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Retinal Capillary Basement Membrane Thickening: Role in the Pathogenesis of Diabetic Retinopathy

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Abstract

Vascular basement membrane (BM) thickening has been hailed over half a century as the most prominent histological lesion in diabetic microangiopathy, and represents an early ultrastructural change in diabetic retinopathy (DR). Although vascular complications of DR have been clinically well established, specific cellular and molecular mechanisms underlying dysfunction of small vessels are not well understood. In DR, small vessels develop insidiously as BM thickening occurs. Studies examining high resolution imaging data have established BM thickening as one of the foremost structural abnormalities of retinal capillaries. This fundamental structural change develops, at least in part, from excess accumulation of BM components. Although BM thickening is closely associated with the development of DR, its contributory role in the pathogenesis of DR is coming to light recently. DR develops over several years before clinical manifestations appear, and it is during this clinically silent period that hyperglycemia induces excess synthesis of BM components, contributes to vascular BM thickening, and promotes structural and functional lesions including cell death and vascular leakage in the diabetic retina. Studies using animal models show promising results in preventing BM thickening with subsequent beneficial effects. Several gene regulatory approaches are being developed to prevent excess synthesis of vascular BM components in an effort to reduce BM thickening. This review highlights current understanding of capillary BM thickening development, role of BM thickening in retinal vascular lesions, and strategies for preventing vascular BM thickening as a potential therapeutic strategy in alleviating characteristic lesions associated with DR.

Keywords

BM thickening; Hyperglycemia; Apoptosis; Angiogenesis; DR

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2. Introduction

2.1. Diabetic Retinopathy

Diabetic retinopathy (DR), a prevalent microvascular complication of diabetes, is the leading cause of blindness and vision loss in the working age population (Duh et al., 2017). DR develops in two stages: background DR (BDR) or non-proliferative DR (NPDR) seen in the early stage, and the late stage represented by proliferative DR (PDR) (Cunha-Vaz et al., 2014; Duh et al., 2017; Simo and Hernandez, 2015; Stitt et al., 2016; Tang and Kern, 2011). BDR is clinically characterized by microaneurysms, intraretinal microvascular abnormalities (IRMA), and vascular permeability. The advanced stage PDR is marked by hypoxia, neovascularization, and angiogenesis. PDR develops as a long-term complication of diabetes generally emerging after 15 years of hyperglycemia (Fong et al., 2004). Importantly, hyperglycemia-driven vascular BM thickening is not only the most common histological hallmark in DR but is also present among the two other diabetic microangiopathies, diabetic nephropathy and diabetic neuropathy (Richner et al., 2018; Williamson and Kilo, 1983). The universal presence of vascular BM thickening in diabetic tissues highlights its importance in DR.

2.2. BM overview

BM is a ubiquitous, multicomponent, ultrastructural layer that provides structural integrity to small blood vessels, and displays diverse functions. The composition of vascular BM has been widely studied and the collective findings indicate presence of several key components including collagen type IV (collagen IV), fibronectin, laminin, and perlecan, a BM specific heparan sulfate proteoglycan (HSPG) (Das et al., 1990b; Jerdan and Glaser, 1986) (Table 1). BM components assemble in a highly organized manner several of those through crosslinking to form a continuous layer that serves as a substratum for attachment of endothelial cells on the luminal surface, and pericytes on the abluminal surface of capillaries. Additionally, BM plays a critical role in cell spreading, migration, growth, repair and differentiation (Ffrench-Constant et al., 1989; Furcht et al., 1984; Morrissey and Sherwood, 2015; Podesta et al., 1997; Yurchenco, 2015). At the ultrastructural level, BM exhibits morphologically distinct zones (Carlson and Bjork, 1990), the *lamina densa* representing the more electron-dense central region with *lamina rara interna* facing the cellular side and *lamina rara externa* facing the stromal side. In individuals with long-term diabetes, the BM becomes significantly thickened in microvasculature of all tissues (Kilo et al., 1972; Siperstein et al., 1968; Williamson et al., 1969) (Table 2). It is important to note that the extent to which vascular BM thickness develops varies in different tissues (Cherian 2009).

2.3. BM thickening as a pathogenetic factor – a historical perspective

The term “basement membrane” was first introduced by the great ophthalmologist, Sir William Bowman (Bowman and Todd, 1858). As early as 1921, Henry Wagener and Russel Wilder from Mayo Clinic observed that DR, then referred to as “retinitis of diabetes”, is almost always accompanied by vascular complications of the retina (Wagener and Wilder, 1921). Long before electron microscopy came into vogue, Norman Ashton using light microscopy observed that the BM of retinal capillaries in diabetic subjects were

intensely stained for PAS (Ashton, 1949), offering early insights that abnormal vascular BM thickening is closely associated with DR. One of the first indications that capillary BM thickening plays a critical role in the development of microvascular complications in diabetes came to light when studies using electron microscopy revealed a close association between BM thickening in glomerular capillaries and diabetic patients with glomerulosclerosis (Bergstrand and Bucht, 1957; Farquhar et al., 1959). Subsequently, a study using electron microscopy investigating ultrastructural changes in the diabetic retina revealed significant BM thickening in retinal capillaries of individuals with DR compared to those of non-diabetic subjects, providing further evidence that the thickened retinal capillary BM is a key histological feature of DR (Toussaint and Dustin, 1963). Furthermore, studies investigating microangiopathy in diabetic dogs revealed significant thickening of capillary BMs in the retina, glomerulus, and muscle suggesting that vascular BM thickening is a prevalent characteristic in diabetic microangiopathy (Bloodworth et al., 1969; Bloodworth and Molitor, 1965). Similarly, BM thickening in retinal capillaries of diabetic rodents (Fig. 1), and in other animal models of diabetes has been reported. Importantly, capillary BM thickening was noted prior to the development of microaneurysms, which are the earliest clinical manifestations in diabetic retinas (Bloodworth, 1963), thus highlighting thickened vascular BMs as an initial change in the pathogenesis of DR. Shortly after, Norman Ashton, a well-known pathologist, suggested that the thickened vascular BM is a likely contributor to the development and progression of DR (Ashton, 1974). During this period, diabetes was considered a “basement membrane disease” (Hayden et al., 2005; Williamson and Kilo, 1977). Although, our current understanding of the role of BM thickening in the pathogenesis of DR has advanced significantly, further studies are needed to gain insights into mechanisms underlying thickened BM-driven cellular abnormalities in the pathogenesis of DR.

3. BM components

Vascular BMs are composed of various components including collagen IV, fibronectin, laminin, and perlecan (Gay et al., 1981; Martinez-Hernandez and Amenta, 1983; Martinez-Hernandez et al., 1982) (Fig. 2). According to some investigators these components are present in specific areas of the BM; however, some disagreement exists in their ultrastructural distribution in the BM.

Collagen IV

Collagen IV are exclusively found in the BM, where each collagen molecule is composed of three α-helical polypeptide chains, that are rich in hydroxyproline and hydroxylysine (Marchand et al., 2019; Martinez-Hernandez and Amenta, 1983). In vertebrates, six distinct chains of collagen IV have been identified, referred to as α1(IV) up to α6(IV) chains (Khoshnoodi et al., 2008; Komori et al., 2018). Traditionally, each triple helical collagen IV molecule is composed of two identical α1 chains and one α2 chain (Timpl et al., 1978). More recently, analyses through advancements in ultrastructural assays, different combinations of α chains have been reported indicating three distinct collagen IV heterotrimers, α1α1α2, α3α4α5, or α5α5α6 (Jayadev et al., 2019; Jayadev and Sherwood, 2017).

Collagen IV chains are incorporated into the BM where the collagen network is connected to laminin polymers via linker proteins, nidogen. Collagen IV undergo crosslinking among four triple-helical type IV chains at unique 7S regions at the NH_2 ends (Jayadev et al., 2019; Jayadev and Sherwood, 2017; Martinez-Hernandez and Amenta, 1983; Risteli et al., 1980; Timpl et al., 1982; Timpl et al., 1981). At the opposite ends of the four collagen IV molecules, each -COOH end joins to a -COOH end of another collagen IV tetramer. This arrangement of four collagen IV molecules to one another leads to the formation of a polygonal network representing the framework of a BM. Collagen IV networks also serve as scaffolds, which can bind to other BM components, enzymes, and growth factors (Brown et al., 2017; Parkin et al., 2011; Wang et al., 2008). Compared to other interstitial collagen molecules, collagen IV molecules have fundamental distinctions. Apart from having glycine in every third position in the primary protein sequence, it has no homology with other collagens (Babel and Glanville, 1984; Khoshnoodi et al., 2008; Killen et al., 1988). In addition, both α1 and α2 chains contain several interruptions of the Glycine-X-Y motif (Brazel et al., 1988; Schuppan et al., 1980; Schwarz et al., 1986), which provides flexibility to the macromolecular structure. Notably, mutations in COL4A3, COL4A4, and COL4A5 genes leading to dysfunctional collagen IV synthesis and assembly have been reported to promote Alport's syndrome, characterized by hematuria, nephritis, hearing loss, and even ocular complications (Khoshnoodi et al., 2008; Savige et al., 2010)

Laminin

Laminins represent one of the most prevalent components of BM. In higher organisms, 16 trimeric laminin isoforms have been identified to date with different extents of cell/tissue specificity (Domogatskaya et al., 2012; Hamill et al., 2009; Hohenester and Yurchenco, 2013). Laminins represent a large family of heterotrimeric multidomain proteins that are composed of one of five α chains, one of four β chains and one of three γ chains (Domogatskaya et al., 2012; Hohenester and Yurchenco, 2013; Yurchenco, 2015). The nomenclature for laminins has undergone recent revision where laminin trimers are referred to by their subunit composition with the exclusion of Greek letters, such that α1β1γ1 laminin, then known as laminin-1, is now referred to as laminin-111 (Hamill et al., 2009), whereas α2β1γ3, then known as laminin-12, is now referred to as laminin-213. Further details are well presented in this review by (Hamill et al., 2009). Importantly, laminins exhibit multiple cell-specific functions including cell adhesion, cell differentiation, cell migration, and cell phenotype maintenance (Domogatskaya et al., 2012). At the C-terminal end of the long arm of laminin, α, β, and γ chains assemble via a coiled coil structure, followed by five large globular domains named LG1-LG5, which represent the cellular binding region (Domogatskaya et al., 2012; Hamill et al., 2009; Yurchenco, 2015). The three chains, α, β, and γ, vary in their sizes: 200–440 kD, ~120–200 kD, and ~120–200 kD, respectively. The resultant trimer formed by these three chains (400–800 kD) can link to form a network, which is connected via nidogen to the collagen IV network. While the homology between the tandem repeats of the three chains are similar, a significant difference exists between α chains on one hand and the β and γ chains on the other in terms of their functionality (Domogatskaya et al., 2012).

Perlecan

Perlecan represents a specific heparan sulfate proteoglycan (HSPG) which is present in the vascular BMs, referred to as HSPG2 (Gubbiotti et al., 2017). Perlecan molecules are composed of three long glycosaminoglycan side chains (100–170 nm in length, 65-kD each) that are covalently linked to a protein core of a single polypeptide chain (80 nm in length, 400-kD) comprised of five globular domains (I-V) (Charonis and Tsilibary, 1990; Farach-Carson and Carson, 2007; Gubbiotti et al., 2017; Martinez-Hernandez et al., 1981b; Yamashita et al., 2018). Notably, domain I is responsible for sequestration of growth factors, while domain III and V represent cell surface binding regions (Gubbiotti et al., 2017). Domain IV of perlecan facilitates ECM scaffold formation and maintenance of ECM integrity (Gubbiotti et al., 2017). The glycosaminoglycans are attached to the protein core through a region containing glucuronic acid, galactose, and xylose (Martinez-Hernandez and Amenta, 1983). In the BM, perlecans are most dense in the lamina rara (Ha et al., 2004; Mynderse et al., 1983), and are known to mediate cell attachment (Martinez-Hernandez and Amenta, 1983) and even influence angiogenesis and autophagy (Gubbiotti et al., 2017).

Fibronectin

Fibronectin represents a prominent primary constituent of BM components. Fibronectin is a multidomain glycoprotein that is contiguous to the connective tissue stroma of the BM (Boselli et al., 1981; Martinez-Hernandez and Amenta, 1983; Martinez-Hernandez et al., 1981a). Fibronectin participates in various intra and extra-cellular activities, and exists as two different forms: a soluble form in the plasma and an insoluble form in the ECM of the cell. Soluble fibronectin is produced by hepatocytes, and exists in the blood, saliva, and other body fluids, and participates during blood clotting (Vaca et al., 2020). In parallel, the insoluble form of fibronectin is primarily synthesized by endothelial cells and fibroblasts which ultimately incorporates into the ECM (Vaca et al., 2020). Furthermore, there are three distinct subunits within each monomer; type 1, type II, and type III subunits. Structurally, fibronectin is a dimer consisting of two identical subunits, which are bound together by disulfide bonds located at the C-terminus of each subunit (Miller et al., 2017; Pankov and Yamada, 2002; Vaca et al., 2020). Dimerization of fibronectin chains via disulfide bond is required for its assembly and function, allowing bending of fibronectin to reveal cryptic sites for other fibronectin molecules to attach (Klotzsch et al., 2009). There are 12 type I repeats, two type II repeats, and 15–17 type III repeats, which accounts for approximately 90% of the fibronectin sequence (Pankov and Yamada, 2002). Notably, the 10^{th} type III repeat contains RGD amino acid residues that interact with $\alpha 5\beta 1$ integrin, allowing cell-fibronectin attachment. In addition, the PHSRN residues on the 9^{th} type III repeat synergizes for efficient attachment. Interestingly, fibronectin also undergoes alternative splicing, which are tissue-specific, producing at least 20 distinct isoforms in humans (Chauhan et al., 2004; Schwarzbauer, 1991; White et al., 2008). Primary functions of fibronectin include cellular adhesion, migration, growth and differentiation (Pankov and Yamada, 2002). In order to maintain a highly organized BM structure, fibronectin facilitates specific binding to collagen, heparan sulfate, and other proteoglycans, and is referred to as the “molecular glue” (Hynes et al., 1984).

Nidogen

Nidogen is a 150 kD sulfated monomeric glycoprotein, a component of the BM, which is also commonly referred to as entactin. The “dumb-bell” structure of nidogen consists of an N-terminal globule, which is linked to a C-terminal globule by a stalk. Vertebrates have two distinct nidogens, a shorter 30 nm nidogen-1, and a longer 40 nm nidogen-2 isoform (Ho et al., 2008; Timpl et al., 1983). Notably, the C-terminal globular domain of nidogen has been reported to bind to the center of the “cross-shaped” laminin as well as the triple-helical domain of collagen IV (Aumailley et al., 1989); therefore, nidogen-1 effectively facilitates the noncovalent molecular connections between collagen IV and laminin (Breitkreutz et al., 2013; Dai et al., 2018; Lakshmanan et al., 2020; Wolfstetter et al., 2019).

3.1. BM assembly—BM components are capable of self-assembly as each of the components harbors information needed for binding to specific sites on other macromolecules. The assembly of BM is a multistep process and is facilitated by the binding of laminin to the cell surface via integrins (Li et al., 2005; Li et al., 2002; McKee et al., 2009; McKee et al., 2007; Smyth et al., 1998). The binding of laminins and polymerization of type IV collagen fibrils produce a scaffold of matrix on which other ECM components are assembled in a supramolecular architecture (Kalb and Engel, 1991; Li et al., 2005; McKee et al., 2007). The primary interaction is initiated by the binding of laminin LG domain to integrins, such as α3β1, α6β1, α7β1 and α6β4 integrins (Hohenester and Yurchenco, 2013), as well as sulfated glycolipids, dystroglycan, and heparan sulfate. The assembly of BM is a carefully regulated process, which involves the local degradation of existing BM by matrix metalloproteinases (MMPs) followed by synthesis and accumulation of new BM components. Assembly of the BM is also achieved through interactions between different BM components as well as ECM interactions with cell surface molecules (Yurchenco et al., 2004). Additionally, cell polarity and tissue shape are established during the assembly of BM (Morrissey and Sherwood, 2015).

3.2. BM function—The primary role of the BM is to provide a substratum for cell attachment and act as a physical barrier between different cell types and tissues, thereby maintaining cell shape or size (Timpl et al., 1981). The assembly of the components into their supramolecular architecture involves collagen IV, laminin, fibronectin, and nidogen and other components (Welling and Grantham, 1972). Alterations in these BM components may influence cell behavior including decreased cell attachment (Chakravarti et al., 1990).

The BM also plays a significant role in the selective permeability of molecules to maintain a homeostatic balance between the inner and outer layers of the retinal vasculature. In the retinal capillaries, the inner layer is lined by endothelial cells, which overlay a thin layer of BM. The BM, thus, serves as a substratum for cell attachment, prevents leakage of growth factors, hormones, and polysaccharides out of the blood stream into the connective tissue. In the capillary endothelial BM, the tight junctions that are formed between the endothelial cells in the paracellular region regulate permeability. Collagen IV and laminin have been shown to contribute to the proper assembly of tight junctions, suggesting that the BM contributes to preservation of selective permeability in the retinal capillaries (Jayadev and Sherwood, 2017).

In addition, the BM has been shown to regulate retinal blood flow. Retinal blood flow is regulated, at least in part, by pericyte contractility and relaxation (Trost et al., 2016). When pericytes relax, the pericyte processes spiraling along the longitudinal axis of the retinal microvessels loosen allowing increase in vessel patency. Inversely, when the pericytes contract, the diameter of retinal capillaries is reduced. Through this relaxation and contraction activity mediated by pericytes, retinal blood flow is regulated. Moreover, pericytes as well as endothelial cells contribute to the synthesis of BM components, and loss of these two cell types as seen in DR can promote hyperdilation of the retinal capillaries which could negatively impact retinal blood flow (Trost et al., 2016). Furthermore, the BM has been found to stiffen in high glucose (HG) conditions (Yang et al., 2016), which could alter the elasticity of the vessels, thereby compromising the ability of the pericytes to regulate retinal blood flow (Yang et al., 2016). Taken together, the BM plays an important role in the maintenance of retinal homeostasis, and HG- or diabetes-induced structural and functional alterations in the BM that can fundamentally lead to retinal dysfunction seen in DR.

4. Vascular BM thickening in diabetes

BM thickening is the most prominent histological hallmark of diabetic microangiopathy. BM thickening as measured by the electron microscopy morphometric techniques have shown not only that there is extra accumulation of BM components, but also that morphological changes in diabetes exist as “Swiss cheese-like” vacuolization and deposition of fibrillar collagen within the homogenous structure of the BM. In retinal capillaries of galactosemic rats, not only is the retinal capillary BM thickness increased (Roy et al., 2003), but also “Swiss cheese-like” changes in the vascular BM have been reported (Frank et al., 1983).

In the context of diabetic nephropathy, there is evidence of glomerular capillary BM thickening, at least in part, due to increased synthesis of ECM components, such as collagen IV by podocytes, as well as expansion of the mesangial matrix and thickening of the tubular BM (Tsilibary, 2003). Additionally, a significant increase in ECM levels was noted in glomerular BMs of diabetic rats (Seon et al., 1999). Interestingly, BM thickening in glomerular capillaries is characterised by an atypical form of thickening, forming nodular deposits called Kimmelstiel-Wilson bodies (Tervaert et al., 2010). Of note, glomerular capillaries are also morphologically different than retinal capillaries in that they consist of fenestrated endothelium underlying the glomerular BM, which participates in the regulation of glomerular filtration barrier. While diabetes-induced changes in retinal pericytes primarily affect retinal blood flow, diabetes-induced insult to podocytes can lead to local renal damage leading to microalbuminuria. Retinal vascular leakage mainly results from the failure of the blood-retinal barrier integrity. Tight junctions involving ZO-1, occludins, and claudins are compromised in retinal vascular cells, which leads to excess permeability. Importantly, BM acts as another layer of barrier that participates in the regulation of retinal capillary leakage. Although the BM itself is thickened, the ultrastructural integrity is compromised, in part due to excess crosslinking of ECM components by lysyl oxidase, resulting in increased interfibrillar space between collagen IV tetramers, ultimately promoting excess permeability. In contrast, the glomerular BM of glomerular capillaries becomes leaky, in part, due to at least two structural changes: (1) the BM integrity is compromised, tilting

in favor of leakage, and 2) loss of podocytes can render the slit diaphragm complex between foot processes of podocytes non-functional, contributing to compromised filtration and ultimately microalbuminuria. While changes in the vascular BM in DR and diabetic nephropathy are similar in many aspects, these findings indicate that vascular BM thickening can compromise tissue functionality in different ways.

Diabetic neuropathy, another well-established diabetic microangiopathy, is also characterized by BM thickening surrounding the endoneurial microvessels, which can lead to degradation of tight junctions and endothelial cell hypertrophy, ultimately resulting in the breakdown of the blood-neural-barrier (Richner et al., 2018). Interestingly, capillary BM thickening has also been reported in the stria vascularis of the inner ear in diabetic rats, demonstrating that the thickened BM in the vasculature contributes, at least in part, to hearing loss in diabetic patients (Smith et al., 1995). Diabetic hepatosclerosis, which is a recently reported form of diabetic microangiopathy, is also characterized by hepatic sinusoidal fibrosis and excess BM deposition with notable increase in laminin and collagen IV (Hudacko et al., 2009). Additionally, a study reported that glomerular, muscular, and retinal capillary BM thickening was evident in diabetic dogs (Bloodworth et al., 1969), supporting the notion that BM thickening is associated with diabetic microangiopathy. Vascular BM thickening also develops in various diseases aside from diabetic microangiopathy, such as congestive heart failure (Longhurst et al., 1975), cystic fibrosis (Rodman et al., 1986), collagen diseases, and atherosclerotic vascular disease (Williamson and Kilo, 1983). In macrovessels, in identical twins, a study demonstrated that quadricep muscle BM thickening developed in the diabetic twin compared to the non-diabetic counterpart (Williamson and Kilo, 1983), clearly suggesting that diabetes is a key contributor in promoting vascular BM thickening. Interestingly, several studies attempted to identify a relationship between muscle capillary BM thickening and clinical manifestations of DR; however, these studies failed to reliably use muscle capillary BM thickening for predicting clinically significant DR (Klein et al., 1987; Williamson et al., 1988). Taken together, vascular BM thickening acts differently in different microenvironments, and requires further investigation to elucidate its role in diabetic disease processes.

5. Vascular BM thickening in DR

5.1. Development of BM thickening in diabetes

To investigate the mechanisms by which BM thickening develops, *in vitro* studies were initially carried out. A pivotal study showed for the first time that collagen IV and fibronectin mRNA expression levels were significantly upregulated in human umbilical vein endothelial cells by HG, showing that HG-induced insult alone is sufficient to mimic the effects of diabetes on overexpression of BM components (Cagliero et al., 1988). A follow-up study then demonstrated that HG promotes gene transcription of BM components in a coordinated manner, which requires several days of HG exposure, through effects exerted intracellularly or at the cell-matrix boundary (Cagliero et al., 1991). Furthermore, relative contributions of retinal endothelial cells and pericytes to the synthesis of ECM components were investigated, which showed that pericytes produce ten-fold more fibronectin than that of endothelial cells but approximately equal laminin

production in both cell types under physiological conditions (Mandarino et al., 1993). However, under HG condition, endothelial cells were reported to show a three-fold increase in fibronectin expression while pericytes showed a generalized but insignificant fibronectin upregulation (Mandarino et al., 1993). Interestingly, an experiment using [^{35}S] methionine radiolabelling immunoprecipitation also demonstrated that endothelial cells and pericytes synthesize different forms of fibronectin, with endothelial cells producing a dimer of two subunits each weighing 220 kD whereas pericytes producing fibronectin consisting of multiple subunits, with one predominant form at 225 kD (Mandarino et al., 1993). Both pericytes and endothelial cells synthesize an α- and two β-chains of laminin of similar size but were reported to have differential post-translational modification in each cell type. It is therefore postulated that due to preferential loss of pericytes under diabetic conditions, and since pericytes and endothelial cells contribute different forms and amounts of fibronectin and laminin to the retinal capillary BM, the net outcome results in BM thickening leading to endothelial cell dysfunction seen in DR. Table 3 shows a list of ECM components known to be upregulated in HG or diabetic conditions. Our previous study has also shown that retinal capillaries of diabetic individuals exhibit fibronectin overexpression (Roy et al., 1996). While vascular BM thickening develops in diabetes, it should also be noted that aging promotes BM thickening as well (Bianchi et al., 2016; Nagata et al., 1986). A recent study showed that retinal Müller cells may deposit BM material and contribute to retinal BM thickening (Bianchi et al., 2016).

In addition to increased synthesis of BM components, studies revealed that BM thickening can be attributed to decreased degradation of the BM components under HG conditions. MMPs, or matrix metalloproteinases, are enzymes responsible for degrading the ECM to allow for ECM remodelling in normal conditions (Nagase et al., 2006). MMPs are also regulated by TIMPs, or tissue inhibitors of metalloproteinases, which are endogenous inhibitors of MMPs (Nagase et al., 2006). In physiological conditions, a critical balance exists between the MMPs and TIMPs to promote ECM remodelling necessary for normal maintenance of the BM. In the context of DR, type IV collagenases, MMP-2 and MMP-9, were detected in the vitreous of diabetic patients with PDR (Brown et al., 1994). A later study demonstrated that MMP-2 and MMP-9 are produced by human retinal microvascular endothelial cells (Grant et al., 1998). *In vitro* studies also demonstrated that MMP-2 activity could be elevated in HG conditions in retinal pericytes, and MMP-9 activity could be elevated in human umbilical vein endothelial cells (Tarallo et al., 2010). Taken together, these findings indicate increased activity of MMP-2 and MMP-9 during late stage development of DR. Therefore, it remains plausible that in early stages of DR, degradation of ECM components is decreased ultimately leading to thickening of the BM, while in the later stages of DR, increased local activity of MMP-2 and MMP-9 allows endothelial cell sprouting during neovascularization leading to angiogenesis (Abreu and de Brito Vieira, 2016; Mohammad and Siddiquei, 2012; Naduk-Kik and Hrabec, 2008). In parallel, several studies suggest that levels of plasminogen activator inhibitor-1 (PAI-1), a serine protease, which can lower matrix degradation of BM components such as laminin and fibronectin (Krag et al., 2005; Levin and Santell, 1987), are elevated in retinal microvessels of diabetic subjects (Lorenzi et al., 1998) and in the vitreous of non-proliferative DR patients (Grant et al., 1996). These findings support the suggestion that in early stages of DR, excess synthesis

of ECM components may also be accompanied by decreased degradation, contributing to the overall thickening of the retinal vascular BM.

In contrast to increased MMP activity in late stage DR, a reverse phenomenon occurs in the pathogenesis of diabetic nephropathy where MMP activity is decreased. Both MMP-1 and MMP-3 mRNA levels were significantly reduced in diabetic rat glomeruli concomitant with TIMP-1 upregulation (Nakamura et al., 1994). MMP-7 and MMP-9 activity were also reduced in human mesangial cells grown in HG conditions with corresponding increase in TIMP-1 activity (Abdel Wahab and Mason, 1996). MMP-2 and MMP-9 activity were also significantly reduced in diabetic rat glomeruli (Song et al., 1999). Interestingly, mesangial cells grown in HG medium exhibited reduced expression of membrane type MMP (MT1-MMP), which is responsible for activating MMP-2, with a corresponding decrease in MMP-2 activity despite a two-fold increase in MMP-2 expression (Boucher et al., 2006; McLennan et al., 2000), suggesting that this decrease in MMP-mediated ECM degradation could contribute to mesangial matrix accumulation even in the presence of MMP gene overexpression associated with diabetic nephropathy. These data suggest that there is decreased degradation of ECM in the process of BM thickening, at least in the context of diabetic nephropathy (Garcia-Fernandez et al., 2020).

While BM thickening develops during early DR, a recent study reported that the thickened BM is also characterized by increased stiffening (Yang et al., 2016). This can promote endothelial cell activation and play a major role in retinal inflammation by producing intercellular adhesion molecule (ICAM)-1 and promote leukostasis (Yang et al., 2016). In addition, connective tissue growth factor (CTGF), a regulatory protein known to modulate several growth factors and ECM proteins, plays a role in contributing to capillary BM thickening and pericyte loss in both early and late stages of DR (Klaassen et al., 2015). In PDR, CTGF participates in switching from neovascularization to a fibrotic phase (Klaassen et al., 2015). These studies suggest a possibility that reducing diabetes-induced CTGF overexpression may have beneficial effects in PDR (Klaassen et al., 2015; Yang et al., 2010).

Advanced glycation and oxidative stress are known to contribute to retinal vascular BM thickening in diabetes. Under hyperglycemic conditions, ECM components, such as collagen IV, are subjected to increased levels of advanced glycation, which can promote stiffening and irreversible thickening of the BM (Beltramo et al., 2003; Hayden et al., 2005). A study showed that administration of anti-glycated albumin reduced diabetes-induced retinal capillary BM thickening despite severe hyperglycemia (Clements et al., 1998), suggesting that glycation alone can contribute to the BM thickening process. In diabetic rats, treatment with aminoguanidine, a non-specific inhibitor of advanced glycation end-products (AGEs), led to significant protection against retinal vascular BM thickening (Gardiner et al., 2003b), suggesting that AGE may contribute to BM thickening associated with DR. Oxidative stress has also been implicated as a contributory factor in promoting retinal capillary BM thickening in diabetes (Obrosova and Kador, 2011). Reactive oxygen species (ROS), known to promote oxidative stress, has been shown to induce collagen IV accumulation and ECM fibrosis (Hayden et al., 2005). Taken together, these studies suggest that glycation and oxidative stress are important factors that can contribute to the process of retinal vascular BM thickening associated with DR.

Pro-inflammatory cytokines may also influence BM thickening, at least in part, by promoting increased synthesis of ECM components. Studies have indicated that diabetes enhances TNF-α production in retinal microvascular cells in diabetic retinopathy (Behl et al., 2008; Huang et al., 2011). In addition, in the presence of TNF-α, fibronectin deposition is increased in endothelial cells, which results in substantial accumulation of fibronectin in the ECM (Lee et al., 2019). Furthermore, TNF-α has been found to be localized in ECM under HG condition (Bianchi et al., 2015; Bianchi et al., 2016; To et al., 2013). Taken together, these findings suggest that pro-inflammatory cytokines can participate promoting ECM synthesis and ultimately contributing to the development of BM thickening.

5.2. Hyperglycemia – a key contributor to BM thickening

Diabetes is primarily characterized by chronic hyperglycemia, which has been identified as the principal contributing factor leading to the thickening of BM seen in DR among other diabetic complications (Frank, 1984). In animal models of diabetes, it was reported for the first time that experimentally induced hyperglycemia mediated by STZ can induce or enhance capillary BM thickening in mouse retinas (Beauchemin et al., 1975). Shortly after, Sima et al. demonstrated that STZ-induced diabetic rats exhibited retinal capillary BM thickening, which was completely prevented in animals that received successful pancreatic islet allografts, clearly indicating that hyperglycemic conditions play a causal role in the development of BM thickening in retinal capillaries (Sima et al., 1988). In humans, a novel study demonstrated that collagen IV expression was upregulated in retinas of patients with background DR compared to that in retinas of non-diabetic subjects (Roy et al., 1994), suggesting that BM thickening can be attributed to excess synthesis of specialized components of the ECM induced by hyperglycemic conditions. Another study demonstrated that retinal capillary BM of patients with DR exhibited accumulations of fibronectin and collagen type I, III, IV, and V compared to that of non-diabetic subjects and diabetic patients without DR (Ljubimov et al., 1996). Interestingly, a porcine model of diabetes demonstrated that hyperglycemic condition, but not high fat diet, promotes retinal capillary BM thickness (Hainsworth et al., 2002) supporting previous reports. In addition, retinal capillary BM thickening was also observed in diabetic dogs compared to non-diabetic controls (Gardiner et al., 2003a). Importantly, a key study demonstrated that there was a strong correlation between retinal and glomerular capillary BM thickening in diabetic rats with elevated HbA1c level, as well as fibronectin overexpression in both tissues (Cherian et al., 2009). Moreover, diabetic rats with tight glycemic control showed protective effects against hyperglycemia-induced fibronectin upregulation as well as retinal capillary BM thickening (Cherian et al., 2009). Interestingly, in a primate model of DR, hyperhexosemia-induced marmosets exhibited retinal capillary BM thickening concomitant with the development of acellular capillaries, pericytes loss, macular edema, and microaneurysms, similar to retinal vascular changes seen in human DR (Chronopoulos et al., 2015). Collectively, these findings clearly indicate that hyperglycemia promotes excess synthesis of ECM components and that it is a key contributor to retinal vascular BM thickening seen in DR.

A significant thickening of the retinal vascular BM was observed using light and transmission electron microscopy in diabetic human eyes compared to those of non-diabetic human eyes (To et al., 2013). Moreover, this thickening was also accompanied

by accumulation of fibronectin and tenascin, an ECM glycoprotein, in the retinal vascular BM of diabetic human eyes compared to that of non-diabetic controls using immunocytochemistry and Western blot analysis (To et al., 2013), suggesting that an increase in BM thickness results not only from excess synthesis of BM components but also from induction of diabetes-specific ECM proteins which are not normally found in the retinal vascular BM. Interestingly, a study using electron microscopy reported that thickening of the retinal capillary BM was prominent at the glial aspect of the retina obtained from diabetic human eyes (Bianchi et al., 2016), suggesting that hyperglycemic conditions affect both inner and outer layers of the BM. It was also shown that retinal vascular BM of diabetic patients exhibited collagen accumulation as well as higher abundance of seventeen other ECM-associated proteins, of which most were implicated in complement-mediated inflammatory process in the diabetic retinal vasculature (Halfter et al., 2017). Specifically, the vascular BM of diabetic patients showed presence of C4 and C9, two members of the complement family involved in inflammation, which were interestingly not detectable in that of non-diabetic donors (Halfter et al., 2017). It is also theorized that this increase in complements localized in the retinal vascular BM could contribute to pericyte loss, a characteristic hallmark of diabetic microangiopathy. In addition, the investigators also reported that there was more than a two-fold increase in the thickness of ocular BMs in both type 1 and type II diabetic patients (Halfter et al., 2017), lending further support that hyperglycemia plays a causal role in the BM thickening process.

Vascular BM thickening depends largely on the dynamic interactions of synthetic and degradative enzymes regulating BM components. It is of interest that in smaller animals, the extent of BM thickening in diabetes is less compared to that of larger animals; however, when compared to the baseline BM thickness, the percent change in thickness is similar. For example, in diabetic mice, 3–4 months of hyperglycemic condition promotes significant retinal capillary BM thickening, whereas in rats, it would take a longer duration for the same to occur. Similarly, in cats and dogs, it takes longer about few years for vascular BM thickening to develop, and in non-human primates, such as rhesus macaques and baboons, about 5–7 years (Gibbs et al., 1966; Stout et al., 1986; Thomson et al., 2008). Generally, in humans, vascular BM thickening is evident after greater than 10 years of hyperglycemia (To et al., 2013). Our studies have shown that the extent to which BM thickening develops, even within the same species, is highly regulated by the level of hyperglycemia. We have previously shown that even within the same diabetic animal, vascular BM thickening can develop at different rates in different tissues, such as in glomerular versus retinal capillaries (Cherian et al., 2009). Table 4 presents retinal vascular BM thickening in diabetic animal models.

5.3. Direct effects of BM thickening

BM is involved in regulating multiple cellular functions (Fig. 3), which can be profoundly affected from its change in morphology, in particular, the thickening of the BM. Specifically, in the context of DR pathogenesis, BM thickening can have significant effects on maintenance of BRB characteristics and cell survival. An emerging role of BM in DR that has gained attention is its participation in retinal neovascularization associated with DR. Furthermore, recent studies suggest that vascular BM thickening can impact cell-cell

communication, and thereby modulate cell metabolism and exchange of small molecules, which, in turn, can impact cell survival.

5.3.1. Thickened BM promotes increased permeability and vascular leakage

—Besides its functional role as a substratum for vascular cells in the retinal capillaries, the vascular BM also serves as a selective barrier to regulate capillary permeability (Roy et al., 2010). When vascular BM thickening develops in diabetes, the ultrastructural integrity of the BM is compromised, and can interfere with the exchange of substances between the inner and outer capillary environments resulting in loss of BRB characteristics (Bianchi et al., 2016). This phenomenon may seem paradoxical in that these remodelled BMs appear thickened on close examination but results in leakage. However, this may be due to the thickened BM's loss of its filtering capacity, or "sieving" function (Hayden et al., 2005).

Conventionally, it was thought that compromised tight junctions and increased vesicular transport were the only factors contributing to excess capillary leakage in DR. While compromised tight junctions, or paracellular pathway, (Antonetti et al., 1998; Barber and Antonetti, 2003; Harhaj and Antonetti, 2004) and transcellular pathway mediated by vesicles (Klaassen et al., 2009; Vinores et al., 1998; Vinores et al., 1993) have been identified in promoting vascular permeability in DR, these changes do not fully account for the excess permeability in totality as they only pertain to the cellular layer of the retinal vasculature. To better understand how the thickened BM may promote excess permeability, a study using cells grown in HG for short-term in which no changes in tight junctions or vesicle formation was observed, showed ECM upregulation concomitant with increased cell monolayer permeability in these cells (Chronopoulos et al., 2011). Thus, this finding demonstrates that excess ECM accumulation can contribute to increased permeability. This observation was further supported by studies in which reducing collagen IV, fibronectin, and laminin resulted in preventing HG-induced cell monolayer permeability and diabetes-induced vascular leakage in rat retinas (Oshitari et al., 2005; Oshitari et al., 2006). These findings indicate that the thickened BM contributes to the development of excess permeability seen in DR.

To better understand how the thickened BM promotes leakiness and excess permeability, the relationship between BM components and tight junctions is of high interest. It has been reported that vascular BM components laminin and collagen IV are critical for proper formation of tight junctions between endothelial cells and for the maintenance of transendotheilal electrical resistance in the blood-brain barrier (Jayadev and Sherwood, 2017). Therefore, it is plausible that excess synthesis of BM components could fundamentally disrupt the natural formation of tight junctions and thus compromise the blood-retinal barrier leading to increased permeability. In addition, it has been reported that alteration in collagen IV and fibronectin can modulate levels of gap junction protein Cx43 (Moon et al., 2011), which is closely associated with the functionality of tight junction proteins ZO-1 and occludin (Tien et al., 2013). Specifically, siRNA-mediated Cx43 downregulation resulted in reduced expression of ZO-1 and occludin in retinal endothelial cells. These findings suggest that excess collagen IV and fibronectin, and the resultant thickened BM may disrupt tight junctions and compromise barrier characteristics, at least in part, through decrease in Cx43 expression (Sato et al., 2002).

The breakdown of the BM's ultrastructural integrity has also been implicated as a key factor in contributing to excess permeability. For proper development and maturation of the BM, collagen fibrils undergo post-translational modification during assembly process and integration into the ECM mediated by lysyl oxidase (LOX), a cross-linking enzyme that aids in the formation of covalent cross-links in the ECM (Rucker et al., 1998). While LOX-mediated cross-linking activity is normal in physiological states, studies have suggested that excess cross-linking in pathological states could lead to formation of overly compact collagen fibrils (Grant et al., 1997; Ortolan et al., 2008), resulting in increased interfibrillar space and ultimately promoting excess permeability. This was further confirmed in retinal endothelial cells grown in HG condition in which elevated LOX expression and activity resulted in excess cell monolayer permeability (Chronopoulos et al., 2010). Similarly, LOX upregulation was noted in retinal microvessels of diabetic rats with corresponding increase in retinal vascular leakage (Chronopoulos et al., 2010; Song et al., 2018). Interestingly, when cells grown in HG medium were transfected with LOX siRNA or treated with β-aminopropionitrile to block LOX expression or activity, respectively, HG-induced cell monolayer permeability was reduced. In addition, diabetic rats that received monthly intravitreal injections of LOX siRNA spanning three months displayed reduced retinal vascular leakage (Song et al., 2018). In an animal model of diabetes using LOX heterozygous knockout mice, we observed that diabetic LOX heterozygous knockout mice exhibited reduced LOX expression as expected but also these animals showed decreased retinal vascular leakage compared to that of diabetic wild type mice (Kim et al., 2019b). Taken together, these findings suggest that abnormal LOX overexpression and excess cross-linking activity could compromise cell barrier functional integrity. Importantly, a recent study provided evidence for abnormal LOX upregulation and its involvement in PDR that shed light to its pathological role in human DR. Specifically, upregulation of LOX expression was found in vitreous of diabetic subjects with advanced DR (Subramanian et al., 2019). This finding showed for the first time that increased LOX levels in the diabetic retina may promote PDR and raises the prospect of targeting LOX overexpression for treatment of PDR.

Studies have also investigated perlecans as a potential contributor to increased permeability seen in DR. Perlecan, an integral component of the BM, is responsible for regulating charge-selective properties pertaining to filtration of polyanions. Therefore, a proposition dubbed the Steno hypothesis stipulated that changes in HSPGs can negatively impact the barrier properties of the BM and compromise charge-specific permeability of molecules (Deckert et al., 1989). A study showed that changes in HSPGs localized to the rabbit glomerular BM are correlated with loss of charge selectivity, and hence increased glomerular permeability, in an animal model of nephropathy (Groggel et al., 1988). In the retina, thickening of the capillary BM was linearly correlated to reduction in anionic site density in diabetic rat retinas, suggesting that diabetes leads to loss of perlecan in the retinal vascular BM and that this may play a role in compromising vascular permeability (Chakrabarti et al., 1991). Moreover, diabetic rat retinas exhibited decreased protein synthesis and mRNA expression of perlecan (Bollineni et al., 1997), suggesting that reduction in perlecan synthesis could potentially account for the apparent loss of anionic sites in retinal vascular BM and contribute to increased capillary BM seen in DR. However, in a study using human donor samples,

it was ultimately determined that increased microvascular permeability seen in DR was not attributed to changes in the expression of perlecan, but rather due to elevated levels of VEGF that promoted overexpression of a perlecan side chain (Witmer et al., 2001). Collectively, these findings suggest that increased retinal vascular permeability in DR is a complex process involving the abnormally thickened BM, where disruption of the BM composition impacts interactions among the BM components. Further studies are needed for better understanding of BM component interactions and their role in the vascular leakage associated with DR.

5.3.2. BM produced under HG condition promotes apoptosis—Studies show that ECM accumulation produced by endothelial cells in HG condition or the thickened BM in diabetes can have detrimental effects on cell survival and barrier characteristics (Chronopoulos et al., 2011; Roy et al., 2011; Roy et al., 2003; Trudeau et al., 2011). To determine whether HG-induced excess ECM triggers apoptosis, matrix laid down by RRECs grown in normal or HG medium were tested. Cells plated on ECM laid down by HG cells (HG-matrix) showed a significantly higher number of apoptotic cells compared to cells grown on N-matrix (337.5±33% of control, $P<0.05$, Fig. 4). These findings suggest that ECM laid down by cells in HG media is abnormal and is capable of promoting apoptosis. Additionally, while LOX is conventionally responsible for crosslinking ECM components for the development and maturation of the BM, studies suggest that excess LOX can also promote apoptosis (Kim et al., 2019a; Kim et al., 2019b; Kim et al., 2017; Song et al., 2018). However, further studies are needed to better understand how the thickened BM and abnormal LOX expression are involved in pro-apoptotic signalling associated with the development and progression of DR.

5.4. Indirect effects of BM thickening on cellular functions

5.4.1. BM alterations and its effects on cell-cell communication—The effects of BM thickening on structural changes are accompanied by functional changes. While BM serves as a substratum for endothelial cell and pericyte attachment and regulates blood-retinal-barrier, it can have a profound regulatory role on cell survival. In DR, HG- or diabetes-induced downregulation of Cx43 has been shown to contribute to the development of vascular lesions in the diabetic retina (Bobbie et al., 2010; Roy et al., 2003; Tien et al., 2014).

BM components, fibronectin, laminin, and collagen IV, have been shown to regulate Cx43 expression and gap junction intercellular communication (GJIC) activity in various cell types. In granulosa cells, when Cx43 was downregulated, a reciprocal effect was seen wherein ECM components, fibronectin, collagen IV, and laminin expression were increased within granulosa cell layers in late stages of follicular atresia (Huet et al., 1998). In alveolar epithelial cells, laminin-332 has been shown to alter Ca^{2+} exchange via gap junction channels, which resulted in Cx43 downregulation and cell morphology change (Isakson et al., 2006). A study using Cx43 knockout mice showed increased collagen expression in late phases of granuloma organization (Oloris et al., 2007), indicating that accumulation of BM components is associated with reduced Cx43 expression. A similar finding reported that increased collagen secretion reduces Cx43 expression in tenocytes

(Waggett et al., 2006). Importantly, in keratinocytes, Cx43 downregulation using antisense oligonucleotides led to a significant increase in collagen type I mRNA and protein expression, further supporting a clear association between Cx43 and BM components (Mori et al., 2006). Cell-cell communication is essential for cell survival and cell growth (Li and Roy, 2009; Roy et al., 2017). Cx43-mediated GJIC is one of the processes through which cell-cell communication is facilitated in the retinal vasculature. A study from our lab showed that HG-induced upregulation of laminin and collagen IV decreases Cx43 expression in microvascular endothelial cells (Pinheiro and Roy, 2007), suggesting that ECM accumulation can impact Cx43 expression. Importantly, reducing HG-induced fibronectin and collagen IV overexpression in retinal endothelial cells positively impacted Cx43 expression, upregulating Cx43 to near normal levels and thereby restoring GJIC (Moon et al., 2011). Such improved intercellular communication was shown to prevent HG-induced apoptosis (Moon et al., 2011). Overall, these reports indicate that HG-induced overexpression of fibronectin, laminin, and collagen IV may influence Cx43 expression and GJIC activity, and thereby compromise vascular BM function towards vascular cell loss seen in DR.

There is a growing realization that in DR pathogenesis, the breakdown of neuronal components along with the vasculature are involved, and that the inter-dependence of endothelium, pericytes, Müller glia, neurons, microglia, perivascular immune cells, and the vascular BM are disturbed as part of the neurovascular unit (NVU) (Simo et al., 2018). In the retinal environment where the vascular BM serves as a common barrier between Müller cells and pericytes, the exchange of critical cell survival factors can be hampered (Muto et al., 2014). Importantly, in the context of cell-cell interactions, HG-driven altered expression of BM components is known to influence CX43 expression (Roy et al., 2017), which can compromise intercellular communication. In addition, aquaporin 4 and Kir4.1 are critically altered in the diabetic retina (Daruich et al., 2018; Zhang et al., 2011), which could negatively impact cell-cell exchange across a modified BM within the neurovascular unit in the diabetic retina. While gap junctions have been observed between Müller cells (Nishizono et al., 1993), currently, it is unclear if BM thickening between the Müller glial end feet and vascular BM plays a role in the pathogenesis of DR. Growing evidence also suggests that neuroprotective drugs may prevent retinal vascular leakage in experimental diabetes (Hernandez et al., 2016; Hernandez et al., 2017; Hernandez et al., 2013); however, whether this effect is mediated by changes in the vascular BM remains to be elucidated. Recent studies indicate that retinal glial cells may be involved in promoting retinal capillary BM thickening (Bianchi et al., 2016; Coorey et al., 2012; Feher et al., 2018; Lee et al., 2010). Notably, a study using electron microscopy demonstrated that BM thickening develops primarily at the glial aspect in the human diabetic retina, and that retinal Müller cells also contribute to excess deposition of ECM components, leading to thickened BM in diabetic human retinal capillaries (Bianchi et al., 2016). These findings highlight vascular BM thickening as a product of pathogenetic cellular processes including those of the NVU in the diabetic retina and underscore the need for further investigation into contributory factors influencing BM thickening.

The vascular BM is also known to sequester growth factors. It has been reported that the vascular BM, namely HSPGs, can sequester growth factors, such as basic fibroblast growth

factor (bFGF), a heparin-binding angiogenic protein, outside endothelial cells (Vlodavsky et al., 1987). A follow-up study further demonstrated that the vascular BM can deposit up to 30% of cellular bFGF into the ECM network, which can then be released in the presence of heparin or upon proteolytic degradation, suggesting that the BM serves as a storage for growth factors which can be released upon BM dissolution (Folkman et al., 1988). In addition, fibronectin and laminin, key components of the BM, have been shown to sequester angiogenic growth factors into the ECM (Bhatwadekar et al., 2008; Ishihara et al., 2018). These findings support the possibility that in later stage DR, as the thickened BM undergoes local dissolution, angiogenic growth factors originally sequestered in the BM become released, initiating the process of neovascularization (Forrester et al., 1993). Interestingly, a study showed that nitritemodified ECM, a model of Bruch's membrane thickening in age-related macular degeneration, led to accelerated release of growth factors such as VEGF and PEDF in the overlying retinal pigmented epithelial cells (Fields et al., 2017), suggesting that the altered BM can affect cellular exchange of trophic and vasoactive growth factors. Whether similar mechanisms occur in the diabetes-modified thickened retinal vascular BM is currently unclear and requires further investigation.

5.4.2. Effects of BM thickening on cell signalling—There is no longer any doubt that interactions between cells and the ECM initiate a flow of information that acts to regulate many fundamental processes throughout development, including cell migration, organ formation, and growth and differentiation of various cell types (Adams and Watt, 1993; Hay, 2013). The BM also relays critical signals to the cell that affects cell viability, cell migration, and adhesiveness. Integrins, a family of transmembrane receptors consisting of α and β subunits that form heterodimers (Hynes, 1992), are the main conduits mediating cell-matrix interactions but other non-integrin receptors such as dystroglycan, syndecans and discoidin domain receptors have also been reported (Short et al., 1998). However, in the context of DR, integrins are of particular interest.

Crosstalk between integrins and ECM allows cells to respond to biochemical and mechanical cues from ECM to the cell and from the cell to the ECM. The α subunits of integrins are specific and can bind to the Arg-Gly-Asp (RGD) motif of fibronectin. This motif was later reported in several other ECM proteins (Ruoslahti, 1996). The presence of RGD motif regulates specific spatial distribution of integrins necessary for cell response (Chen et al. 1997). Similarly, the α3 subunit of integrin is involved in communicating signals specifically from collagen IV to and from the cells. When BM composition is altered in HG condition, the communication between ECM components and the actin cytoskeleton via integrins is compromised. It has also been reported that excess HG-induced upregulation of integrins can have detrimental effects including firmer cell-matrix adhesion and compromised cell survival.

BM components have also been reported to influence cell shape as reported recently (Randles et al., 2020). Cells plated on Collagen IV may promote a round morphology, with large adhesion complexes rich in actin-binding proteins, whereas, cells plated on laminin can assume a polygonal shape with small adhesion complexes. To what extent these shapes are dictated by BM ligands and how they influence cell signaling and functionality are currently under investigation.

Cooperation between integrins and soluble mitogens is essential to allow efficient propagation of signals to downstream kinases (Short et al., 1998). Specifically, α5β1 integrin-dependent adhesion of endothelial cells has been reported to promote tyrosine phosphorylation of important cellular targets including focal adhesion kinase and paxillin (Short et al., 1998). Anchorage to the ECM through α2, α3, and α5 integrins has also been shown to be necessary for efficient MAPK activation by cytokines in endothelial cells (Davis and Senger, 2005). It has been reported that β1 integrins communicate cell-matrix signaling through focal adhesion kinase and integrin-linked kinase, which then modulate MAPK and PI3K/AKT signaling pathways (Davis and Senger, 2005). Of particular note, it has been indicated that integrins α1β1 and α2β1 play a role in the regulation of collagen and MMP synthesis and are thus of special importance for ECM turnover or BM degradation (Niland and Eble, 2012). Taken together, these findings highlight the importance of integrins in relaying cellular signaling between the BM and the cell and more studies will be necessary to further elucidate how vascular BM thickening impacts signaling pathways leading to the pathogenesis of DR.

Currently, several clinical trials are ongoing assessing the use of integrin antagonists for treatment of diabetic retinal diseases. In a Phase 2 trial, intravitreal injection of ALG-1001 (Risuteganib), a novel peptide targeting α5β3, α5β5, α5β1, and α5β2 integrins, has shown greater efficacy over Avastin therapy against diabetic macular edema (DME) (ClinicalTrials.gov Identifier: NCT02348918) (Bhatwadekar et al., 2020). Topical administration of SF-0166, an inhibitor of α5β3 integrin, was shown to reduce VEGF-induced retinal vascular leakage in animal studies (Askew et al., 2018), and led to a reduction in retinal thickness with concomitant improvements in visual acuity in 53% of DME patients conducted in a Phase 1/2 trial (ClinicalTrials.gov Identifier: NCT02914613 (Bhatwadekar et al., 2020). Use of another agent called THR-687, an inhibitor of α5β3, α5β5 and α5β1 integrins, led to reduced retinal vascular permeability in animal studies while attaining safety endpoints and showing promising improvements in visual acuity in DME patients in a Phase 1 trial (ClinicalTrials.gov Identifier: NCT03666923) (Bhatwadekar et al., 2020; Hu et al., 2019). The common denominator of these aforementioned clinical trials is the targeting of α5β3 integrin, which has been implicated in activation of VEGF receptor-2 associated with the initiation of neovascularization and angiogenesis (Soldi et al., 1999). Therefore, further studies are necessary to determine whether these integrin antagonists provide beneficial effects against retinal vascular leakage through interactions with the thickened BM associated with DR.

5.4.3. Downregulation of HG-induced overexpression of BM components modulates integrin expression in retinal endothelial cells—Impaired cell-matrix interactions under HG condition have been shown to promote apoptosis (Beltramo et al., 2002). Studies suggest that HG altered cell-matrix interactions may compromise pericyte adhesion to the matrix, and thereby contribute to pericyte loss (Beltramo et al., 2003; Beltramo et al., 2002), and that overexpression of ECM components may play a critical role in HG or diabetes-induced apoptosis (Roy et al., 2015; Roy et al., 2011). Currently, it is unknown whether excess ECM synthesized by cells grown in HG mediates apoptotic cell death through modulation of integrin expression.

Integrins act as the key signalling conduits between ECM and the internal components of the cell. Integrins are transmembrane receptors present as heterodimers and consist of an α and a β subunit (Hynes, 2002). They are widely distributed in various tissues and mediate diverse biological functions, including interactions between cells and matrix via induction of signal transduction cascades regulating among other functions the recruitment of structural and signalling molecules to adhesion sites (Schoenwaelder and Burridge, 1999). Such interactions are mediated through integrin-signalling, which has been shown to regulate ECM synthesis in a feedback dependent manner (Beekman et al., 1997). Addition of fibronectin to chondrocytes increased expression of α5 integrin subunit (Beekman et al., 1997), and synthesis of the α2β1 integrin was selectively upregulated when fibroblasts were seeded on type I collagen gels (Klein et al., 1991). Beekman et al reported that through integrin-mediated mechanism, extracellular collagen accumulation downregulated collagen synthesis in a negative feedback manner (Beekman et al., 1997). These studies suggest that feedback mechanisms are operative between cells and ECM via integrin in various cell types. However, currently it is unknown whether feedback mechanisms are affected by HG-induced modulation of ECM component expression and its corresponding integrin subunit expression in retinal endothelial cells.

Despite the knowledge in aberrant ECM synthesis in diabetes and its contribution in the pathophysiology of diabetic complications, its role in the control of specific integrin subunit content related to retinal vascular cell loss remains to be determined. Having downregulated specific fibronectin and Collagen IV overexpression with antisense oligos (Oshitari et al., 2006; Roy et al., 1999) and siRNAs (Oshitari et al., 2005; Roy et al., 2011), we investigated whether modulation of fibronectin or Collagen IV expression alters expression of corresponding integrin subunits associated with fibronectin or Collagen IV in RRECs, and whether the abnormal ECM produced by cells grown in HG mediates apoptosis by modulating the expression of specific integrin subunits.

Western blot data revealed that cells grown in HG medium exhibited increased fibronectin (218±65% of control, $P<0.05$, Fig. 5) and integrin α5 expression (233±23% of control, $P<0.01$, Fig. 5) compared to those of cells grown in N medium. In cells grown in HG medium and transfected with AS-fibronectin (As-FN) oligos, expression levels of fibronectin (107±42% of control, $P<0.05$, Fig. 5) and integrin α5 subunit were significantly reduced (161±30% of control, $P<0.01$, Fig. 5) compared to that of cells grown in HG condition. Cells grown in HG medium and transfected with random oligos showed no effect in either integrin α5 subunit or fibronectin expression. Results investigating the effects of Collagen IV levels on integrin α3 protein expression through Western Blot analysis revealed that cells grown in HG medium exhibited a significant increase in ColI IV (133.0±17.0% of control, $P<0.01$, Fig. 5) and integrin α3 expression (142.5±13.9% of control, $P<0.01$, Fig. 5) compared to cells grown in N medium. In cells grown in HG medium and transfected with AS-Col IV oligos, Col IV expression (101.6±20.7% of control, $P<0.05$, Fig. 5) and integrin α3 expression (116.5±9.1% of control, $P<0.05$, Fig. 5) were significantly reduced compared to cells grown in HG medium. Cells grown in HG medium and transfected with random oligos showed no effect in either integrin α3 subunit or Collagen IV expression.

Immunostaining data revealed increased fibronectin (132±9% of control, P<0.05, Fig. 6) and integrin α5 in RRECs grown in HG medium (140±11% of control, P<0.01, Fig. 7) compared to cells grown in N medium. α5 integrin immunostaining was markedly reduced (101±10% of control, P<0.05, Fig. 7) in cells transfected with As-FN oligos compared to cells grown in HG condition. Cells grown in HG medium and transfected with random oligos showed no effect. Additionally, increased immunostaining of Collagen IV (124±6% of control, P<0.05, Fig. 6) and integrin α3 (140±22% of control, P<0.05, Fig. 7) was observed in RRECs grown in HG medium compared to cells grown in N medium. In cells grown in HG medium and transfected with AS-Col IV oligos, immunostaining of integrin α3 (105±20% of control, P<0.05, Fig. 7) was significantly reduced compared to cells grown in HG medium. Importantly, reduction in collagen IV overexpression using As-Collagen IV oligos resulted in a significant decrease in the number of TUNEL-positive cells (2.3±0.7) compared to that of cells grown in HG medium (3.7±1.3, P<0.05, Fig. 8) or cells grown in HG medium transfected with random oligos (4.3±0.9, P<0.05, Fig. 8). Similarly, cells grown in HG medium and transfected with As-FN oligos exhibited a significant decrease in the number of TUNEL-positive cells (2.8±0.8, Fig. 8) compared to that of cells grown in HG medium (4.1±0.7, P<0.05, Fig. 8) or cells grown in HG medium transfected with random oligos (3.8±1.3, P<0.05, Fig. 8).

Findings from this study indicate that the ECM participates in cell-matrix interactions via integrins. Since HG or diabetes upregulates fibronectin and Collagen IV with concomitant increase in fibronectin-specific α5β1, and Collagen IV specific α3β1 receptors, a strategy that simultaneously reduces both ECM components and integrin subunits could be useful in thwarting DR progression. As reported in the above-mentioned study, reducing ECM component expression decreases the corresponding integrin, whereas another study reported inhibiting α5 integrin reduces fibronectin accumulation in lung BMs (Lu et al., 2020), suggesting that targeting integrin subunits can modulate ECM levels and thus ameliorate BM thickening. Also, integrin-mediated interactions between the cells and the ECM are critical for cell survival, and abnormal overexpression of ECM components and integrins may compromise such interactions with profound consequences including cell death. Thus, reduction of integrin levels may be beneficial for maintenance of normal interaction between cell and BM.

5.5. Neovascularization involves the local dissolution of BM – a critical step in PDR

BM thickening likely occurs not only due to excess synthesis of ECM components but also due to decreased degradation. Studies were conducted to follow-up on *in vitro* findings on MMPs and their inhibitors, TIMPs, in diabetic human patients. It was first revealed that MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, TIMP-2, and TIMP-3 were present in human vitreous samples (De La Paz et al., 1998; Plantner et al., 1998), suggesting that normal remodelling of the BM occurs regularly even in non-diabetic subjects. In retinas of 12-week STZ-induced diabetic rats, MMP-2, MMP-9, and MMP-14 mRNA expression was found to be upregulated concomitant with increased retinal vascular leakage (Giebel et al., 2005). MMP-9 expression was also significantly elevated in retinal endothelial cells grown in HG condition (Giebel et al., 2005). In addition, cells treated with purified MMP-2 or MMP-9 led to degradation of occludin, a tight junction protein, suggesting that MMPs

disrupt tight junctions and thus facilitate the breakdown of the blood-retinal barrier, leading to vascular leakage associated with DR. Interestingly, in diabetic patients with retinopathy, an elevated circulating level of MMP-9 was observed as well as increased MMP-9/TIMP-1 ratio compared to those of diabetic patients without retinopathy (Abu El-Asrar et al., 2017; Jacqueminet et al., 2006; Jayashree et al., 2018), suggesting that serum levels of MMP-9 could potentially serve as a biomarker of retinopathy in diabetic patients. Furthermore, significant elevation in the plasma level of MMP-2 was noted in type I diabetic patients with proliferative retinopathy compared to those without retinopathy (Peeters et al., 2015). In epiretinal neovascular membranes obtained from human diabetic patients, both pro- and active forms of MMP-2 and MMP-9 as well as high- and low-molecular weight forms of urokinase, a serine proteinase involved in the degradation of ECM, were significantly increased (Das et al., 1999), indicating that degradation of the ECM mediated by these molecules promotes neovascularization clinically seen in late stages of DR. Taken together, these data suggest that plasma levels of MMP-2 and MMP-9 are associated with the severity and progression of DR.

For angiogenesis to occur, local dissolution of BM is essential for new tip cells to sprout and undergo endothelial cell invasion. Participation of MMP-2 and MMP-9, in part mediated by PI3K/Akt and ERK1/2 pathways (Mohammad and Siddiquei, 2012), orchestrates local dissolution of BM allowing the initiation of controlled neovascularization. Studies have shown that diabetes-induced VEGF production correlates with MMP-2 and MMP-9 upregulation indicating that MMPs are closely associated with VEGF-driven angiogenesis. Furthermore, in hypoxic condition, Müller cells produce VEGF, which in turn induces MMP-2 expression and activity in retinal endothelial cells (Rodrigues et al., 2013). Presence of MMP-2 on invading endothelial cell surface has also been reported (Brooks et al., 1998; Kowluru and Mishra, 2017), suggesting that MMP-2 is associated with neovascularization. The involvement of other MMPs in neovascularization has recently been reported showing MMP-10 and MMP-14 to have significant involvement in patients with PDR (Drankowska et al., 2019). While further studies are necessary to better understand the role of different MMPs, findings from these recent reports provide substantial evidence that implicate MMPs as critical players in the angiogenic process seen in late stages of DR.

Uncontrolled neovascularization is the hallmark of PDR. Initiation of neovascularization involves complex interactions of several factors, enzymes, and local dissolution of vascular BM (Fig. 9). In response to hypoxia, VEGF produced by Muller cells activate endothelial cells to release MMP-2, MMP-9, and urokinase, which orchestrate local degradation of the BM, allowing endothelial cells to breakout from vessel lumen, and exhibit sprouting. Sprouting angiogenesis is led by endothelial tip cells with their filopodia, which possess highly enriched actin filaments capable of responding to cues from growth factors. This process is dictated, at least in part, by the binding of growth factors to endothelial cell receptors on pre-existing vessels, and ultimately results in stromal invasion. As cells proliferate, migrate, and lay collagen IV scaffolds, other ECM molecules assemble on the collagen framework in a highly organized manner, eventually leading to the development of a matured BM. Simultaneously, cells keep migrating to the site of angiogenesis, and facilitate the extension and formation of the neovessel. During PDR, fibronectin plays a major role in the assembly of the neovessel BM, which is facilitated in part by PDGF

and TGF-β. However, the neovessel architecture is characterized as aberrant due to altered matrix assembly. Furthermore, PDGF-driven pericyte recruitment (Caporarello et al., 2019) may result in reduced pericyte coverage in the neovessels. Taken together, the aberrant neovessel lacks functional and blood-retinal barrier characteristics contributing to leakiness. Intense research is underway to identify mechanisms underlying regulation of sprouting angiogenesis in diabetes. Specifically, studies have focused on identifying distinct responses by specific endothelial cells involving sprouting. For example, tip cells were found to be capable of sensing and responding to guidance cues from growth factors, such as VEGF (Ruhrberg et al., 2002), exhibiting spatially restricted patterning cues in blood vessel branching morphogenesis. Other studies indicate filopodial extension to be mediated by VEGFR2 expressed on endothelial tip cells (Fruttiger, 2002; Gerhardt et al., 2003). Taken together, neovessels that develop from pre-existing vessels are abnormal and contribute to vascular leakage, retinal detachment, and other retinal complications in DR.

6. BM remodelling – a biomarker of DR

As biomarkers become more available for the diagnosis and identification of DR development and progression, not much is known about the role of abnormal vascular BM and its components as biomarkers in DR.

Effective diagnosis and treatment of DR depends on reliable biomarkers. Hyperglycemia-driven vascular BM thickening is known to play a key role in the pathogenesis of DR (Fig. 10). However, the abnormal levels of BM components have not yet been fully studied as potential biomarkers of DR. A study suggests that collagen IV, a major component of the BM, could serve as a potential biomarker of DR, in that plasma levels of collagen IV was found to be significantly elevated in patients with DR compared to those of diabetic patients without DR (Simo-Servat et al., 2016). Additionally, in retinas of patients with DR, there was evidence indicating excess accumulation of fibronectin and collagen types I, II, IV (α1-α 2), and V in the inner limiting membrane (Ljubimov et al., 1996).

Moreover, age- and gender-adjusted plasma level of fibronectin was found to be increased in diabetic patients with DR compared to that of non-diabetic subjects or diabetic subjects without DR (De Giorgio et al., 1984). Another study demonstrated that collagen IV levels were significantly elevated in serum and in the vitreous samples of patients with DR compared to those of non-diabetic subjects with non-inflammatory retinopathy such as those with macular holes or retinal detachment (Kotajima et al., 2001). Studies have also suggested that serum laminin-P1 levels may serve as a useful biomarker of DR but not in the early stages of DR pathogenesis (Masmiquel et al., 2000; Masmiquel et al., 1999). Furthermore, serum laminin-P1 levels were correlated with an increase in retinal thickness in subjects with type 2 diabetes, suggesting that circulating levels of laminin-P1 may be a useful tool in monitoring early stages of DR (Hernandez et al., 2020). A recent report also found that elevated levels of circulating oxidized low-density lipoprotein immune complexes could serve as a potential biomarker to predict risk for severe non-PDR and PDR (Simo et al., 2013). Furthermore, another study showed that MMPs, zinc-dependent proteinases responsible for degrading ECM proteins, specifically, MMP-2 and MMP-9, were elevated in plasma samples of patients with PDR (Beranek et al., 2008; Maxwell et al., 2001).

As discussed previously, plasma levels of MMP-2 and MMP-9 (Peeters et al., 2015), and urokinase levels in epiretinal neovascular membranes (Das et al., 1999), were found to be significantly elevated in DR patients. However, further studies are needed to definitively establish reliable biomarkers that are clinically indicative of DR.

7. BM thickening – a potential therapeutic target for treatment of DR

Given that current treatments for DR are limited in scope, and that BM thickening is a characteristic hallmark of this disease process, there have been attempts to ameliorate the effects of thickened BM for inhibiting retinal vascular lesions. A study by Gardiner et al. showed that oral administration of Sulindac, a non-steroidal anti-inflammatory drug, prevented retinal capillary BM thickening in diabetic dogs, suggesting that thickening of the BM can be halted in mechanisms independent of the polyol pathway activity, advanced glycation, or oxidative stress (Gardiner et al., 2003a). Another study reported that tight glycemic control with insulin therapy resulted in complete prevention of BM thickening in both deep and superficial capillary beds of the diabetic rat retina, whereas treatment with ponelrestat, an aldose reductase inhibitor, reduced BM thickening in the deep capillary bed but not in the superficial capillary bed of the diabetic retina (Chakrabarti and Sima, 1989). Similarly, tight glycemic control was found to normalize diabetes-induced fibronectin overexpression and also reduce vascular BM thickening in retinal and glomerular capillaries of diabetic rats (Cherian et al., 2009). Interestingly, treatment with another aldose reductase inhibitor, Sorbinil, was found to prevent collagen IV and laminin overexpression and thereby reduce capillary BM thickening in galactose-fed rat retinas (Das et al., 1990a). A different aldose reductase inhibitor, Tolrestat, was found to attenuate glomerular capillary BM thickening (Donnelly et al., 1996) and retinal capillary BM thickening in diabetic rats (McCaleb et al., 1991; Robison et al., 1986; Robison et al., 1983; Robison et al., 1988). While Tolrestat showed beneficial effects in animal models of diabetes, it was deemed ineffective in ameliorating microaneurysms, soft exudates, capillary closures, and neovascularization in patients with DR as part of a clinical trial (van Gerven et al., 1994).

One of the pioneering efforts to modulate abnormal vascular BM thickening in the diabetic retina was the use of antisense oligonucleotides via intravitreal delivery targeting fibronectin overexpression (Roy et al., 1999). This led to downregulation of fibronectin expression, reduced retinal capillary BM thickness, and also led to a decrease in the number of pericyte ghosts and acellular capillaries (Roy et al., 2003). This suggests that targeting BM components, which are overexpressed in the diabetic retina, is at least in part, beneficial towards preventing retinal vascular lesions associated with DR. The antisense oligonucleotides administered via intravitreal injection were shown to localize in the vascular cells of retinal capillaries.

Once it was determined that fibronectin antisense oligonucleotides were effective in reducing fibronectin overexpression and ultimately BM thickness, a more powerful approach to fully reduce BM thickness towards normal level was attempted. To achieve this, a combined antisense oligonucleotide approach was strategized using an antisense oligonucleotide cocktail targeting fibronectin, collagen IV, and laminin, three prominent BM components which are overexpressed in diabetes (Oshitari et al., 2006). This

combined antisense oligonucleotide approach was more effective than a single antisense oligonucleotide approach targeting one component of the BM. Ultimately, the proof of this approach proved effective in reducing BM thickening and preventing retinal vascular permeability (Oshitari et al., 2006). Overall, findings from this study indicate that BM thickening is a potential therapeutic target in DR.

Since DR is a long-term complication, administering intravitreal injections on a regular basis is undesirable. A long-term strategy that will allow longer intervals between intravitreal injections is therefore important. To design such an approach that can have the same type of effect in reducing BM thickening, short interfering RNAs (siRNAs) were developed. siRNAs were considered for this purpose of long-term duration of efficacy because it works on the principle of targeting the RISC complex of the mRNA, disrupting the mRNA, and importantly the siRNA is “re-usable”, as opposed to antisense oligonucleotides that are of “one-time” use. In contrast to antisense oligonucleotides, siRNAs were tested as an alternative modality to reduce BM thickening as they do not require a vector such as that used for antisense oligonucleotide delivery. Importantly, a long-term regimen involving intravitreal injections of siRNA targeting fibronectin was found to be effective in reducing diabetes-induced retinal capillary BM thickening, as well as preventing vascular cell loss (Roy et al., 2011) with no untoward effects. Of note, antisense oligonucleotide administration required monthly intravitreal injections (Roy et al., 2003; Roy et al., 1999) while siRNA administration was efficacious for up to 6 weeks (Roy et al., 2011). As such, the siRNA approach for gene modulation is robust and has recently been preferred over antisense oligonucleotides as a potential treatment modality due to its long duration of efficacy and ease of use.

Interestingly, data obtained from the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial has shown that administration of fenofibrate, a drug designed to treat hyperlipidemia and hypercholesterolemia, markedly blocked the development of PDR and alleviated macular edema in diabetic patients by 30% (Keech et al., 2005; Keech et al., 2007). Although it is well documented that fenofibrate lowers cholesterol by reducing the levels of low-density lipoprotein, very low-density lipoprotein, and triglycerides while increasing levels of high-density lipoprotein, how this drug protects against the progression of non-PDR to PDR is not well known. PDR is characterized by uncontrolled neovascularization accompanied by vascular leakage, at least in part, due to abnormally thickened BMs which contribute to excess leakiness. Studies conducted from our lab have demonstrated that fenofibric acid can reduce HG-induced fibronectin and collagen overexpression (Roy et al., 2015; Trudeau et al., 2011) concomitant with a decrease in cyclooxygenase-2, a pro-inflammatory molecule, as well as improving tight junction functionality by upregulating zonula occludens-1 (ZO-1) expression (Roy et al., 2015). Additionally, retinal endothelial cells grown in HG condition exhibited increased cell monolayer permeability whereas cells grown in HG and treated with fenofibric acid showed reduced cell monolayer permeability, indicating fenofibric acid’s beneficial effects against vascular permeability (Roy et al., 2015). Moreover, in an *in vivo* environment, oral administration of fenofibrate halted retinal capillary BM thickening, downregulated VEGF and ROS expression, and prevented retinal vascular permeability in diabetic rats (Li et al., 2018), suggesting that fenofibrate can preserve BM functionality and prevent retinal

vascular leakage associated with DR. Taken together, while further studies are necessary to better characterize fenofibrate efficacy and other gene modulatory strategies in human DR, targeting abnormal vascular BM thickening holds promise amidst the growing need for therapeutic strategies to treat DR.

8. Conclusions

Novel targets associated with BM thickening are being assessed for better understanding of the pathogenesis of DR. In particular, LOX has gained attention for its abnormal levels and activity in hyperglycemic condition and for its unusually high level in vitreous samples of patients with PDR. Findings support the possibility that altered LOX levels promote BRB breakdown and retinal vascular cell loss associated with DR. Based on current studies LOX may be useful in accurately classifying disease stage of DR patients and designing therapeutic strategies accordingly, and this raises the prospect of targeting abnormal LOX overexpression as a therapeutic target for PDR treatment.

A balance between ECM formation and the level of expression of the LOX isoforms is critical, as factors that disrupt this balance could lead to BM thickening and compromise BM function. Intense research is currently underway attempting to establish if a link exists between LOX isoforms and the early stage of DR. Further studies are needed to determine if pharmacological inhibition of LOXs is feasible in clinical practice to slow the progression of DR.

The role of integrins in mediating signals from ECM to intracellular action and vice versa are critical in understanding how abnormal BM mediates changes to its microenvironment and affects cell function and survival. Collagen is known to activate $\alpha 2\beta 1$ integrin-regulated signal transduction pathway through focal adhesion kinases, and signals that sustain cell survival and initiate cell proliferation are elicited by a synergistic action of growth factors and ECM components. Anchorage of cells with their surrounding ECM through integrins is necessary for MEK/MAPK and PI3K/Akt driven pathways and cell survival. Thus, maintenance of proper ECM structure and function could prove to be a therapeutic target for supporting growth factor action and importantly, cell survival.

Our current findings also highlight an association between altered expression of ECM components and its influence on Cx43 expression in mediating apoptosis in retinal vascular cells under HG condition. Thus, identifying mechanisms underlying HG-induced changes in ECM expression and its effects on cell-cell communication may provide novel insights into the deleterious role of abnormal BM thickening in DR.

Vascular BM thickening may not be the only contributory factor to the pathogenesis of the vasculature in tissues targeted by diabetes (Bae et al., 1987; Weynand et al., 1999). Hyperglycemia-driven changes could be quite complex as it is still unknown why some individuals with 50 years of diabetes have not developed any pathological microvascular changes (Sun et al., 2011). Whether tissues have genetic protection against certain pathological changes are not well understood. Some studies indicate that, for example, retina, kidney, and nerves succumb to pathological changes from hyperglycemia, while

other tissues are spared against hyperglycemia-induced insult. It is unclear why no lung complications are associated with diabetes despite pulmonary capillaries exhibiting BM thickening. While BM thickening clearly plays an important role in the development and progression of DR, other pathological factors are at play in contributing to the development of characteristic lesions associated with DR (Duh et al., 2017; Stitt et al., 2016). Biochemical mechanisms underlying the development of BM thickening are now better defined and the detrimental effects of the thickened vascular BMs are better understood during the development and progression of DR - the challenge now is to test whether preventing vascular BM thickening has potential merit in treating DR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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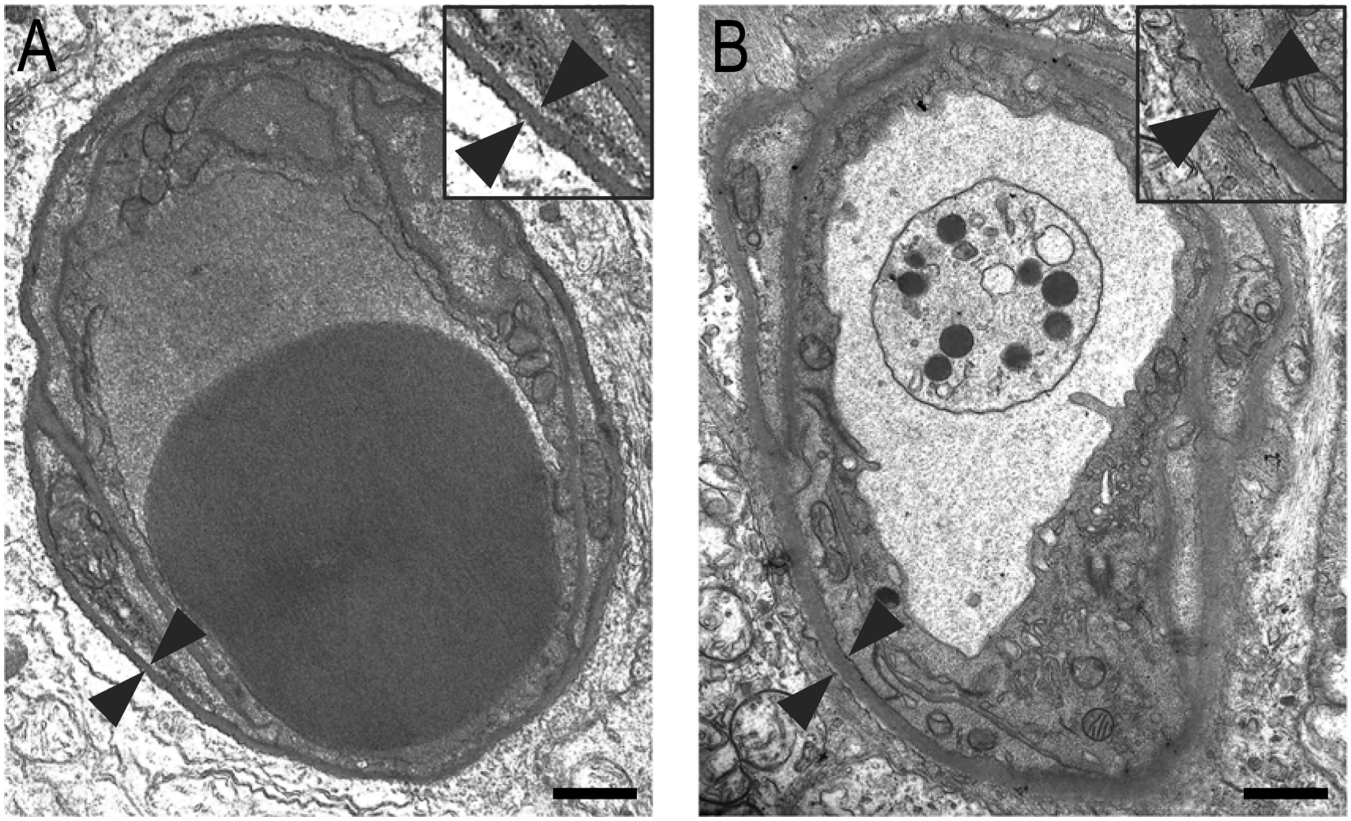


Figure 1. Transverse section of a retinal capillary from a normal rat and a diabetic rat.

Compared to retinal capillary BM of the (**A**) normal rat, note significant thickening of vascular BM in the (**B**) diabetic rat retina. Arrowheads indicate a representative area of the retinal capillary BM from outer plexiform layer. Insets represent enlarged view of corresponding fields. Scale bar: 1.0 μm.

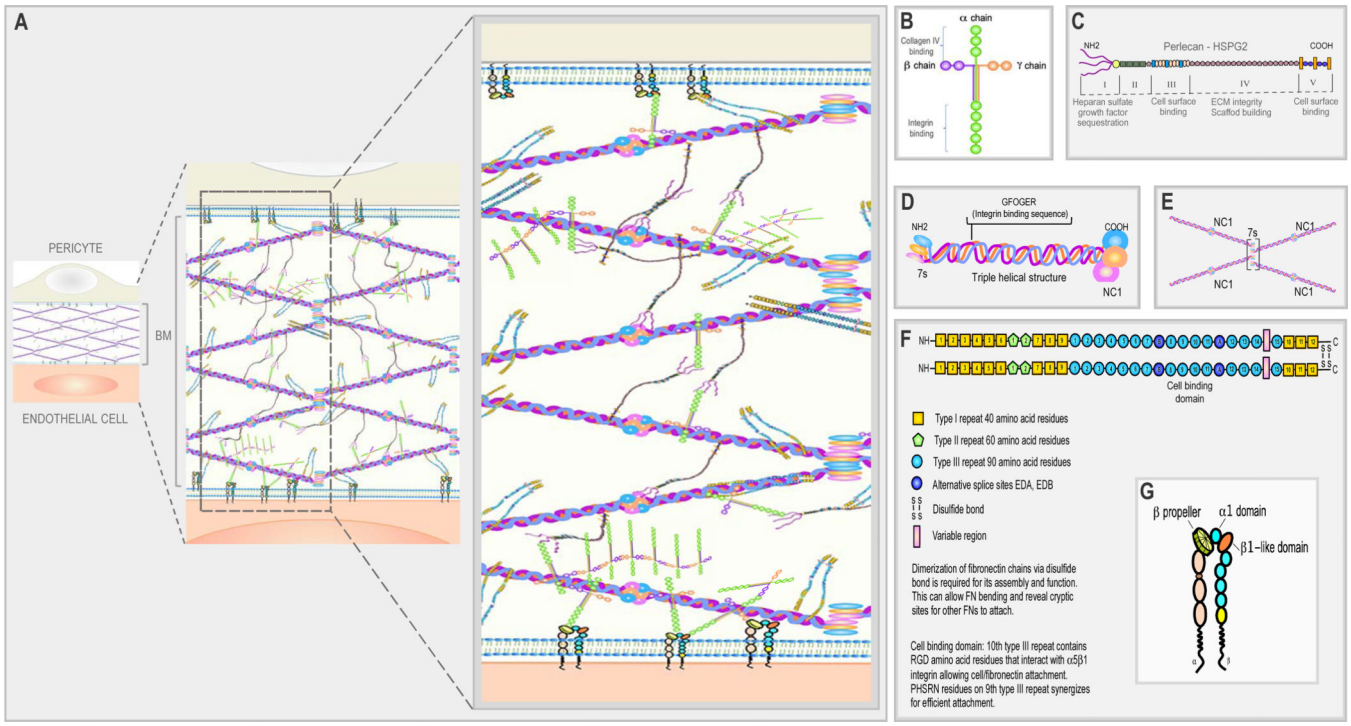


Figure 2. Schematic illustration of retinal vascular BM structure and its components.

(A) BM is a highly organized structure composed of various components including collagen IV, laminin, fibronectin, and HSPG (perlecan). **(B)** General structure of laminin and its domain-binding sites. The NH2 end of laminin is represented by three “arms” of α chain, β chain, and γ chain, followed by a coiled-coil structure and five large globular domains at the COOH end representing the integrin binding region. **(C)** Structure of perlecan, a BM-specific HSPG. Perlecan has five globular domains, which are connected by a protein core of a single polypeptide chain. The NH2 end of the first globule (domain I) is attached to three HSPG side chains responsible for sequestration of growth factors. Domains III and V represent cell surface binding regions while domain IV facilitates ECM scaffold formation and maintenance of ECM integrity. **(D)** Triple helical structure of collagen IV. A protomer of collagen IV is made up of three α-helical polypeptide chains with different combinations; most commonly two α1, and one α2 chains, each with a 7s domain at the NH2 end and a NC1 domain at the COOH end. **(E)** Formation of collagen IV tetramer: four triple-helical collagen IV molecules undergo cross-linking at the 7s domains, while the NC1 domains dock to another set of NC1 domains, forming a NC1 hexamer and the formation of a “chicken wire” network. **(F)** Fibronectin exists as a dimer consisting of two similar subunits, which are bound together by disulfide bonds located at the COOH end of each subunit. Dimerization of fibronectin chains is required for its assembly, function and bending that allows cryptic site for other fibronectin polypeptides to attach. Each subunit has 12 type I repeats, two type II repeats, and 15–17 type III repeats. These domains represent specialized areas for cell binding including RGD sites for integrin binding. **(G)** Schematic diagram of integrin structure: integrins are transmembrane receptors that consist of one α and one β chain forming heterodimers. The “coil” structure represents the transmembrane domain

while the “tail” represents the cytoplasmic domain. The specificity of integrins binding to their ligand is primarily conferred through the α chain.

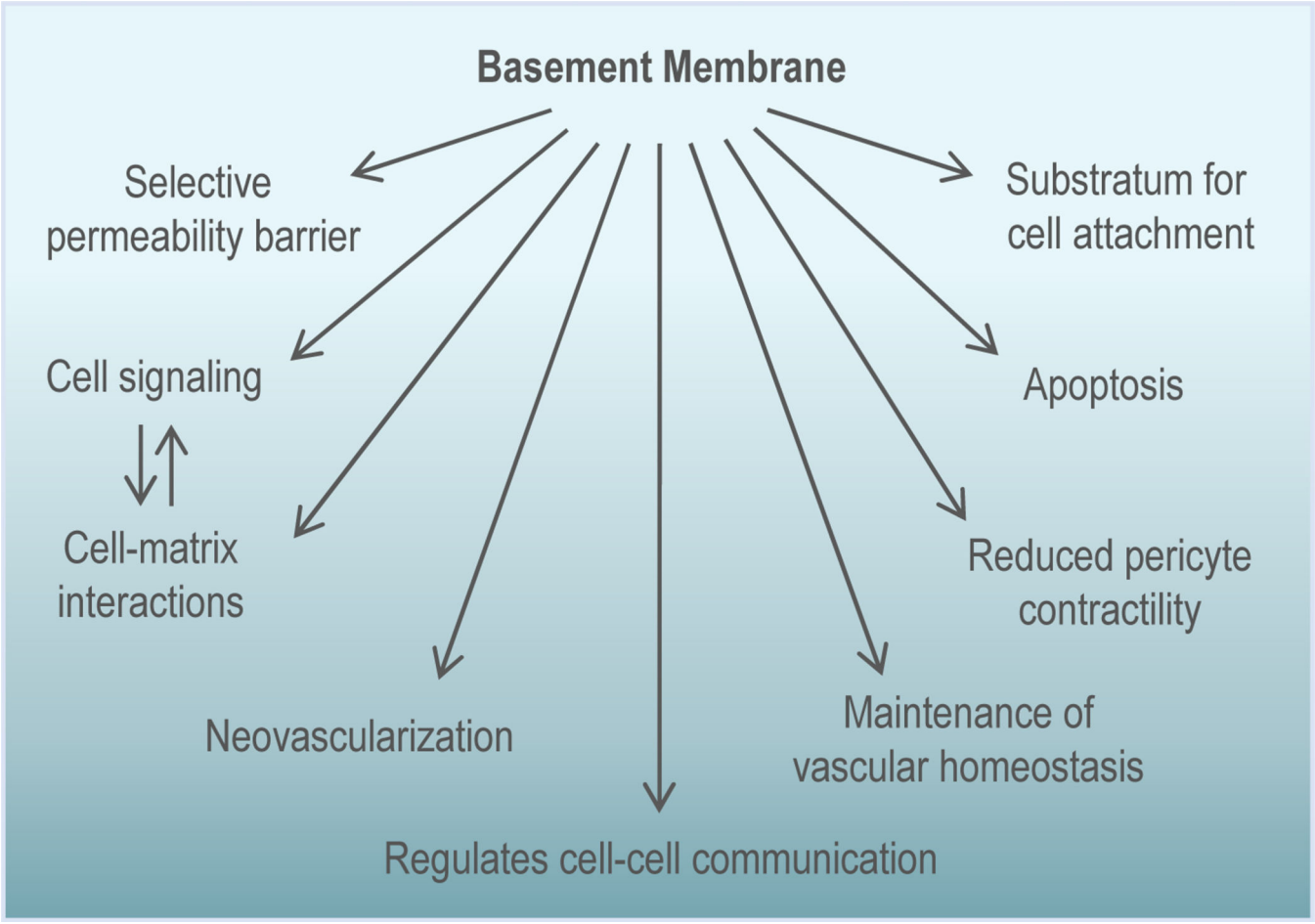


Figure 3. Multi-faceted functions of the BM.

The vascular BM is a multifunctional unit that serves as a selective permeability barrier, mediates cell-matrix interactions, acts as a substratum for cell attachment, and is also involved in regulating apoptosis, pericyte contractility, vascular homeostasis, neovascularization, and cell-cell communication.

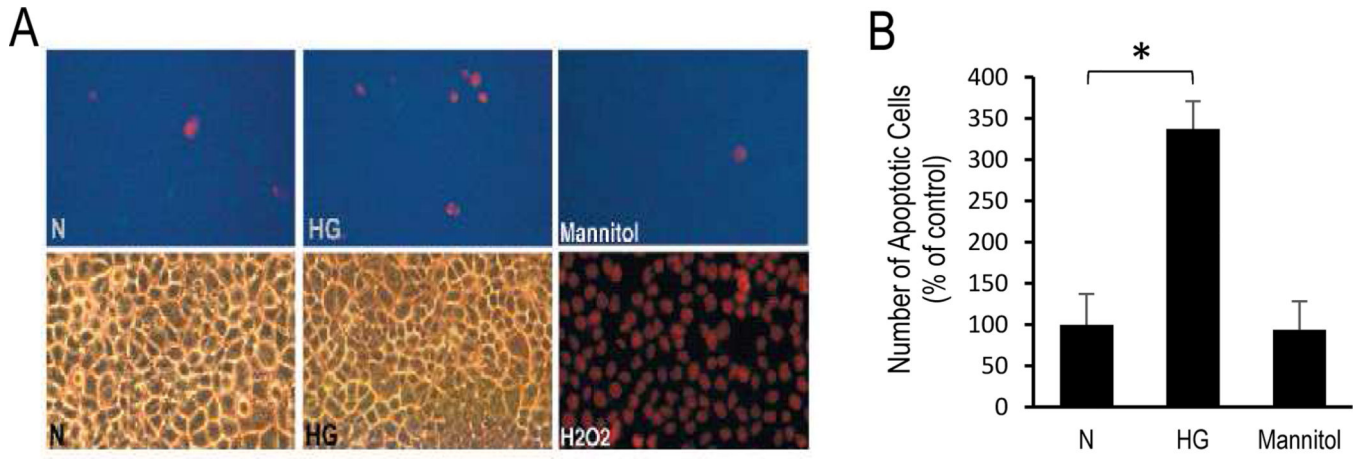


Figure 4. HG-induced excess ECM promotes apoptosis.

(**A**) Representative images show RRECs plated on HG-matrix and grown in N condition exhibit increased number of apoptotic cells (red) compared to those plated on N-matrix and grown in N condition. Cells exposed to hydrogen peroxide (H_2O_2) served as positive control. (**B**) Bar graph shows cumulative data indicating that ECM produced by cells grown in HG condition (HG-matrix) is capable of promoting apoptosis in cells grown in N medium. Cells exposed to mannitol exhibited no difference in apoptosis compared to cells grown in N medium. *$p<0.05$; n=6.

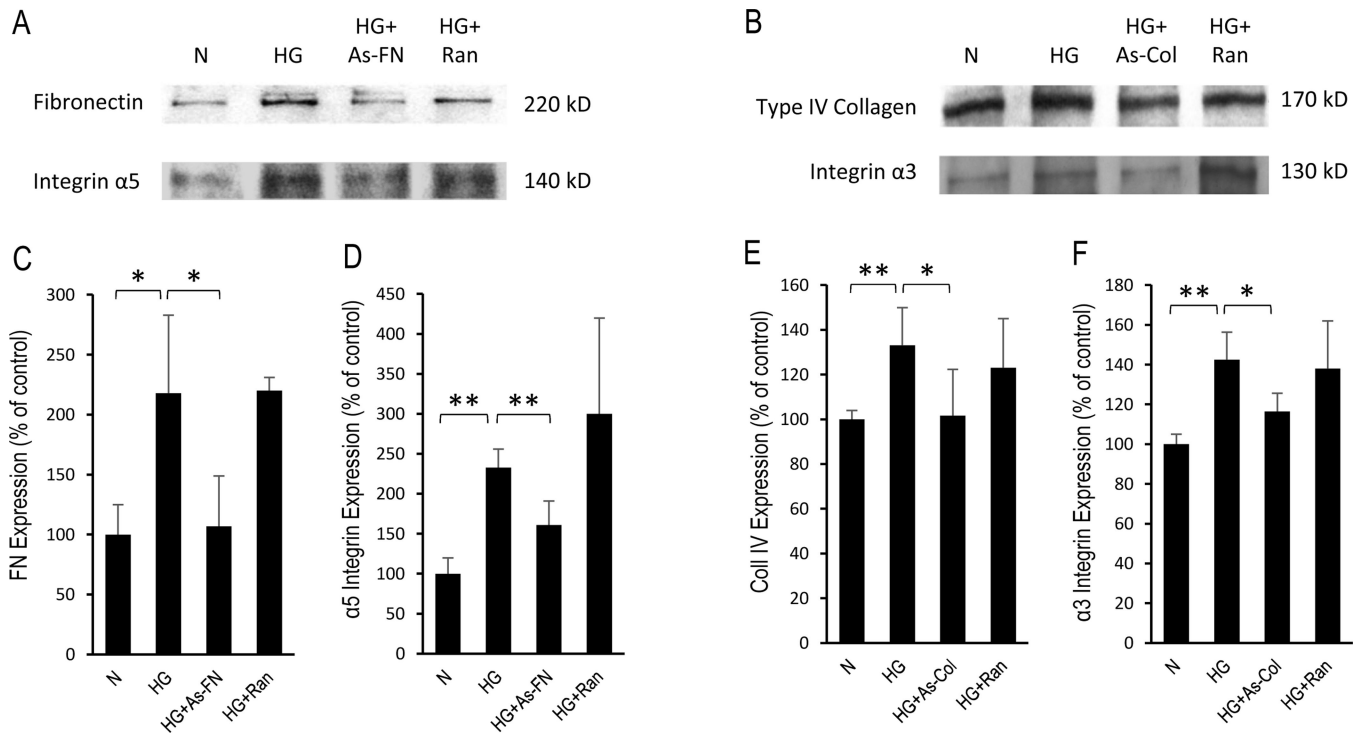


Figure 5. Effect of FN and Col IV antisense oligos on integrin α5 and α3 expression in RRECs.

Representative western blot image demonstrates that expression levels of (**A**) FN and α5 integrin, as well as those of (**B**) Coll IV and α3 integrin, are increased in cells grown in HG medium compared to that of cells grown in normal medium. Bar graphs representing cumulative data indicate that reducing (**C**) FN and (**E**) Col IV overexpression using antisense oligos decrease (**D**) α5 and (**F**) α3 integrin levels, respectively. Data is presented as mean ± standard deviation; *p<0.05. **p<0.01. N: normal media; HG: high glucose media; As-FN: HG media + antisense Col IV oligos; As-Col: HG media + antisense Col IV oligos; HG+Ran: HG medium + random oligos.

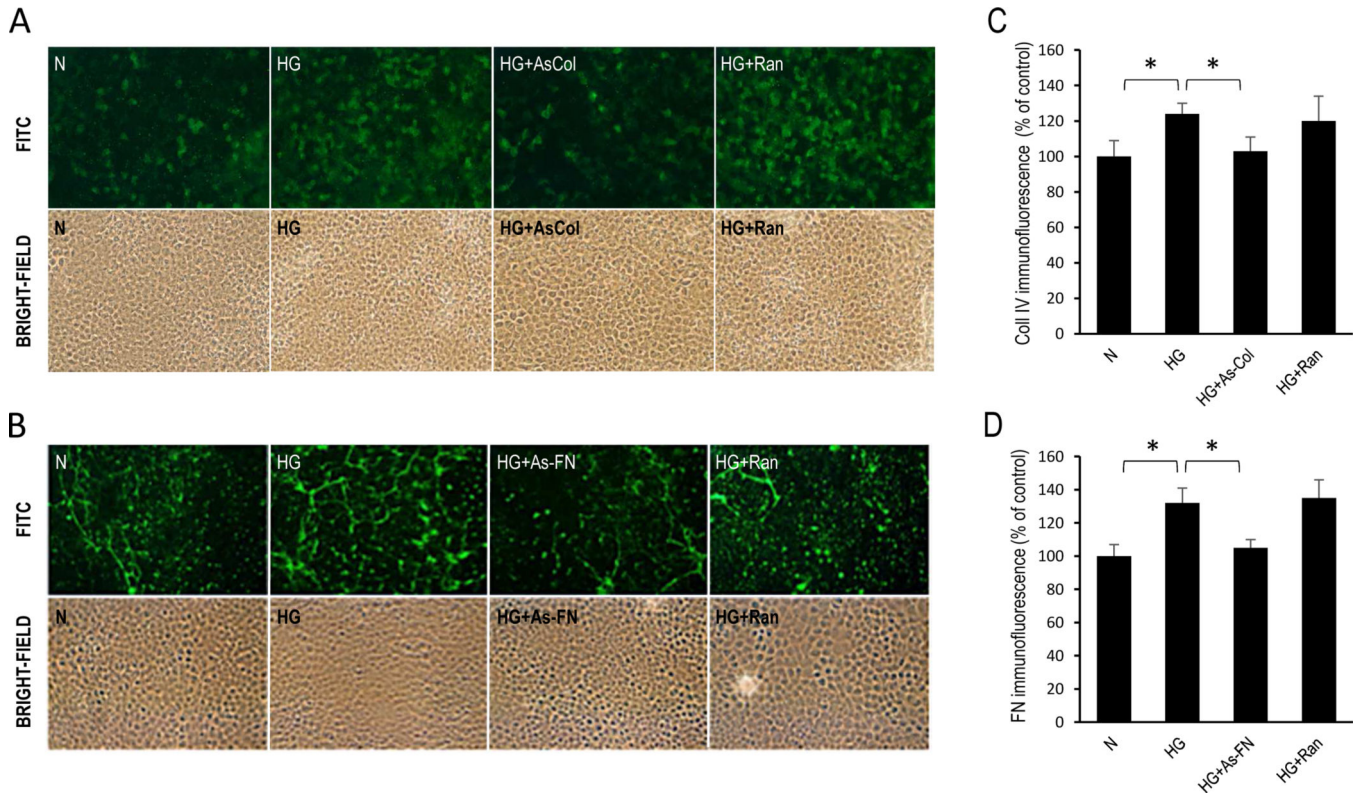


Figure 6. Effect of As-Coll IV and As-FN oligos on Coll IV and FN immunostaining in RRECs.

Immunohistochemical analysis show efficacy of (**A**) AS-Col oligos and (**B**) AS-FN oligos in reducing Col IV and FN levels, respectively, in RRECs grown in HG condition. Corresponding bright-field image is shown below each FITC-labeled photomicrograph. Bar graphs of cumulative data show AS-Col-oligos and AS-FN-oligos downregulate (**C**) Coll IV and (**D**) FN, respectively. Data is presented as mean ± standard deviation; *p<0.05. N: normal media; HG: high glucose media; As-FN: HG media + antisense FN oligos; As-Col: HG media + antisense Col IV oligos; HG+Ran: HG medium + random oligos; n=6.

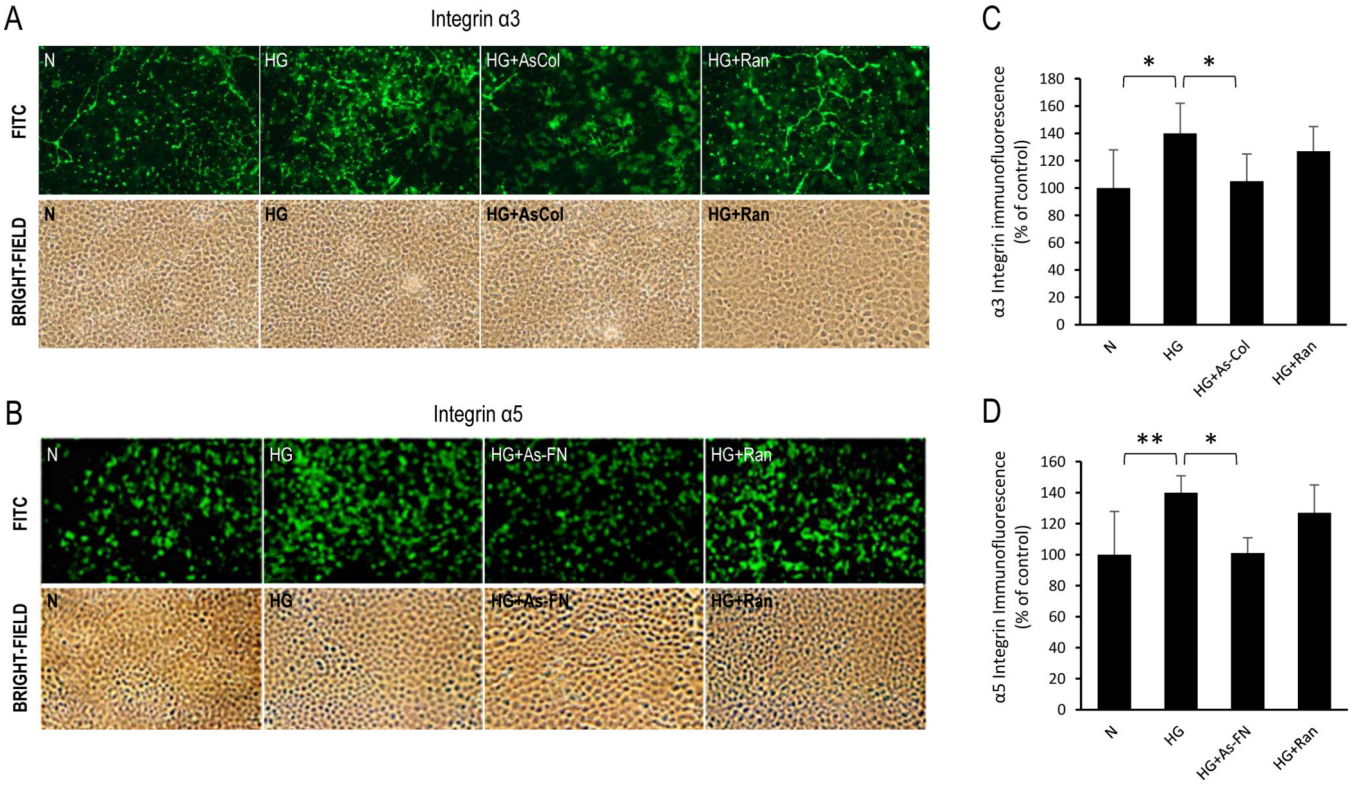


Figure 7. Effect of Coll IV and FN antisense oligos on α3 and α5 integrin immunostaining in RRECs.

Immunohistochemical analysis show (**A**) α3 integrin and (**B**) α5 integrin immunoreactivity in RRECs. Corresponding bright-field image is shown below each FITC-labeled photomicrograph. An increase in integrin α5 and integrin α3 immunoreactivity were observed in cells grown in HG medium compared to that of cells grown in normal medium. Bar graphs of cumulative data show that Coll IV and FN downregulation using antisense oligos decreases immunostaining of integrin α3 and integrin α5, respectively. Data is presented as mean ± standard deviation; *p<0.05, **p<0.01. N: normal media; HG: high glucose media; As-FN: HG media + antisense FN oligos; As-Col: HG media + antisense Col IV oligos; HG+Ran: HG medium + random oligos; n=6.

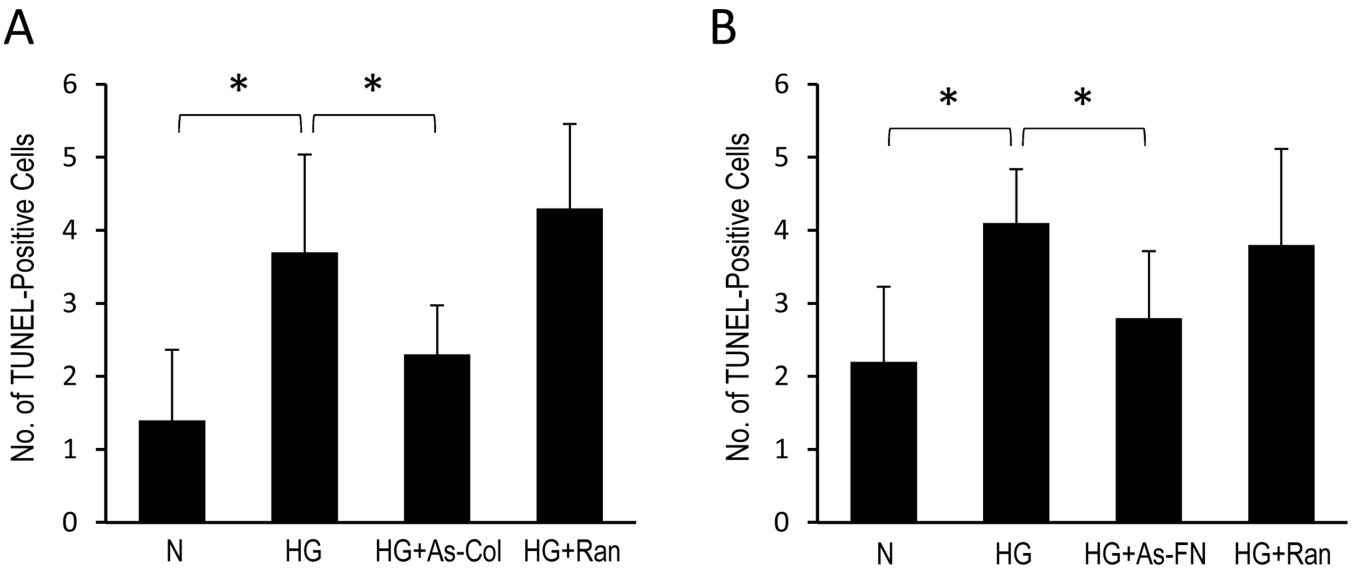
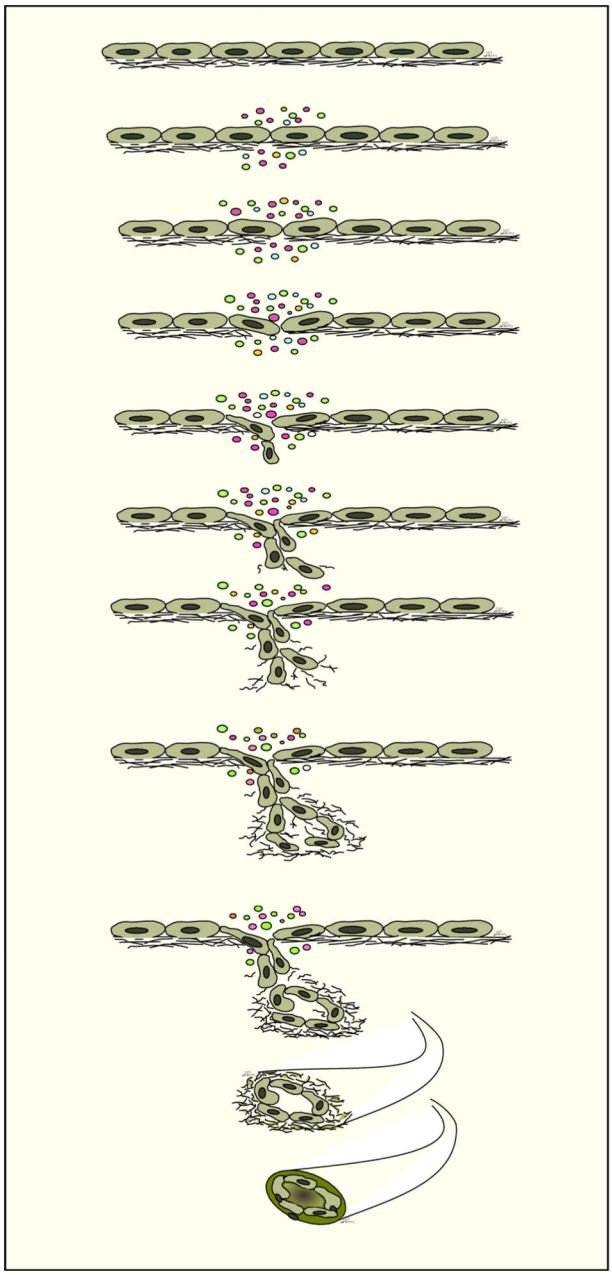


Figure 8. Effect of Coll IV and FN antisense oligos on apoptosis.

Bar graphs of cumulative data indicate that cells grown in HG medium exhibit increased number of TUNEL-positive cells. Interestingly, cells grown in HG medium and transfected with antisense Coll IV oligos or antisense FN oligos exhibit a decrease in the number of TUNEL-positive cells compared to that of cells grown in HG medium or cells grown in HG medium transfected with random oligo. Data is presented as mean ± standard deviation; *$p<0.05$. N: normal media; HG: high glucose media; As-FN: HG media + antisense FN oligos; As-Col: HG media + antisense Col IV oligos; HG+Ran: HG medium + random oligos; n=6.



(1) Endothelial cells anchored to vascular BM.

(2) Hypoxia induces production of angiogenic factors.

(3) MMPs/urokinase initiate local degradation of vascular BM.

(4) BM degradation compromises cell attachment.

(5) Endothelial tip cells and their filopodia exhibit sprouting.

(6) Stromal invasion by endothelial cells.

(7) Endothelial cell proliferation and ECM production

(8) Endothelial cell migration, proliferation, and BM scaffold formation allowing ECM molecules to assemble.

(9) Organized ECM assembly and BM maturation

(10) BM development and formation of a tubular structure.

(11) Formation and maturation of neovessel.

Figure 9. A schematic illustration showing major steps in retinal neovascularization.

This multi-step process is hypoxia-driven and involves endothelial cell participation, local BM dissolution, endothelial cell sprouting, ECM synthesis, ECM assembly, BM maturation, and neovessel formation.

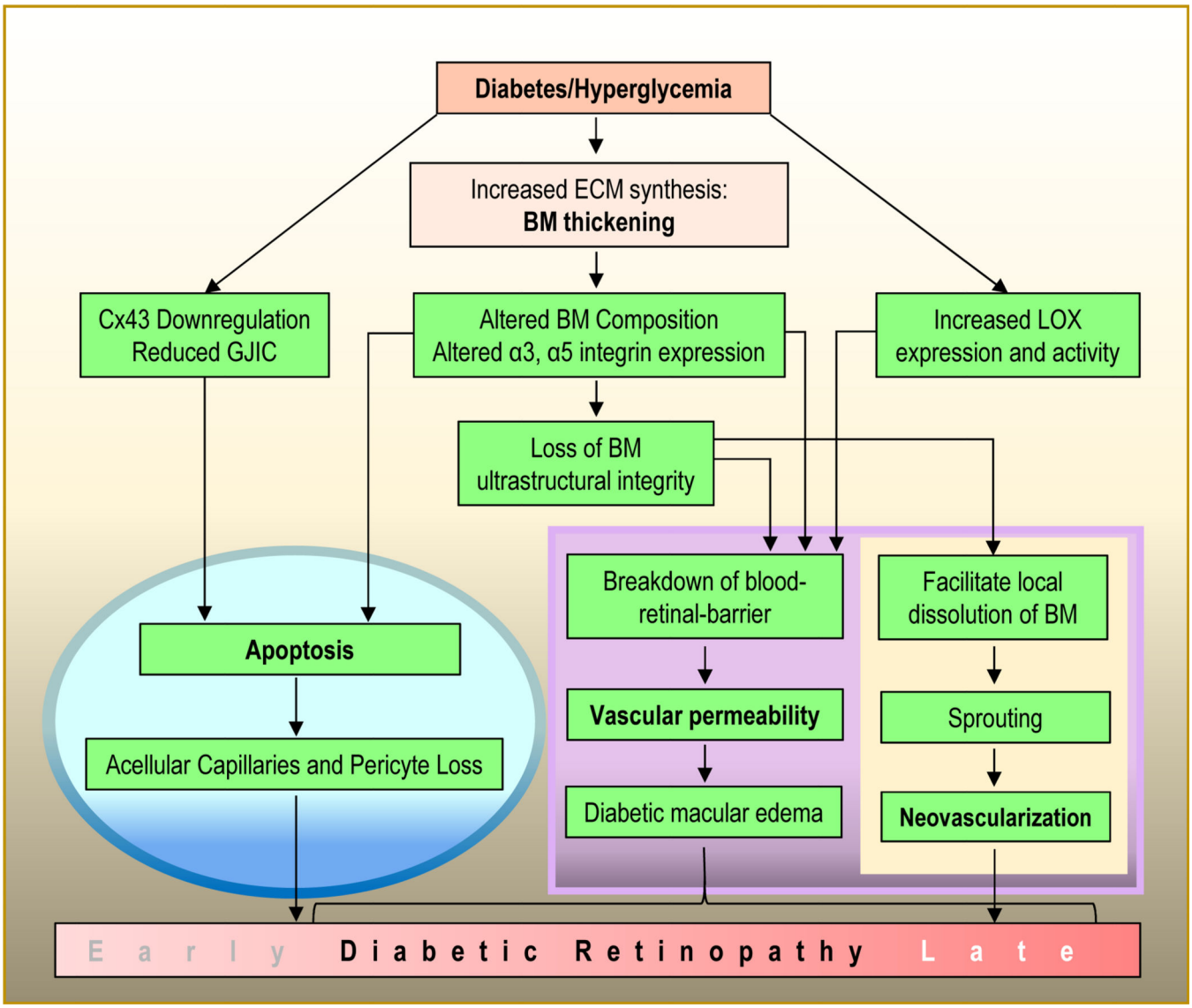


Figure 10. Hyperglycemia-induced retinal vascular BM thickening contributes to the development and progression of DR.

Schematic diagram highlights major steps in the development of vascular lesions characteristic of DR. GJIC: gap junction intercellular communication.

Table 1.

Diabetes-induced changes in ECM component levels associated with BM thickening

ECM Component	Level	Species	References
Fibronectin	Increased	Human	(Ljubimov et al., 1996; Ljubimov et al., 1998; Monnier et al., 1999; Monnier et al., 1986; Nwomeh et al., 1998; Risteli et al., 1987; Roux et al., 1977; Roy et al., 1996; Salmela et al., 1989)
Laminin-111 (α1, β1, γ1)	Increased	Human, Rat	(Bek and Ledet, 1996; Das et al., 1990)
Vitronectin	Increased	Human	(Bek and Ledet, 1996; Weller et al., 1991)
Collagen type I	Increased	Human	(Salmela et al., 1989)
Collagen type III	Increased	Human	(Ljubimov et al., 1998; Salmela et al., 1989)
α1 Collagen type IV	Increased	Human, Mouse, Rat	(Cooper et al., 1998; Das et al., 1990; Ljubimov et al., 1996; Rasmussen and Ledet, 1993; Roy et al., 1994; Rumble et al., 1998)
α2 Collagen type IV	Increased	Human, Rat	(Ljubimov et al., 1996)
Collagen type V	Increased /Unchanged	Human	(Labat-Robert and Robert, 1988; Rasmussen and Ledet, 1993; Salmela et al., 1989)
Collagen type VI	Increased	Human, Rat	(Bek and Ledet, 1996; Shinoda, 1992; Spiro and Crowley, 1993)
Tenascin	Increased	Human	(Monnier et al., 1999; Roux et al., 1977; Salmela et al., 1989)
Chondroitin sulfate	Decreased/ Increased	Human	(Anderson, 1993; Hagedorn et al., 1993)
Hyaluronic acid	Increased	Human	(Brenner, 1994)
Heparan sulfate	Increased/Unchanged/ Decreased	Human, Rat	(Anderson, 1993; Chowdhury et al., 1995; Das et al., 1990; Huijberts et al., 1994; Monnier et al., 1999; Nakamura et al., 1993)

Prog Retin Eye Res. Author manuscript; available in PMC 2022 May 01.

Table 2.

Vascular basement membrane thickening in human diabetic microangiopathies

Tissue	Normal BM Thickness (nm)	Diabetic BM Thickness (nm)	References
Retina	292 ± 24	583.1 ± 38.52	(Bianchi et al., 2016)
Glomerulus	321 ± 21	482 ± 151	(Rayat et al., 2005)
Skeletal Muscles	108 ± 2.7	240.3 ± 11.9	(Longhurst et al., 1975)
Quadricep Muscles	118 ± 2.7	203.1 ± 18.7	(Lindsay et al., 1994; Quabbe et al., 1983; Siperstein et al., 1968)
Skin	250 ± 34	353.3 ± 38	(Bae et al., 1987)
Lungs	164 ± 14	223 ± 27	(Weynand et al., 1999)
Perineurium	405.86 ± 4.2	524.55 ± 5.9	(Hill and Williams, 2004)

Table 3.

Increased synthesis of BM components in HG or diabetic conditions

BM Components	Cell Type / Tissue	References
FN, Coll IV	Rat Retinal Endothelial Cells	(Nguyen et al., 2020)
FN, Coll IV	Rat Retinal Endothelial Cells	(Roy et al., 2015)
FN	Human Retinas	(To et al., 2013)
FN	Rat Retinas	(Roy et al., 2011)
FN, Coll IV	Rat Retinal Endothelial Cells	(Chronopoulos et al., 2011)
FN, Coll IV, LM	Human Retinal Pigment Epithelial Cells	(Trudeau et al., 2011)
FN	Rat Retinas	(Cherian et al., 2009)
FN, Coll IV, LM	Rat Microvascular Endothelial Cells	(Oshitari et al., 2006)
FN	Rat Retinas	(Roy et al., 2003)
FN	Rat Mesangial Cells	(Noh et al., 2002)
FN, Coll IV	Rat Retinas	(Roy and Lorenzi, 1996)
FN	Human Retinas	(Roy et al., 1996)
Coll IV	Human Retinas	(Roy et al., 1994)
FN	Bovine Retinal Endothelial Cells and Pericytes	(Mandarino et al., 1993)
FN receptor	Human Umbilical Vein Endothelial Cells & Human Retinal Capillaries	(Roth et al., 1993)
FN	Human Umbilical Vein Endothelial Cells & Rat Retinas	(Roy et al., 1990)
Coll IV	Rat Retinas	(Das et al., 1990)
FN, Coll IV	Human Umbilical Vein Endothelial Cells	(Cagliero et al., 1988)
Coll IV	Bovine Retinal Pericytes	(Li et al., 1984)
FN	Human Glomeruli	(Parthasarathy and Spiro, 1982)

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Table 4.

Retinal vascular basement membrane thickening in diabetic animal models

Species	Mode of diabetes Induction	BMT in Non-Diabetic (nm)	BMT in Diabetic (nm)	References
Porcine	Alloxan-induced diabetes with normal diet	97.7±11.2	152.2 ± 16.3	(Hainsworth et al., 2002)
Porcine	Alloxan-induced diabetes with high fat diet	97.7 ± 11.2	120.2 ± 13	(Hainsworth et al., 2002)
Wistar Kyoto (WKY) rat	Galactose	160.4 ± 31.2	203.3 ± 376	(Frank et al., 1983)
Spontaneously hypertensive (SHR) rat	Galactose	159.4 ± 36.1	230.7 ± 623	(Frank et al., 1983)
Marmosets	Galactose	147 ± 34	244 ± 30	(Chronopoulos et al., 2015)
Dog	Alloxan-induced diabetes	141 + 25	236 ± 46	(Engerman and Kern, 1984; Engerman et al., 1993; Gardiner et al., 2003)
Dog	Galactose	141 ± 25	258 ± 88	(Engerman and Kern, 1984; Engerman et al., 1993)
Human	Diabetic subject	292.4 ± 24.3	583.1 ± 38.52	(Bianchi et al., 2016)
Yorkshire swine	Streptozotocin	2.6 ± 15.0	121.5 ± 21.8	(Lee et al., 2010)
Spiny mice	Streptozotocin	124.2 ± 1.7	150.8 ± 1.4	(Beauchemin et al., 1975)
Cat	Partial Pancreatectomy	72 ± 12	114 ± 15	(Mansour et al., 1990)
Sprague-Dawley rats	30% Galactose	159 ± 23	227 ± 37	(Kern and Engerman, 1994; Robison et al., 1986)
Sprague-Dawley rats	50% Galactose	159 ± 23	276 ± 226	(Kern and Engerman, 1994)
Sprague-Dawley rats	50% Galactose	159 ± 23	216 ± 36	(Kern and Engerman, 1994)
BALB/c mice	Streptozotocin	149 ± 45	295 ± 72	(Cuthbertson et al., 1989)