney and Sensebe 2013). The message of the authors is: a) MSCs differ based on their tissue origin and b) when isolated from different organisms, c) MSC are heterogeneous, d) the current panel of surface "markers" is insignificant for function, e) MSCs function in vitro may/will differ from their in vivo, thus f) only clinical data will be able to give insight into the mode of action.

In this context, at least minimal criteria regarding MSC characterization were proposed by the International Society for Cellular Therapy (ISCT). Following ISCT guidelines, MSC are defined as multipotent progenitor cells with a fibroblast-like, spindle shape morphology and a robust proliferation capacity. They possess a strong plastic adherence, and trilineage mesenchymal differentiation potential (adipocytes, osteoblasts and chondroblasts) as well as a panel of surface antigens that define their phenotype (Dominici, et al. 2006). In

the meantime, modifications of these criteria have been agreed on to characterize MSC from other tissue sources, e.g. ASC (Bourin, et al. 2013).

Table 1. Main MSCs trophic mediators

Growth factor	Receptors	Some functions
FGF (FGF1-24) (Fibroblast growth factor)	FGFR1-4	Reported as a mitogen. Regulates and induce angiogenesis. Involved in to inflammatory and immune responses.
VEGF (Vascular endothelial growth factor)	VEGFR1, VEGFR2	One of the key regulators of angiogenesis. Inhibits apoptosis. Activate protein kinases activity.
NGF (Nerve growth factor)	Trk A	Maintenance and growth of some neurons.
BNDF (brain-derived neurotrophic factor)	Neurotrophin TRKB	Positive regulation of sprouting. Regulation of proteins on cell surface. Cell-cell signaling.
GDNF (glial-derived neurotrophic factor)	i.e. Toll-like receptor binding	Regulation of stem cells differentiation. Regulation of gene expression.
CNTF (ciliary neurotrophic factor)	Interleukin-6 receptor binding	Regulates retinal cells apoptosis. Signal transduction. Positive regulation of cells proliferation.

Interest in MSC as therapeutic cells has been steadily increasing, supported by an improved understanding of their functions and

properties. In addition, their beneficial behavior in contexts of different diseases such as graft versus host disease (Le Blanc Katarina, et al. 2004; Le Blanc Katarina, et al. 2008), wound healing processes (Gaur, et al. 2017; Kim, et al. 2017), tumors (Blogowski, et al. 2016; Cammarota and Laukkanen 2016; Koliaraki, et al. 2017), and autoimmune diseases (Pistoia and Raffaghello 2017; Tyndall 2008) has been documented. Taking into account the multiplicity of studies and diseases in which MSC treatment has been applied, at least three mechanisms can be considered predominant in the MoA of MSC in a diseased context.

First and most documented MoA is represented by the paracrine and trophic potential. MSC, in fact, are able to secrete a broad range of growth factors that can modulate their surrounding microenvironment, favoring cell-cell interaction and communication with different cell types (Salgado, et al. 2010).

Secondly, MSC retain an immunological profile that makes them hypo-immunogenic. Because of low surface expression of HLA class I, inducible HLA-class II expression and the lack of costimulatory molecules such as CD40, CD80 and CD86 they may survive in an allogeneic environment (Haddad and Saldanha-Araujo 2014). In addition, MSC are strong immunomodulators (Najar, et al. 2016). Immunomodulation can be mediated by both cell-cell interaction and the release of soluble factors. The most reported mechanism is the MSC-mediated inhibition of T-cell proliferation, and the promotion of a regulatory T-cell subpopulation (Treg) (Haddad and Saldanha-Araujo 2014; Luz-Crawford, et al. 2013). However, MSC can interact also with B-cells, natural killer cells, monocytes/macrophages and dendritic cells. The production of immunoregulatory factors such as TGF-β, HGF, prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO)

and nitric oxide (NO) has been widely documented in several studies and directly sustains their immunological potential (Yan, et al. 2014a; Yang, et al. 2009).

Lastly, MSC have a differentiation potential that is clearly related to their multipotency. This potency would enable them to differentiate towards the damaged or lost cell types in order to replace cells at least transiently at the site of injury. However, the understanding of the underlying mechanisms of homing, proliferation, differentiation and functional engraftment of MSCs is in its infancy, in particular with respect to treatment of DR. The mere fact that e.g. in rodent models for retinal injury, MSC co-localized with and acquired neuronal and glial markers (rhodopsin resp. GFAP) or pericytic markers (Rajashekhar G, et al. 2014 Mendel TA, et al. 2013) does confirm that the respective functions were acquired too after intravitreal administration. Cell fusion may have happened resulting in co-expression of different marker molecules (Park 2016; Tomita, et al. 2002).

These combined functions render MSC one of the most interesting and promising tool for a potential therapeutic cell-based approach for a number of diseases.

MSC for DR treatment – potential modes of action

Given the potential of MSCs and the characteristics of early DR in models, the promotion of cell repair and the defense against stress cell damage evolves as experimental focus. The functional restoration of damaged tissue by repair or regeneration can be accomplished by stem cells in three ways or a combination. Firstly, stem cells are constructive via their differentiation and engraftment into tissue. Secondly, stem cells are instructive which means that via the secretion of trophic factors, stem cells direct the local microenvironment to a

proregenerative state. Thirdly, stem cells may act in a reconstructive manner via the remodeling of the extracellular matrix (ECM) by secretion of e.g. matrix metalloproteinases (MMPs) and a host of structural (ECM) proteins. The reconstructive function of, in particular MSC, is frequently neglected, but it should be noted that the ECM is the prime extracellular reservoir of growth factors as well as of diabetic triggers for inflammation (AGEs).

MSC as trophic mediators

MSC are well known to secrete a broad range of pro-regenerative, mitotic, angiogenic, anti-apoptotic, anti-fibrotic factors, such as basic fibroblast growth factor (bFGF), VEGF, insulin-like growth factor (IGF), HGF, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) (Table 1) (Caplan 2008; Ezquer, et al. 2014; Griffin, et al. 2016). These factors appear to modify the local microenvironment from an adverse injury to a pro-regenerative milieu. In addition to secreted growth factors, cytokines and chemokines, which will be influential, but most likely in local distance and short-lived, MSC are able to pack trophic mediators into extracellular vesicles (exosomes, microvesicles) (Doeppner, et al. 2015; Ophelders, et al. 2016). These extracellular vesicles cannot only transport proregenerative factors, but also mRNA and microRNA but even mitochondrial components over a long distance (Rani, et al. 2015; Yanez-Mo, et al. 2015).

It is not yet clarified whether these trophic functions require MSC to home and engraft to the sites of injury as there are also indications that conditioned medium, extracellular vesicles or other shuttled factors have been functional (Gao, et al. 2014; Nagaishi, et al. 2016; Park, et al. 2010). Migration to pancreatic islets or organs suffering from

diabetic complications has been shown (Lee, et al. 2006; Sordi, et al. 2005). Tissue regeneration, revascularization and sustained normoglycemia were achieved. In NOD-SCID and STZ diabetic mice MSC injection restored glycemia by increasing β cell mass and promoting renal protection (increased proliferation, decreased apoptosis, increased levels of proregenerative factors, and anti-inflammatory cytokines, decreased macrophage infiltration and oxidative stress damage) (Ezquer, et al. 2015; Ezquer, et al. 2008). Priming of MSC appears to even further improve these effects (Xu, et al. 2009). MSC, genetically-engineered to express VEGF or pancreatic-duodenal homeobox 1 (PDX-1), improved survival and reversed hyperglycemia. The effect, however, was only sustained when injecting VEGF-MSC and involved recovery of β cell mass (Milanesi, et al. 2012).

In models of diabetic neuropathy, MSC have been shown to act via induction of neurotrophic factors, e.g. NGF and neurotrophin-3 (NT-3). Both were significantly increased 2 weeks post-infusion, but no more 4 weeks post-infusion (Kim, et al. 2011). A recent study indicates that MSC injection in diabetic neuropathy has additional effects by reducing inflammatory cytokines, apoptosis, calcium and ROS levels (Oses, et al. 2017).

To date, the mode trophic mode of action of, proangiogenic, MSCs appears paradoxal, in particular with respect to DR in which angiogenesis should be dampened or normalized instead of promoted. The caveat in this reasoning, is that MSCs secrete a plethora of paracrine factors, that partly have opposite functions, while MSCs may act to normalize aberrant microvasculature via juxtacrine mechanisms too (described in the next section). Nevertheless, a number of factors secreted by MSC and exerting proregenerative functions in other

disease modalities may have adverse effects in DR. Many of these factors, in fact, are considered as biomarkers for DR and have been attributed to disease progression. VEGF, for instance, is a target of therapeutic interventions in DR. There is evidence that anti-VEGF antibodies (bevacizumab, Avastin or ranibizumab, Lucentis) have protective effects and can slow down disease progression (Li, et al. 2017b; Martinez-Zapata, et al. 2014). These anti-VEGF-A antibodies recognize all isoforms of VEGF, although it might be advantageous in the future to target only those specific isoforms known to cause trouble. For instance, a recent study addressed the expression of the two isoforms of VEGF, $VEGF_{165a}$ and $VEGF_{165b}$, considered to represent pro- or anti-angiogenic VEGF, and their receptors in plasma of patients with DR, with diabetes or controls (Paine, et al. 2017). With increasing disease stages, $VEGF_{165a}$ and VEGFR-2 concentrations were increased. A variety of other intravitreal growth factors, cytokines and chemokines have been attributed to disease progression and considered as potential biomarkers or even as therapeutic targets. However, it has to be taken into account that vitrectomy is only performed in only a minority of patients and mostly at severe disease stages and that thus samples might be contaminated with serum/plasma proteins, especially in severe disease stages (Abcouwer 2013). Similar to VEGF, further angiogenic factors such as angiopoietin-2 (Ang-2), osteopontin, PDGF, erythropoietin (EPO), stromal cell derived factor 1 (SDF-1) have been found to be increased, and vice versa anti-angiogenic factors decreased, such as pigment epithelium derived growth factor (PEDF), endostatin, angiostatin and tissue kallikrein (reviewed in (Abcouwer 2013)). A recent meta-analysis identified VEGF, IL6, IL8, EPO, PDGF-BB, NO, endothelin-1 (ET-1), monocyte chemotactic protein 1 (MCP-1), TGF-β, and TNF-α to be increased in the vitreous of patients with proliferative DR independent of T1D or T2D. PEDF and HGF were

decreased (McAuley, et al. 2014). In this meta-analysis, VEGF was the only biomarker which was able to significantly discriminate patients with and without nonproliferative DR. Since a variety of these molecules have dual functional roles, the may not only represent disease but also the attempt to regenerate injured tissue. IL6, for instance, is considered as proinflammatory molecule but it is known to orchestrate functions, not only in the immune system, but also in the nervous system. Here it can increase inflammation and thus tissue damage, but acting as neuropoietin it can also support neuronal regeneration (Suzuki, et al. 2009).

Because DR is a multifactorial and multistep disease, protection or slow-down of progression requires a well-orchestrated milieu balancing intrinsic repair attempts against adverse stress events promoting injury. MSC are highly plastic and adapt their repertoire of secreted factors, to cues of the local microenvironment (Phinney 2007). Accordingly, these cells secret a variety of factors, which are considered to contribute to DR progression, MSC may assist to change track from a vicious cycle to a proregenerative response.

MSC to act as pericytes

Pericytes are cells that wrap around microvessels throughout the body and which are of mesodermal origin. Ever since the first light microscopy studies in the late 19th century, little has changed on this description. In a landmark publication, Zimmermann published detailed light microscopy studies on pericytes and their intimate contact with capillaries (Zimmermann 1923). Currently, the working definition of a pericyte is a (peri)vascular, basement membrane-embedded cell (Sims 1986). In the microvasculature three types of contractile perivascular cell types, pericytes, smooth muscle cells and supra-adventitial stromal cells appear to form a continuum of cell types

that can (trans)differentiate into each other and which may be derived from a common $CD146^+$ endothelial precursor (Zimmerlin, et al. 2013). This high plasticity and the lack of definitive pericyte markers, as well as rapid phenotypic changes *in vitro*, render 'pericytes' a heterogeneous population at best (reviewed in (Armulik, et al. 2011)).

Pericyte loss is considered a key event in the initiation of vasoregression (Hammes 2005). Very recently it has been shown that selective pericyte loss is not enough to mediate blood retinal barrier (BRB) disintegration. However, loss of pericyte coverage sensitizes retinal vascular EC to VEGF-A. Via FOXO1 this leads to Ang-2 upregulation and triggers a positive feedback loop as seen in the pathogenesis of DR (Park, et al. 2017a).

MSC share several features with pericytes, such as their morphology and function but also their anatomic localization as perivascular cells. It was long debated which in situ origin MSC may have and where these are localized. Crisan et al. documented that in a magnitude of tissues MSC derive from a perivascular origin (Crisan, et al. 2008). These data suggest that pericytes might be the in vivo progenitor of MSC (Caplan 2008; Caplan 2017). Recent data however, challenge this idea. Guimarães-Camboa et al. used lineage tracing of pericytes/smooth muscle cells expressing the transcription factor Tbx18 to demonstrate that perivascular cells do not behave as tissue-specific progenitors in various organs, neither in aging nor in pathological settings (Guimarães-Camboa, et al. 2017). Tbx18 cells kept their identity and failed to differentiate to other cell types. Sorted *in vitro* cultured Tbx18 pericytes, however, showed MSC potential, such as phenotype and differentiation potential. In line with this, a variety of data indicate that pericytes can be differentiated out of cultured MSC. In a recent systematic review Xu et al. compared

protocols from 20 reports (Xu, et al. 2017). The markers most commonly used to identify pericytes were PDGFR-β, alpha-smooth muscle actin (α-SMA), neural/glia antigen 2 (NG2), desmin and regulator of G-protein signaling 5 (RGS5) (Diaz-Flores, et al. 2009). However, expression patterns on pericytes *in vivo* can differ at different developmental stages and in different tissues and organs. Accordingly, a combination of phenotypic markers, perivascular location, morphology, and functionality shall be used to define pericyte identity. These are all animal-based results; thus, the translation to human clinical applications warrants caution.

ASC have been shown to differentiate to pericytes *in vitro* and *in vivo*. Based on surface markers, the group of Bruno Peault classified adipose tissue derived MSC into two major groups: CD31- /CD45- /CD34+ /CD146- cells (adventitial stromal/stem cells [ASC]) and CD31- /CD45- /CD34- /CD146+ cells (pericytes [PCs]). Using single-cell quantitative polymerase chain reaction they recently showed that aldehyde dehydrogenase (ALDH) activity can identify subclasses. ALDHbright-ASC were classified as the most primitive cells, followed by ALDHdim-ASC, ALDHbright-PC and finally ALDHdim-PC as least primitive, suggesting ASC at the basis of the differentiation hierarchy (Hardy, et al. 2017; Herrmann, et al. 2016). Transcriptomic analysis of highly purified non-cultured adipose tissue derived cells support this notion that perivascular MSC are precursors of pericytes (da Silva Meirelles, et al. 2016). One study performed a direct comparison of MSC and pericytes (isolated based on CD34+, CD146+ expression from adipose tissue and bone marrow and retinal and placental pericytes. This comparison revealed reduced proliferative capacity of pericytes. MSC markers were comparable and highly expressed on all cell populations (CD44+CD90+CD73+CD105+ expressed on at least 75% of all cells). Pericyte markers were highly

variable, CD146+NG-2+PDGFRβ+ on 1.3% and 3.6% of retinal and placental pericytes and 48% to 80% on bone marrow or adipose tissue-derived MSC and pericytes. Interestingly, PDGFRβ expression appeared to be highly variant, but not discriminatory for MSC vs. pericytes (Herrmann, et al. 2016). This study showed higher osteogenic but slightly reduced adipogenic differentiation potential of the CD34+, CD146+ sorted pericytes from both tissues. Chondrogenic potential was only seen in cells from BM but reduced in the pericyte population. Angiogenic potential in a Matrigel tube formation assay did not reveal any differences between the different cells.

The large phenotypical and functional overlap with pericytes renders MSC an ideal candidate in the cure and/or prevention of DR (Traktuev, et al. 2008). There are numerous indications that injected MSC acquire a perivascular fate within injured tissues. Examples for DR are given below. Of note, pericytes, also retinal pericytes, have been shown to exert immunomodulatory function such as MSC (Navarro, et al. 2016; Tu, et al. 2011). Loss of immunomodulatory functions by disappearance of pericytes may add to vasoregression by impaired control of immune responses (see below).

MSC to restore the extracellular matrix

It is well documented that hyperglycemia, e.g. AGE modification, leads to changes in the retina, including the extracellular matrix (ECM) composition, matrix metalloprotease activity and basement membrane thickening (Hammes, et al. 1996; Yang, et al. 2007). Resulting changes of interaction of EC and pericytes, as well as induced apoptosis, can lead to abnormal function, pericyte migration, endothelial cell apoptosis and finally increased permeability (Preissner, et al. 1997). Since pericytes and EC are important components of the neurovascular unit, this is likewise affected causing disruption of the BRB.

The basement membrane is mainly composed of laminin, collagen type IV, fibronectin as structural components and proteoglycans such as perlecan and nidogens 1 and 2. It allows cell attachment and binds growth factors. Especially the heparan sulfate attached platelet derived growth factor (PDGF) appears to be relevant for the maintenance of cell-cell/cell-matrix interaction. PDGF is required for the BRB formation by recruiting pericytes and inducing their maturation (Park, et al. 2017b).

ECM degradation is required for vessel sprouting, thus there is a tight regulation of expression of matrix metalloproteases (MMPs) and their inhibitors (van Dijk, et al. 2015). In mesangial repair MSC have been shown to reverse the alterations of ECM proteins and by this contribute to repair (Herrera, et al. 2016).

Furthermore, glycated matrix can trigger inflammation. AGEs are sensed by the receptor for advanced glycation endproducts (RAGE). RAGE activation results in pro-oxidant and pro-inflammatory pathways and an activation of the innate immune system additionally contributing to the vicious cycle of vasoregression (Zong, et al. 2010). In a hyperoxia-induced lung injury model, MSC reduced signs of damage, associated with reduced RAGE/NFkB expression and TNF-α secretion, suggesting an impact on RAGE/NFkB signaling (Tian, et al. 2013). AGEs can affect MSC (Aikawa, et al. 2016; Kume, et al. 2005; Stolzing, et al. 2010). However, COMP-Ang, a chimeric form of Angiopoietin1 fused to the coiled-coil domain of rat cartilage oligomeric matrix protein, prevented negative effects of AGEs and improved viability and differentiation properties (Kim and Kwon 2013).

MSC as scavengers of reactive oxygen species

Molecular oxygen acquires oxidant property if an unpaired electron is excited and changes its spin. Several molecules and free radicals derive from the reduction of oxygen, these molecular modifications are defined as ROS (Liochev and Fridovich 1999). If the cellular metabolic pathways involved in ROS detoxification are impaired, oxidants indiscriminately react with proteins and DNA (Turrens 2003). Cellular oxidative stress may originate from mitochondria via dysfunction of the electron transport chain or from the cytoplasm via NADH-oxidases (Nox) among others. Pathways that further dysregulate ROS production include increased polyol pathway flux, increased AGE formation, and activation of protein kinase C (PKC) isoforms (Brownlee 2000). Diabetes causes ROS accumulation with serious complications (Giacco and Brownlee 2010) including DR (Guzman, et al. 2017; Kowluru and Chan 2007; Kowluru and Mishra 2015).

Obviously, the reversal of either retinal ROS production or the scavenging by ROS neutralizing molecules has been put forward in therapeutic context. Hyperglycemia-induced ROS, via the TGF-β-activated kinase 1 (TAK1) –NFκB signaling axis, also perpetuates inflammation in cells (Ajibade, et al. 2013; Hoesel and Schmid 2013; Morgan and Liu 2011).

MSC are equipped with a full array of mechanisms that neutralize oxygen and nitrogen radicals. These include glutathione peroxidases (GPx1-3), thioredoxin reductases (TrxR1, TrxR2), and superoxide dismutases (SOD1, SOD2), as well as DNA repair systems (Ezquer, et al. 2014; Valle-Prieto and Conget 2010a). Several of these redox balancing systems, however, depend on co-factors e.g. supplementation of culture medium with selenium augments MSC's potential to scavenge radicals (Ebert, et al. 2006). Despite built-in

protective mechanisms, ROS may promote apoptosis (Hajmousa, et al. 2016) and senescence (Chen, et al. 2015; Jeong and Cho 2015; Ko, et al. 2012) of MSCs *in vitro*. ASC from obese mice have severe mitochondrial dysfunction and increased ROS production (Perez, et al. 2015) which might compromise the use of ASC from adipose diabetics for cell therapy. NOX-induced ROS reduces the (longterm) proliferative capacity of ASC (Sela, et al. 2015). On the contrary, anti-oxidant pathways e.g. regulated via Nuclear Factor E2-related factor (Nrf2) cross-talk with pro-inflammatory NFκB signaling which balances ROS responses in ASC (Chen, et al. 2016). Peterson and co-workers, report that acute hypoxic exposure of pro-inflammatory-primed (IFNγ) ASC, dramatically increased intracellular ROS, while longterm exposure render these ASC in a pro-angiogenic state (Peterson, et al. 2011). Hypoxic preconditioning alone also promotes survival of MSC in a ROS-dependent fashion (Valle-Prieto and Conget 2010b). This suggests that ROS-based preconditioning of ASC is beneficial for cell therapy, provided the right timing and conditions are determined and selected. In fact, hypoxically preconditioned ASC, accompanied by increased intracellular ROS, promoted survival primary hepatocytes via extracellular matrix-derived signaling, more than via soluble trophic factors (Qin, et al. 2015).

Yet, how does this affect the influence of MSC on exogenously produced ROS or on intracellular ROS production of target cells? For the past decade, results accumulated that MSC harbor anti-oxidative features in rodent models of diabetes, such as diabetic nephropathy, diabetic neuropathy and DR (Ezquer, et al. 2015; Fang, et al. 2012; Ho, et al. 2012; Lv, et al. 2014; Ren, et al. 2013; Sukpat, et al. 2013). For instance, conditioned medium from umbilical cord MSC (UC-MSC) induced the production of anti-oxidant enzymes such as copper

superoxide dismutase, zinc-superoxide dismutase, manganese superoxide dismutase, glutathione peroxidase and catalase by ROS-producing muscle cells (Park, et al. 2016). In addition, oxidative stress provoked by hyperglycemia did not compromise the glucose uptake and only partly abrogated mitochondrial functionality of ASC (Hajmousa, et al. 2016). Furthermore, ASC capacity to function as vascular supportive cells was maintained. One of the strategies that MSC acquire to modulate oxidative stress might involve mitochondria transfer to target cells (Melcher, et al. 2017). In this study, in vitro cultured fibroblasts deficient of mitochondrial complex one (CI) accepted MSC mitochondria. ROS could be further reduced by either treating MSC with TNF-α, or MSC conditioned medium.

The aforementioned studies did not directly evaluate the ROS metabolism in MSC *in vivo*. Reports which address the balance of ROS guided by MSC injection in animal models affected by DR are lacking. Li and co-workers showed a first strategy to attenuate ROS during retinal ischemia. In this study, MSC were engineered to overexpress heme oxygenase-1 (HO-1), which catalyzes heme to biliverdin, free iron, and carbon monoxide (CO) and alleviates the oxidative stress. A reduction in apoptosis caused by the activation of antiapoptotic proteins Akt-1 and Bcl-2 was observed (Li, et al. 2017a). The intravitreal injection of ASC in the STZ-induced T1D mice suppressed oxidative damage in the retina, which indicates that ASC influence ROS levels directly or indirectly (Ezquer, et al. 2015).

Overall these studies showed that MSC are capable to regulate ROS production in a variety of target cells. The molecular mechanisms comprise direct regulation of ROS with anti-oxidant enzymes, normalization of mitochondrial function, reprogramming of glucose metabolism, upregulation of anti-oxidant signaling (Nrf2) as well as

upregulation of MAP kinase signaling. In general, suppression of ROS coincides with suppression of a pro-inflammatory status of cells, although it may occur that inflammation is suppressed while ROS is maintained.

MSC to target inflammation

Chronic low-grade inflammation (also called paraflammation) appears to be involved in the pathogenesis of DR, however it is most likely not involved in incipient stages of DR (Karlstetter, et al. 2015; Wang, et al. 2014). Hyperglycemia and hypertension induce oxidative stress, apoptosis, fatty acid metabolism and AGE/RAGE interactions. Increased levels of pro-inflammatory mediators, such as IL1α, IL1β, IL6, IL8, MCP-1, TNFα, and IL18 and chemokines, such as CCL2, CCL5, CXCL8, CXCL10, and CXCL1 have been detected in the vitreous of patients with advanced DR and proliferative DR, correlated to increased VEGF concentrations (Murugeswari, et al. 2008; Wakabayashi, et al. 2010). Inflammasome activation has been observed both in patients with proliferative DR and in the STZ animal models (Capitao and Soares 2016; Chen, et al. 2017). Resulting macrophage/microglia activation and M1 polarization fosters endothelial, glial and neuronal stress and sometimes degeneration (Abcouwer 2011; Ma, et al. 2014). Modeling endothelial activation, in human umbilical vein endothelial cells, co-stimulated with hyperglycemia and high doses of palmitic acid, to mimic DR-associated glucolipotoxicity, UC-MSCs suppressed inflammation (An, et al. 2016). Both NFκB activation was suppressed as well as downstream activation of glucolipotoxicity-related TNF-α-induced protein 6 (TSG-6). The endothelial phenotype appeared restored, because tube formation was normalized, but unexpectedly endothelial ROS production was not affected (An, et al. 2016). T cells may rather

play a minor role for local activities, as they are typically not found as infiltrates. Nevertheless, there are some indications, that patients with advanced DR stages show changes in the balance of T helper cell cytokines in serum and vitreous. Furthermore, T cell infiltrates in vitreous of patients with proliferative DR have been observed (Capitao and Soares 2016).

MSC have proven broad immunomodulatory potential in various disease entities involving inflammatory and immune processes. However, since inflammation and immune responses at different stages in the development and progression of DR may play a distinctive role, the functions of MSC in this respect remain open. As mentioned, animal models are limited in modeling all stages of the human disease and thus do not represent the late stage disease in humans. Nevertheless, we would like to speculate on possible contributions of MSC in inflammation and immune responses.

MSC and microglia

Microglia are the resident macrophages of the CNS in the retina. They become activated in the concourse of diabetes (Wang, et al. 2014). This has been observed both in humans and in animal models. Plasticity is a very well-known property of macrophages. In fact, they are able to respond to changes in their microenvironment polarizing towards an M1 or M2 phenotype. M1 macrophages are playing the major role in inflammation; producing pro-inflammatory cytokines and a large quantity of NO, and triggering the activation of the Th1 mediated immune response. On the contrary, M2 macrophages possess an immunosuppressive phenotype correlated to the production of anti-inflammatory cytokines like IL-10 (Gordon and Martinez 2010; Mantovani, et al. 2002). It appears, that in early phases of DR, activated microglia are polarized towards an anti-inflammatory/tolerogenic M2

phenotype, whereas at later stages the balance changes towards pro-inflammatory M1 phenotypes. Wang et al. identified CD74 as a microglia activation marker which increased upon both glucose and methylglyoxal administration in a rat model (Li and Lin 2012; Wang, et al. 2014). CD74 is involved in the formation and transport of MHC/HLA-class II molecules, and is thus important for antigen presentation. Furthermore, it acts as receptor for Macrophage Migration Inhibitory Factor (MIF). Recent data in glioma models suggest that tumor-secreted MIF acting on CD74-activated microglia promotes a shift towards M2 macrophages (Ghoochani, et al. 2016).

So far there is no study published analyzing the interaction of MSC with retinal microglia in the context of DR. One study showed that MSC engineered to express CX3CL1 exert neuroprotective and immunomodulatory function in a model of light-induced retinal degeneration (Huang, et al. 2013). Here microglia activation and migration were reduced in MSC-transplanted animals. Modulation of microglia activation has also recently been shown by Jaimes et al. Here, BV-2, a murine microglia cell line, and primary murine brain microglia were cultured with microvesicles obtained from MSC supernatant. LPS induced activation of microglia was prevented (Jaimes, et al. 2017). This fits to own unpublished data analyzing the effect of human ASC supernatant on primary human macrophages (K. Widmanm, unpublished). We observed a clear reduction of M1 in favor of a M2 polarization. An elegant study conducted by Melief et al. showed how Treg induction (see below) may be dependent on the skewing of monocytes toward a M2 phenotype. They observed that Treg formation was increased in MSC-PBMC cocultures more than MSC-CD4+ cocultures, suggesting the involvement of monocytes in Treg induction. Analyzing the monocyte fraction of cocultures they found that: 1) MSC increased survival of monocytes towards M-CSF

secretion and 2) monocytes from cocultures have a decreased mRNA expression of pro-inflammatory cytokines with a concomitant increase in mRNA expression of anti-inflammatory cytokines and CCL18. CCL18 turned out to be key cytokine in driving Treg generation, suggesting that the modulation of monocytes towards an M2 phenotype may be a crucial mechanism in MSC mediated Treg formation (Melief, et al. 2013).

All these observations found a surprisingly confirmation in a murine animal model of allo- and autoimmunity in the eye. Although it does not belong to the specific topic of this review, we briefly summarize these data as they maybe informative for future studies applying stem cells in DR considering immunogenicity of transplanted cells. In fact, Ko et al. demonstrated that intravenous injections of MSC were able to protect from rejection of corneal allotransplantation and from an experimental autoimmune uveitis (EAU). In the corneal allotransplantation model, MSC prevented histological abnormalities and reduced pro-inflammatory cytokines in the eye. Similarly, in EAU, MSC injection reduced IL2, IL1β, IL6 and TNF-α in the eyeball. A B220+CD11b+ monocyte population found in the lung as well as in blood, spleen and lymph nodes mediated these effects. In addition, increasing levels of Treg where found one day after MSC infusion, and lower level of Th1/Th17 cells were induced in coculture of B220+CD11b+/CD4+ T cells. Collectively these results suggest that monocytes, which were primed by the infusion of MSC, were able to promote Treg proliferation and inhibit the pro-inflammatory phenotype of T cells. More interestingly, intravenous infusion of MSC induced-B220+CD11b+ cells were able to prevent corneal rejection and EAU as well, highlighting the fact that they are crucial in the MSC mediated tolerance (Ko, et al. 2016).

MSC and T-cells

As previously introduced, MSC affect a variety of immune cells and appear to regulate immune responses in favor of a pro-regenerative rather than an adverse injury-response. It is highly speculative whether the MSC effects on T cell responses may contribute to therapeutic efficacy in DR, nevertheless, we will summarize the known and provide possible links to DR.

In diabetes there have been reports, that MSC can exert anti-diabetic effects by affecting T cells. An improvement in the Th1/Th2 balance, decreased Th17 and increased Treg numbers have been reported in models of T1D (Bassi, et al. 2012; Ezquer, et al. 2012; Favaro, et al. 2014). Volarevic et al. have shown a disbalance of T-cell derived IL-1 and its receptor antagonist (IL-1Rα) as a cause of β-cell impairment in T2D. In a mouse model, the β-cell damage has been ameliorated by MSC-derived IL1-Rα (Volarevic, et al. 2010). Whether MSC-mediated modulation of the T helper cell balance could be a MoA in DR has not been investigated yet.

MSC exert a strong suppressive effect on T cell proliferation, when T cells are triggered by different mitogenic stimuli such as PHA or anti-CD3/anti-CD28 monoclonal antibodies (Mohammadzadeh, et al. 2014; Nicola, et al. 2002; Yan, et al. 2014a). On the contrary, they seem to support their survival when T cells are in a quiescent state (Benvenuto, et al. 2007). Mechanisms that regulate the interaction with T cells still need elucidation; however, many studies revealed that both cell-cell interaction and production of soluble factors may drive MSC immunomodulation (Haddad and Saldanha-Araujo 2014). In co-culture with naïve or activated T cells, MSC inhibited T cell proliferation principally by soluble factors. In one study, MSC conditioned media led to a 2-fold increase in apoptosis, while direct contact with MSC

caused cell arrest in G0/G1 phase. The major factors responsible for T cell energy were IL-10 and IDO (Yang, et al. 2009). Yan at al. reported MSC inhibition on activated T cells in both direct and indirect coculture system. Here, the axis PD-1/B7-H1 was partially involved in inducing T cell apoptosis, with a dispensable role of IL-10 and TGF-β in the up-regulation of PD-1 on T cells (Yan, et al. 2014b).

Furthermore, MSC can affect the balance of T helper (Th) cell subsets Th1, Th2 and Th17 cells and can induce Treg cells (Maccario, et al. 2005). Th cells have been classified into Th1 cells, which produce IL2 and IFNγ and are involved in cellular immunity; Th2 cells which produce IL-4, IL-5 and IL-13 and are involved in humoral immunity; and Th17 cells producing the proinflammatory cytokine IL-17. Treg are commonly described as a CD4+CD25+Foxp3+ cells capable of suppressing other immune cells (von Boehmer 2005). They can originate from thymus as a mature subpopulation or be induced from a circulating naïve T cell population (Sakaguchi, et al. 2008). Treg formation seems to be triggered more by soluble factors than cell-cell interaction. A tight balance between Th1/Th2 and Th17/Treg has to be maintained for homeostasis (Zhang, et al. 2014). Generation of ASC-induced functional Treg was observed in coculture by Engela et al. and resulted to be IL-2 pathway-dependent (Engela, et al. 2013). In another study, MSC derived PGE2 was involved in Treg generation, as a PGE2 inhibitor resulted in reduced Fox-P3 mRNA expression in CD4+ T cells (English, et al. 2009). It has been shown that MSC are able to inhibit Th1 and Th17 differentiation, depending on the differentiation stage and coculture ratio (Luz-Crawford, et al. 2013). High levels of PGE2, TGFβ and IL-10 were found in coculture of MSC with differentiated CD4+ T cells, i.e. Th1 and Th17.

There are few indications that T cells may play a role in DR. There are no T cells in the retina, thus effects on T cells may be related to rather systemic (autoimmune) than local responses. Some studies report a dysregulated Th1/Th2/Th17/Treg balance in favor of a pro-inflammatory Th1 and Th17 cytokine profile in serum and vitreous of patients with proliferative DR (Cao, et al. 2016; Takeuchi, et al. 2015). One study analyzed Th1/Th2 cytokines in the serum of 25 healthy subjects compared to 35 T2D patients without DR and 30 T2D patients with DR. In patients with DR the Th1 cytokines IL-2 and TNFα were strongly upregulated, even stronger in the T2D without DR group. Th2 cytokines were lower in T2D patients with DR, whereas T2D patients without DR showed no difference to the control cohort (Cao, et al. 2016). In another study, a pro-inflammatory skewed profile with expanded Th1/Th17 cells and reduced Treg has been observed. It correlated to an increased urine albumin:creatinine ratio indicative of diabetic nephropathy (Zhang, et al. 2014). In an experimental model of STZ-induced diabetes, blockade of IL-23, a cytokine critical for survival and proliferation of Th17 cells, significantly improved structure and tightness of the BRB (Xu, et al. 2015). However, the impact of these findings for human disease are unknown, as this model never develops proliferative DR and has also no relevant BRB disruption compared with humans.

Interestingly Th1/Th2 cytokines have been related to angiogenesis, by either controlling it directly or indirectly by modulating other factors in the micromilieu (Naldini, et al. 2003). Pro-inflammatory Th1 cytokines appear to be inhibitory to endothelial cell and vessel growth, whereas anti-inflammatory T cells appear to be positive regulators of angiogenesis. Since MSC have profound properties in restoring the Th1/Th2 Th17/Treg balance this may be a further mechanism linking immunity and angiogenesis.

MSC, autoantibodies and the complement system

The DR-mediated cell degeneration can induce adaptive immune responses. For instance, auto-antibodies reactive against EC and pericytes have been detected in the serum of patients with diabetes and DR. This correlated to increased concentration of serum complement factors C3a and C5a, but not 4a. These autoantibodies conferred complement-mediated pericyte toxicity (Abcouwer 2013; McAuley, et al. 2014; Paine, et al. 2017). Antibody-injured pericytes were also affected in their efficacy to reduce T cell proliferation (Suzuki, et al. 2009). Upon disease progression, and due to increased pericyte loss, pericyte-reactive autoantibodies become decreased (McAuley, et al. 2014). In vitro, hyperglycemic culture increased the susceptibility of pericytes to become attacked. CD38, a known diabetes-associated autoantigen, was upregulated by IFNγ and TNFα and was involved in antibody-dependent pericyte damage (Suzuki, et al. 2009).

Complement factors 3a and 5a are chemotactic for MSC and can recruit and retain MSC at sites of injury (Schraufstatter, et al. 2009). MSC constitutively secrete the important complement regulatory factor H and constitutively express the three complement regulatory receptors CD46, CD55 and CD59 (Li and Lin 2012; Tu, et al. 2010). Studies showed that MSC can prevent- via factor H- complement activation and cell damage (Li, et al. 2016; Tu, et al. 2010). However, despite expression of these regulatory factors, MSC *per se* can be targeted by the complement system once they are in contact with serum (Li and Lin 2012; Li, et al. 2016). This has to be taken into account when planning a therapeutic intervention with MSC, considering local versus systemic administration.

MSC efficacy in DR

Various adult cell therapies have been explored, i.e. CD34 hematopoietic stem/progenitor cells, endothelial progenitor cells and MSC (Lois, et al. 2014; Park 2016). Animal models used to investigate the impact of cell therapy in the context of diabetic retinopathy are summarized in Table 2.

Intravitreal administration of multipotent stromal cells of mesenchymal origin gave encouraging results for the treatment of early phase of DR (Rajashekhar 2014). Vascular leakage, apoptosis and inflammation were drastically downregulated. Ezquer et al. focused on the ROS-induced damage to the neurovascular unit of the retina, to find that retinal ganglion cells death was attenuated and neurotrophic factors such as NGF, bFGF and GDNF increased when MSC were injected (Ezquer, et al. 2016). Interestingly, the ASC did not acquire neural-like or perivascular-like phenotypes.

A similar localization pattern of ASC was also found in another study after intravitreal injection in oxygen-induced retinopathy (OIR) NOD-SCID mice. In this study, DiI labeled ASC were localized alongside the central retinal microvasculature up to eight weeks after injection, adopting a pericyte-like perivascular location and wrapping around vessels. These cells were also positive for the pericytic markers NG2 and alpha-SMA. ASC treatment pre and post induction of OIR reduced avascular areas in the retina, suggesting that even with the time dependent decrease of ASC, modulation of the retinal microenvironment with soluble/paracrine factors may mediate retinal microvessel protection. Of note, having established that ASC can acquire a pericytic phenotype and protect against OIR, the treatment was repeated on Akimba mice, recapitulating the findings of OIR mice (Mendel, et al. 2013).

Tassoni and coworkers addressed the potential risks associated with cell injection. They addressed retinal glial responses (graft-induced reactive gliosis) upon intravitreally injected rat BM-MSC (Tassoni, et al. 2015). Inflammation and extensive reactive gliosis driven by JAK/STAT3 and MAPK cascade were accompanied by macrophage infiltration and retinal detachment upon BM-MSC intravitreal transplantation. Müller cells expressing NGF receptor and neurotrophic tyrosine kinase receptor type 1 (TrkA) were observed in the retinas of rats transplanted with rBMSC. Factors downstream of NGF/TrkA signaling, such as p-PI3K, p-Akt and p-CREB, were also increased in Müller cells (Jian, et al. 2015).

Cell therapy requires knowledge of the retinal pathological microenvironment prior to cells implantation to be able to ensure safety and efficacy. To this regard, the role of pericytes-EC interaction in the retina has been further elucidated. Partial depletion of pericytes resulted in a lack of spontaneous normalization of pericyte-EC associations in adult animals. Moreover, this caused an inflammatory response and macrophages infiltration, ultimately leading to a negative vascular damage loop via VEGF, PIGF and Ang-2 (Ogura, et al. 2017). This study suggests that hyperglycemia alone can initiate the progression of DR, however parallel pathological routes independently and irreversibly damage the retina. In line with this study, the role of ASC-EC communication has been linked to Notch signaling. Specifically, NOTCH2 is a transmembrane ligand which mediates ASC migration and their communication with EC (Terlizzi et al., submitted). Moreover, PDGFRβ downregulation was concomitant to NOTCH2 protein downregulation in ASC. Interestingly, hyperglycemia did not quantitatively influence the expression of Notch ligands and receptors in ASC. Modulation of evolutionary conserved mechanisms, such as Notch signaling, might help to reduce the

proangiogenic capacity of ASC and maintain their regenerative capacity.

In summary, MSC, specifically ASC exert at least a dual role in DR. First, these seem to resume a pericyte function, acquiring perivascular localization and endothelial cell enwrapping. By this, MSC/ASC appears to adopt pericytic regulatory functions, controlling survival and proliferation. Second, they secrete a variety of trophic factors which modulate the local adverse milieu by regulating oxidative stress, inflammation and integrity of the neurovascular unit. With the exception of the immunomodulatory function, the few animal data existing so far documented that MSC can exert the MoA, we proposed herein. Further studies are needed to elucidate efficacy (and MoA), and the safety/risk profile. Although it is expected that it takes its time to get enough knowledge to be able to move from bench to bedside, we think it is imperative to consider important questions:

(1) optimal dose and
(2) route of administration regarding the low survival of transplanted cells,
(3) choice of animal models to model therapy-relevant DR stages (Fig. 1),
(4) autologous or allogeneic approach due to impaired potency of MSCs from diabetes (see below),
(5) safety versus risk of adverse events (cataract, accelerated disease progression due to induced and non-regulated angiogenesis, tumor formation, adverse differentiation),
(6) identification of MoA (Fig. 2) and establishment of potency assays to predict therapeutic efficacy
(7) clinical end points to assess the efficacy of MSC therapy.

Table 2. Effects of injected mesenchymal and adipose stromal/stem cells in animal models relevant to diabetic retinopathy

Animal model	Cell type	Effects	Disease stage	Reference
C57BL6 mice streptozotocin-injection	2×10^5 mouse ASC, passage 2, in 2 µL saline	- reduced retinal ganglion cells (RGCs) loss - do not differentiate into neural-like or perivascular-like cells - increased intraocular levels of neurotrophic factors - reduced retinal oxidative damage - no provasculogenic microenvironment changes induced	NPDR - acellular capillaries - loss of pericytes	(Ezquer, et al. 2016)
C57BL/6 mice -Hes5-GFP transgenic mice -Glia fibrillary acidic protein (GFAP)-STAT3-cKO	Mouse BM-MSC 1×10^5 in 1 µL	-Extensive reactive gliosis and Inflammation - LCN2 production and activation of STAT3 and ERK in retinal Müller Glia	NPDR - vasoregression	(Tassoni, et al. 2015)
oxygen-induced retinopathy (OIR)/ Akimba mouse	hASC, mASC, or hBMSC were suspended in 0.5 to 1.5 uL of PBS and injected into the vitreous of one eye with a 33G Hamilton needle through the pars plana	- ASC migrated and integrated with the retinal vasculature - 16% reduction in avascular area - TGF-β1 enhanced hASC pericyte function	PDR - retinal ischemia - proliferative vascular damage	(Mendel, et al. 2013)
Streptozotocin (STZ) induced diabetic athymic nude rats	ASC 5×10^5 to 25×10^5 of GFP-labeled ASC in 2 µL saline	- improved neuronal function (ERG) - alleviated vascular leakage - alleviated apoptosis - ASC incorporated into the host vasculature	NPDR	(Rajashekhar 2014)
RCS (RCS-Pþ, dystrophic rats with pigment)	1×10^5 CM-Dil-labeled rat BMSC in 3 µl of PBS	- promoted the proliferation and dedifferentiation of Müller cells - Müller cell dedifferentiation via the NGF induced activation of PI3K and its association with the NGF receptor TrkA		(Jian, et al. 2015)
Akimba mouse	mASC, healthy and diabetic mice	- ASC from diabetic sources have an impaired ability to stabilize the microvasculature in diabetic retinopathy	PDR - microaneurysms - leaky capillaries . capillaries dropout	(Cronk, et al. 2015b)

Are ASC impaired by diabetes?

The fact that ASC could be easily isolated in a large quantity and with non-invasive procedure, as well as a relatively easily expansion, makes them an impressive suitable candidate for autologous treatment options (Bieback, et al. 2012; Moonen, et al. 2012; Spiekman, et al. 2017; Wuchter, et al. 2015). However, in a specific disease context like diabetes, which is well known to affect the metabolism and adipose tissue, it is extremely important to assess whether these cells become affected by the disease. In fact, this would implicate a possible failure and/or impairment of the treatment.

There are contradictory data. Some authors report similar functions and potency, whereas others identified differences between healthy and diabetic donors (Davies, et al. 2016; de Lima, et al. 2016; Ko, et al. 2015; Yaochite, et al. 2016). Many studies investigated phenotypical and functional differences between healthy and diabetic ASC, mostly isolated from animal models (Cianfarani, et al. 2013; Cronk, et al. 2015b; Rennert, et al. 2014). Cianfarani et al. observed differences in the expression of ASC markers in the stromal vascular fraction (SVF) isolated from diabetic mice fat pads, suggesting that a depletion of ASC can reflect their reduced survival and the functional impairment in secreting VEGF, HGF and IGF-1. Moreover, skin wounds treated with diabetic SVF resulted in reduced efficacies in wound healing compared to healthy SVF (Cianfarani, et al. 2013).

Rennert et al. isolated ASC from inguinal fat pads of STZ C57BL mice and demonstrated their impaired angiogenic potential both *in vitro* and *in vivo.* In fact, ASC failed to induce tube like structures in coculture with HUVEC, although they assumed a perivascular position. This effect was also recapitulated *in vivo,* where gel plugs seeded with diabetic ASC and implanted in healthy mice, displayed significantly

reduced levels of vascularization compared with healthy ASC. The authors also analyzed the composition of the freshly isolated SVF. As previously stated, they found a depletion of the global ASC population (here described as a CD45-CD31-CD34+ subpopulation of the SVF) (Rennert, et al. 2014).

Similar findings regarding the impairment of the angiogenic potential were also assessed in model of DR (Cronk, et al. 2015a). While healthy ASC were able to improve vascular length density and numbers of branch points in the superficial vascular plexus of the retina of Akimba mice, diabetic ASC failed, although they reached the perivascular location. Analysis of cell bioenergetics revealed that healthy and diabetic ASC had a comparable rate of basal, ATP-dependent, uncoupled and non-mitochondrial respiration.

Systems biologists contributed significantly to our understanding of metabolic shifts in diseases such as diabetes. Mardinoglu and Nielsen found that respiratory metabolism was significantly reduced in abdominal fat tissues of diabetic patients (Mardinoglu and Nielsen 2015). Consequently, oxidated tryalglycerols were linked to slower dynamics of lipid molecules in the patients affected by diabetes. Mitochondrial pyruvate carrier 1 and 2 (MPC1-2) were strongly reduced in diabetic patients. Consequently, acetyl-CoA, central metabolite for biosynthesis of fatty acids, isoprenoids and involved in processes including protein prenylation and N-glycosylation, was also reduced (Pearce, et al. 2013). The authors concluded that limitation of condensation of oxaloacetate (OAA) which in turn, downregulated pyruvate and acetyl-CoA, interrupted tricarboxylic acid (TCA) cycle leading to mitochondrial dysfunction (Mardinoglu, et al. 2014).

The prolonged exposure of ASC to hyperglycemia and the consequences caused by the associated oxidative stress, formation of ROS and AGEs such as inflammation likely contribute to their dysfunction such as their disturbed protective capacity in angiogenesis. Although, the underlying mechanisms are poorly understood, dysfunctional ASC from diabetics could be partially normalized by augmenting their capacity to clear AGEs i.e. via genetical modification with methylglyoxal-metabolizing enzyme glyoxalase-1 (GLO-1). Introduction of GLO-1 augmented the viability, migration and proangiogenic capacity of ASCs from diabetics. Moreover, in an STZ BALB/c mice model of limb ischemia, Glo-1 expressing ASC restored blood reperfusion, with an overall effect comparable to healthy ASC (Peng, et al. 2017).

The alternative to an autologous setting is allogeneic MSCs. In a variety of clinical settings, MSCs are derived from allogeneic donors (without HLA-matching), mostly based on logistic reasons, rarely based on better efficacy (Ankrum, et al. 2014). In a variety of preclinical experiments, human cells have been applied to animal models of DR with signs of efficacy. In these models, no signs of alloreactivity/xenoreactivity have been reported so far. Since MSC application in DR in general is still in its infancy, safety aspects have to be well defined first before discussing autologous versus allogeneic use and HLA-matching or mismatching. As for a proper timing, very early DR preferentially needs systemic rather than local administration, with a safety profile in the range comparable to general antidiabetic and antihypertensive drugs, while routes of administration may change with disease progression (Griffin, et al. 2016). Other aspects such as the use of autologous versus allogeneic use and HLA-mis/-matching may be subordinate.

The future of cell therapy?

As example of novel concepts, the next generation approaches may involve differentiation of cells before transplantation into the retina, as shown for age-related macular degeneration (AMD) (Kuriyan, et al. 2017). Embryonic stem cells (ESC) differentiated to a cone phenotype survived and matured within a model of advanced retinal degeneration. Cell differentiation involved a controlled inhibition of Notch pathway to enhance cone differentiation. Inhibition or activation of specific signaling proteins involved in MSC differentiation might hold the key to effective cell-therapy. For example, retinal cells derived from hESC, migrate, settle and, differentiate into mice retinas. (Lamba, et al. 2009). Furthermore, hESC-derived retinae have been demonstrated to engraft and matured in two different primate models of retinal degeneration (Shirai, et al. 2016). A similar concept, but different approach, seeds mouse induced pluripotent stem cells (miPSCs) cultured in a 3D retinal tissue *in vitro* (Assawachananont, et al. 2014). Subsequent to transplantation into the sub-retinal space, iPSCs were able to reconstruct the outer nuclear layer of the retina in an advanced model of retinal degeneration. However, another study that combined the transplantation of human retinal pericytes (hRPCs) and human BM-MSC in a rat model of retinal degeneration, showed great improvements in the overall health of the retinas. Integration and migration of both transplants increased compared to single cells transplantation. Importantly, activation of microglia and the gliosis of Müller cells were suppressed indicating an effective immunomodulatory effect (Qu, et al. 2017).

Both human and murine autologous cells used for transplantation have been reported to selectively differentiate and integrate in the retinal microenvironment (Hirami, et al. 2009; Ikeda, et al. 2005). Meyer et al.

demonstrated that human iPSC can generate retina-specific cell types, setting the bases for studying molecular mechanisms involved in cell-differentiation (Meyer, et al. 2009).

Besides this targeted differentiation, other approaches investigate whether local cell injection is needed or whether systemic infusion followed by local homing or even systemic effects may be efficacious. In other disease entities, conditioned medium or extracellular vesicles/exosomes were as efficient as direct cell injections (Marote, et al. 2016). Intraperitoneal infusion was able to prevent experimentally induced autoimmune uveitis by altering the Th1/Th17 response (Oh, et al. 2014).

Clinical trials

To date, investigations of MSC therapy in patients with DR are missing. There are a few cell therapy trials investigating intravitreal cell injections. A phase I clinical trial to assess the feasibility and safety of intravitreal injection of autologous CD34+ BM-cells is currently enrolling participants (NCT01736059). A first-in-human study to examine the safety of an intravitreal injection of autologous BM-MSC has been published recently (Satarian, et al. 2017). The patients in this study had advanced retinitis pigmentosa and were treated with 10^6 BM-MSC per 0.1 ml behind the limbus in the phakic and pseudophakic eyes. This pilot resulted in a temporary improvement in perception of light. Three months later, fibrous tissue proliferation was observed with a tractional retinal detachment. Since biopsy could not be obtained from patients, MSC were transplanted in mice to investigate the shift in the cellular microenvironment provoked by the MSC implantation. The authors reported no detection of MSC after ten months. They concluded that MSC lead to macrophage infiltration and retinal folding as reported by others (Tassoni, et al. 2015). Autologous ASC have been

used for injection to a complementary study (Kuriyan, et al. 2017). Although the number of ASC injected was not reported, 60-ml of liposuction aspiration was processed to isolate the stem cells. The resulted pellet was resuspended in platelet-rich plasma and immediately used for intravitreal injection in both eyes. The patients in this study had severe visual loss as well as hemorrhagic retinopathy and retinal detachment. The authors concluded that such a complication had to be analyzed from the point of view of cells' preparation rather than the injection procedure. Because of these negative results from these early clinical trials, cell-therapy research is in need of information with regard to safety for human trials. In this respect, several clinical trials are to be initiated (NCT03011541, NCT01920867, NCT01736059). Furthermore, animal models capable of assessing efficacy and risk profile need to be established and careful studies need to be performed, controlling for benefits versus risks.

It also has to be taken into account at which disease stage therapy shall be undertaken in terms of a benefit-to-risk assessment. Currently, patients at advanced disease stage receive intravitreal drug injections to slow down progression and prevent vision loss. Accordingly, the benefit-to-risk assessment has to weigh the risk of adverse events versus the beneficial effect preventing vision loss. MSC-based cell therapy, however, based on the MoA we summarized above, can be considered as an early treatment option to entirely prevent or delay DR onset and progression to pathological end stages. Here the benefit-to-risk assessment is not yet weighing against vision loss. Accordingly, an extremely careful risk-to-benefit assessment is required, based on more experimental and preclinical models.

Conclusion

To date, MSC belong to the most intensely investigated cell types for cell-based therapies. Due to their diverse - and admittedly still incompletely understood – MoAs (Fig. 2), the efficacy of MSCs is challenged in various randomized clinical trials to treat a plethora of diseases. Overall, these trials are phase I safety trials and report that the use of MSCs is safe. Efficacy, nevertheless, remains to be documented. MSC-based therapy for eye diseases is still in its infancy. Preclinical data suggest beneficial effects in retinopathy, including DR. As mentioned before, more data are needed to be able to perform a thorough risk-to-benefit assessment prior to clinical trials and in bench-to-bedside-and-back approaches. Only these will help to understand potential risks and relevant, and possibly disease-stage dependent mode of action.

This review discusses the potential mode of action of MSC in DR (Fig. 2), highlights that a delicate interplay between the different cell types and the diabetic microenvironment needs to be taken into consideration, and requires appropriate models.

In DR, there is preclinical evidence that MSC

1) adopt pericyte functions regulation vascular proliferation and survival,
2) secrete trophic factors in favor of a proregenerative shift within the micromilieu,
3) reduce oxidative stress.

The regulation of immune/inflammatory responses to expand favorable phenotypes (M2 macrophages and Treg respectively) has not been shown yet and may relate rather to systemic than local effects in the immuneprivileged retina.

The complexity of diabetic microvascular complications, together with the limitation of animal models and, not yet proven safety for human clinical trials, requires a careful analysis from fundamental biology to patients' susceptibility to improve cell therapy.

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Conflicts of Interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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