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The Unfolded Protein Response in Retinal Vascular Diseases: Implications and Therapeutic Potential Beyond Protein Folding

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Abstract

Angiogenesis is a complex, step-wise process of new vessel formation that is involved in both normal embryonic development as well as postnatal pathological processes, such as cancer, cardiovascular disease, and diabetes. Aberrant blood vessel growth, also known as neovascularization, in the retina and the choroid is a major cause of vision loss in severe eye diseases, such as diabetic retinopathy, age-related macular degeneration, retinopathy of prematurity, and central and branch retinal vein occlusion. Yet, retinal neovascularization is causally and dynamically associated with vasodegeneration, ischemia, and vascular remodeling in retinal tissues. Understanding the mechanisms of retinal neovascularization is an urgent unmet need for developing new treatments for these devastating diseases. Accumulating evidence suggests a vital role for the unfolded protein response (UPR) in regulation of angiogenesis, in part through coordinating the secretion of pro-angiogenic growth factors, such as VEGF, and modulating endothelial cell survival and activity. Herein, we summarize current research in the context of endoplasmic reticulum (ER) stress and UPR signaling in retinal angiogenesis and vascular remodeling, highlighting potential implications of targeting these stress response pathways in the prevention and treatment of retinal vascular diseases that result in visual deficits and blindness.

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Keywords

endoplasmic reticulum stress; unfolded protein response; retina; angiogenesis; VEGF; endothelial cells

1. Introduction

Pathogenic retinal angiogenesis or neovascularization (NV) is the principle cause of blindness in several common sight-threatening diseases, such as diabetic retinopathy (DR), retinopathy of prematurity (ROP), central retinal vein occlusion (CRVO), branch retinal vein occlusion (BRVO), and sickle cell retinopathy (reviewed in (Zhang and Ma 2007)). Neovascularity is often observed on the optic disc or at the junction of non-perfused and perfused areas of the retina. The newly formed blood vessels are composed of abnormal cellular components and have impaired barrier function. Hence, these vessels are fragile and leaky; without proper treatment, this often leads to vitreous hemorrhage, retinal edema and exudation, and tractional retinal detachment, eventually resulting in sudden or even permanent vision loss in the patients (Zhang and Ma 2007). Despite recent advances in the development of new anti-angiogenic agents and the availability of several highly efficient inhibitors against vascular endothelial growth factor (VEGF), a large percent of patients do not show satisfactory responses to such treatments (reviewed in (Waisbourd, Goldstein et al. 2011, Holz, Schmitz-Valckenberg et al. 2014)). This implies the existence of diverse VEGF-dependent and -independent mechanisms, potentially some yet to be discovered, in the pathogenesis of retinal NV. In addition, in most circumstances, retinal NV develops at the advanced stage of a disease as a result of severe retinal ischemia and vasodegeneration caused by endothelial dysfunction and cell death. Moreover, the process of pathogenic angiogenesis may be accompanied by active retinal vascular remodeling and intraretinal revascularization. These paradoxical, but interdependent and possibly concurrent, events indicate the complexity of retinal NV formation and further lead to great challenges in the treatment of vision-threatening ischemic/neovascular retinal diseases.

The unfolded protein response (UPR) is a sophisticated set of signaling pathways activated upon accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER), a condition known as ER stress. Intriguingly, a large body of studies has shown that the UPR is activated in vascular endothelial cells when exposed to one or more angiogenic stimuli, *e.g.*, hypoxia/ischemia, inflammation, and oxidative stress, and the activation of the UPR is vital for endothelial cell survival and activity (Ozawa, Tsukamoto et al. 2001, Wouters, van den Beucken et al. 2005, Dong, Ni et al. 2008, Luo and Lee 2013). Although the mechanisms are yet to be elucidated, it has been confirmed that the molecular chaperones that normally function to facilitate protein folding in the ER regulate angiogenic factor production and are actively involved in angiogenesis (Ozawa, Kondo et al. 2001, Ozawa, Tsukamoto et al. 2001, Dong, Stapleton et al. 2011). These chaperone proteins also have been found to be expressed in the retina and in retinal endothelial cells and are up-regulated by the UPR (Li, Li et al. 2008, Li, Wang et al. 2009, Chen, Wang et al. 2012). For instance, the 78-kDa glucose-regulated protein (GRP78), also referred to as the immunoglobulin binding protein BiP or the 70-kDa heat shock protein (Hsp70), is abundantly expressed in

endothelial cells and acts as a critical mediator of tumor angiogenesis by controlling endothelial cell proliferation, survival, and migration (Dong, Stapleton et al. 2011). Likewise, oxygen-regulated protein 150 (ORP150), an inducible ER chaperone, has been shown to regulate VEGF transport and secretion and is, thereby, implicated in both tumor angiogenesis and wound healing (Ozawa, Kondo et al. 2001, Ozawa, Tsukamoto et al. 2001).

Consistent with the findings in the cancer field, emerging evidence suggests that the UPR pathways and ER chaperones are involved in both physiological and pathological retinal angiogenesis during retinal development as well as in retinal neovascular diseases, likely through regulation of angiogenic factors, endothelial function, and cellular events such as inflammation and oxidative stress that are closely related to angiogenesis (Li, Wang et al. 2009, Li, Wang et al. 2012, Liu, Qi et al. 2013, Zeng, Tallaksen-Greene et al. 2013). Given the importance of the UPR as the most conserved mechanism in cellular stress response, understanding how ER stress-associated molecules and signaling pathways of the UPR modulate the angiogenic process may not only shed light on new mechanisms of retinal vasculature development, but also may provide critical insights toward the identification of novel therapeutic targets for treatment of neovascular retinal diseases. In the current review, we discuss the role of the UPR in regulation of retinal angiogenesis and interrelated processes, such as vasodegeneration, vascular remodeling, angiogenic progenitor function and vascular repair, highlighting the novel implication of ER-related signaling pathways in the retinal vascular system. For a recent review on the role of ER stress and the UPR in the context of the pathobiology of retinal degenerations, see Zhang et al. (Zhang et al, 2014).

2. Endoplasmic reticulum: structure and function

The ER is a major endomembrane compartment consisting of an extensively folded tubulovesicular membrane network supported by the gel-like cytoplasmic matrix (reviewed in English and Voeltz, 2013). There are two types of ER in a cell: the smooth ER and the rough ER. While the membrane composition of both is similar, the smooth and the rough ER have distinct morphologies and functions. The smooth ER is tubular, while the rough ER appears (as the name implies) like bumpy sheets stacked on top of each other, due to the presence on its surface of ribosomes, where protein synthesis occurs. The ribosomes are not a stable, permanent part of the ER; rather, they bind transiently to the ER through a receptor named ribophorin when actively synthesizing proteins, and are constantly being released from the ER surface (Marcantonio 1982). When protein synthesis becomes more active, the rough ER expands and forms a branched reticulum, thereby providing more surface area for the ribosomes to occupy. In addition, the cisternae of the rough ER dilate and form large membranous saccules in the cell. In contrast, the smooth ER forms its network of tubules and vesicles for the storage of key enzymes and the biogenic products of those enzymes; it is also the major compartment where cellular lipids are synthesized. This network of smooth ER provides increased surface area allowing for maximum storage capacity. Notably, the morphology and number of smooth and rough ER in a cell vary significantly as a function of organism, tissue, and cell type, and can be altered quickly to subserve specific physiological needs. For example, the cytoplasm of retinal pigment epithelial (RPE) cells from the frog retina has been observed to be filled with a compact lattice of tubular smooth ER, but is

relatively sparse with regard to rough ER, consistent with other cell types known to engage in the production of lipid-rich secretions (Porter and Yamada 1960).

While protein folding is widely considered to be a major function of the rough ER, it also occurs in the cytosol (in cytosolic ribosomes) and in the mitochondria (Christis, Lubsen et al. 2008). In the process of protein folding, newly synthesized proteins exit as unfolded polypeptide chains from the ribosomes and are pushed into the deep cisternal space of the ER. In the cisternae, these linear polypeptide chains are folded and oligomerized, disulfide bonds are introduced, and (if destined to become a glycoprotein) N-linked oligosaccharides are attached. The latter two events, *i.e.*, formation of disulfide bonds, which covalently link two cysteine residues, and N-linked glycosylation of the folding proteins, are unique features of protein folding in the ER, which distinguish the process from folding in the cytosol (Christis, Lubsen et al. 2008). These processes are catalyzed by specialized folding enzymes and chaperone proteins, some of which exert functions exclusive to folding in the ER, while others have homology to cytosolic and mitochondrial folding factors. The involvement of dolichylphosphate-linked oligosaccharide formation and transfer of the oligosaccharide to nascent polypeptide chains via oligosaccharyl transferase are separate biochemical events that occur in the ER, concomitant with or temporally associated with these folding and disulfide bond formation events (reviewed in (Breitling and Aebersold 2013)). Major chaperones and folding enzymes in the ER include: 1) chaperones of the heat shock protein family, such as GRP78 and its co-chaperone partners (*e.g.*, p58^{IPK}); 2) chaperone lectins, such as calnexin and calreticulin; and 3) thiol oxidoreductases of the protein disulfide isomerase (PDI) family (Nishikawa, Brodsky et al. 2005).

Upon completion of folding, mature proteins are released from ER chaperones and then are transported to the Golgi apparatus. However, a large fraction of proteins cannot be correctly folded; these proteins are retained in the ER, whereupon they are recognized by ER chaperones such as GRP78 and are then targeted for the refolding process (Schubert, Anton et al. 2000). If the refolding fails, terminally misfolded proteins will be cleared from the ER by the ER-associated degradation (ERAD) machinery to maintain ER homeostasis (Schubert, Anton et al. 2000, Nishikawa, Brodsky et al. 2005). The exposure of hydrophobic residues, which may occur during any protein folding event, whether in the cytosol, ER, or mitochondria, results in undesirable interactions within or between different polypeptide chains, leading to protein misfolding and often aggregation (Christis, Lubsen et al. 2008). A critical role for ER chaperones, therefore, is to prevent nonproductive or inappropriate interactions, and to prevent protein misfolding and aggregation. For example, heat shock protein family chaperones (*e.g.*, Hsp70) present in the cytosol, mitochondria, nucleus, and ER of eukaryotic cells (and, in addition, in the chloroplasts of plants) assist protein folding by shielding exposed hydrophobic stretches and keeping newly synthesized proteins in a folding-competent state (Christis, Lubsen et al. 2008). Disturbed chaperone function would lead to formation of cytotoxic protein aggregates, such as amyloid, which have been seen in many human diseases and have been implicated as a primary cause of various neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and AMD (Broadley and Hartl 2009, Ding, Johnson et al. 2011).

In addition to protein synthesis and folding, the ER also possesses many important functions vital for cell survival. For instance, the ER is responsible for ion storage (*e.g.*, calcium ions, phosphate, etc.) and serves as a dynamic pool of calcium, governing the process of intracellular calcium homeostasis (Gorlach, Klappa et al. 2006). Uncontrolled calcium release from the ER increases cytoplasmic calcium concentration, resulting in activation of calcium/calmodulin-dependent protein kinase (CaMK)II, which triggers multiple pro-apoptotic pathways mediated by the death receptor Fas, release of apoptogenic factors from the mitochondria, signal transducer and activator of transcription-1 (STAT1), and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived reactive oxygen species (ROS) (Timmins, Ozcan et al. 2009, Tabas 2010). Severe calcium dysregulation is also involved in other types of cell death, such as necrosis and necroptosis (Pinton, Giorgi et al. 2008, Webster 2012). Other major functions of the ER include lipid and steroid hormone synthesis, carbohydrate metabolism, and drug detoxification (*e.g.*, via cytochrome P450 enzymes). The ER also is the initial site for synthesis of sphingolipids, which are transferred to the Golgi apparatus, where their synthesis is completed, *e.g.*, glycosylation to form neutral glycosphingolipids and gangliosides (Futerman 2005). The ER is also the primary compartment of the cell where sterols, such as cholesterol, are synthesized (Schroepfer 1981); the peroxisome is thought to be an auxiliary compartment where sterol biosynthesis also takes place (Kovacs and Krisans 2003). As well, the ER works in conjunction with the mitochondria to produce steroid hormones (Issop, Rone et al. 2013). In hepatocytes, the ER is responsible for production of lipoprotein particles, which function as specialized cargo vehicles that carry lipids throughout the blood stream to the extrahepatic tissues (Fielding and Fielding 1982, Havel 1987). It also plays a central role in carbohydrate metabolism, including glucagon metabolism, gluconeogenesis, and glycogen storage diseases (Robertson, Kim et al. 2006). Consistent with these important functions in cellular metabolism, the ER constantly senses subtle changes in nutrient status and responds to multiple nutrient-associated signals, such as those induced by fatty acids, glucose, free cholesterol, insulin, and amino acids (Hotamisligil and Erbay 2008). Furthermore, through an endomembrane network, the ER is closely connected with other key organelles that regulate cellular metabolism, such as the Golgi apparatus, mitochondria, lysosomes, and peroxisomes. Indeed, the interconnection of the Golgi apparatus, ER, and lysosomal compartments was recognized four decades ago using cytochemical approaches (Novikoff 1976, Spater, Novikoff et al. 1978), and this multi-organelle network has been formally termed “GERL”. Thus, disturbed function in any of these organelles can evoke a stress signal that can be shared and spread throughout this system, resulting in exacerbated cellular stress during chronic disease conditions, such as obesity and diabetes, as well as natural aging (Hotamisligil and Erbay 2008). These stress signals activate a variety of molecular pathways to regulate cellular activity and mediate the balance between cell survival and cell death.

3. The unfolded protein response (UPR)

The ER is the foremost important organelle in controlling protein production in the cell. Regulation of the amount of unfolded and misfolded proteins in the ER is critical for ER homeostasis and is tightly controlled by the interrelated processes of protein translation, protein folding, and protein degradation (*i.e.*, by ERAD). The accumulation of excessive

unfolded or misfolded proteins, *i.e.*, ER stress, can be observed both in normal physiological conditions, where the protein synthesis rate surpasses the folding capacity, and in disease conditions related to gene mutations resulting in protein misfolding, impaired function, and expression of chaperones and foldases, disturbance in metabolic and redox status, and dysregulated calcium homeostasis (Kim, Xu et al. 2008). ER stress can be sensed by three ER transmembrane proteins: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1 α (IRE1 α), and activating transcription factor 6 (ATF6). Activation of these proteins triggers the UPR, which consists of complex signaling pathways that aim to mitigate ER stress and restore ER homeostasis (Brewer, Hendershot et al. 1999). The UPR enhances protein folding by up-regulation of ER chaperones, facilitates unfolded protein degradation by promoting ERAD, and reduces the amount of new proteins entering the ER by slowing down protein translation. These efforts eliminate ER stress, which is critical for the cell to function and survive.

3.1 The IRE1/XBP1 pathway

The IRE1/XBP1 (X-box binding protein 1) branch (**Figure 1**) has been considered as the conserved core of the UPR, which widely exists from yeast to human and is essential for mammalian developmental processes (Newton and Ron 2007). Knockout of IRE1 or XBP1 causes embryonic lethality in the early developmental stage (by 12.5 days of gestation) (Reimold, Etkin et al. 2000, Iwawaki, Akai et al. 2009, Hetz, Martinon et al. 2011, Sano and Reed 2013). IRE1 is an ER membrane protein whose luminal domain is involved in the sensing of unfolded proteins, whereas the cytoplasmic domain contains a kinase domain and an RNase domain. There are two different isoforms of IRE1 in mammalian cells: IRE1 α and IRE1 β . IRE1 α is ubiquitously expressed, while IRE1 β is tissue-specific (Tirasophon, Welihinda et al. 1998, Wang, Harding et al. 1998). When unfolded proteins become overloaded in cells, bound GRP78 is released from IRE1, resulting in its activation through autophosphorylation and dimerization (**Figure 1**) (Bertolotti, Zhang et al. 2000, Ali, Bagratuni et al. 2011). Activated IRE1 splices a 26-nucleotide intron from the mRNA encoding XBP1, which generates a more stable and potent transcriptional factor of UPR genes, known as spliced XBP1 or XBP1s (**Figure 1**) (Yoshida, Matsui et al. 2001). XBP1s induces downstream genes, including ER chaperones, such as GRP78, p58^{IPK}, ERdj4, PDI-P5, and HEDJ, and proteins involved in ERAD, such as HRD1, EDEM, Derlin-2, and Derlin-3; these proteins are thought to play a central role in restoring ER homeostasis and promoting cell survival (Lee, Iwakoshi et al. 2003, Yoshida, Matsui et al. 2003, Kanemoto, Kondo et al. 2005). Ablation of XBP1 has been shown to result in the severe loss of function of pancreatic β cells, hepatocytes, and plasma B-cells, all of which produce large amounts of secretory proteins, suggesting an essential role for XBP1 in maintaining the protein secretory machinery (Glimcher 2010).

Besides acting as an endoribonuclease, activated IRE1 also functions as a kinase and binds to tumor necrosis factor receptor-associated factor 2 (TRAF2), which recruits apoptosis signal-regulating kinase 1 (ASK1) and activates the c-Jun N-terminal protein kinases (JNK) pathway (**Figure 1**) (Urano, Wang et al. 2000). IRE1-dependent JNK activation is an important signaling mechanism involved both in the inflammatory response and in apoptosis (reviewed in (Jing, Wang et al. 2012, Sehgal and Ram 2013)).

3.2. The PERK pathway

PERK is a type I transmembrane protein situated in the ER that senses the accumulation of unfolded proteins in the lumen of the ER (Bertolotti, Zhang et al. 2000). Like IRE1, the luminal domain of PERK senses unfolded proteins, whereas the cytoplasmic domain contains a kinase motif. In the absence of ER stress, GRP78 binds to the luminal domain of PERK and prevents its activation. Upon ER stress and dissociation from GRP78, PERK is activated through oligomerization and trans-phosphorylation (**Figure 2**) (Bertolotti, Zhang et al. 2000). Activated PERK phosphorylates and inactivates the α -subunit of eukaryotic initiation factor (eIF2 α), resulting in arrest of protein synthesis, which reduces the further influx of ER client proteins. Furthermore, the phosphorylation of PERK is tightly controlled through feedback regulation by specific phosphatases, such as constitutive repressor of eIF2 α phosphorylation (CReP) and its regulatory subunit GADD34 (growth arrest and DNA damage-inducible protein-34) (**Figure 2**) (Jousse, Oyadomari et al. 2003), and by its endogenous inhibitor, p58^{IPK} (Yan, Frank et al. 2002). The expression of GADD34 and p58^{IPK} is induced under ER stress by the PERK and ATF6 pathways, respectively, whereas CReP is constitutively expressed in the cell (Donnelly, Gorman et al. 2013). In contrast to global translational attenuation, phosphorylation of eIF2 α enhances the translation of activating transcription factor 4 (ATF4), which subsequently induces its target genes, including C/EBP homologous protein (CHOP), a transcription factor involved in the induction of apoptosis, and proteins involved in amino acid metabolism, such as asparagine synthetase, or those involved in resistance to oxidative stress (anti-oxidants) (**Figure 2**) (Harding, Zhang et al. 2003). The phosphorylation of eIF2 α is also regulated by other kinases, such as dsRNA-dependent protein kinase (PKR), general control of amino acid synthesis 2 (GCN2), and heme-regulated inhibitor kinase (HRI) (Lu, Han et al. 2001). The activation of these kinases can be triggered by both physiological and pathological conditions in addition to ER stress, such as viral infections, amino acid deprivation, and heme deficiency (Wek 1994).

3.3. The ATF6 pathway

ATF6 is a type II transmembrane protein, the luminal domain of which is accountable for the sensing of unfolded proteins (**Figure 3**) (Shen and Prywes 2004). It also has a DNA-binding domain containing the basic-leucine zipper motif (bZIP) and a transcriptional activation domain towards its cytoplasmic portion. In the absence of ER stress, binding of GRP78 to the ATF6 luminal domain hinders the Golgi-localization signal, which inhibits its translocation to the Golgi apparatus (Ye, Rawson et al. 2000). Upon ER stress, ATF6 is dissociated from GRP78 and translocates to the Golgi apparatus by vesicular transport. In the Golgi apparatus, a pair of processing proteases, called site 1 protease (S1P) and site 2 protease (S2P), sequentially cleave ATF6, releasing its cytoplasmic portion (pATF6(N)), an active form of ATF6 (**Figure 3**). The released pATF6(N) translocates into the nucleus, where it activates the transcription of ER chaperone genes, such as GRP78, GRP94, and calreticulin (Yoshida, Haze et al. 1998), as well as components of the ERAD pathway, by binding to a *cis*-acting element, the ER stress response element (ERSE) (Newton and Ron 2007, Wu, Rutkowski et al. 2007, Yamamoto, Sato et al. 2007).

4. Retinal vascular development and angiogenesis

Retinal vascularization commences in the innermost layer (superficial layer) of the retina at the nerve head and extends outward towards the periphery. In humans, the retinal vascularization begins at approximately 16 weeks of the gestational age. The hyaloid vasculature originating at the optic nerve provides the vascular supply partially during early stages of vasculature development and traverses the vitreous toward the anterior segment (reviewed in (Stahl, Connor et al. 2010)). When ocular development advances, the hyaloid vasculature must regress so as not to obstruct the path of incident light entering the eye en route to the retina (Mitchell, Risau et al. 1998). In humans, the regression of the hyaloid vasculature is completed before 34 weeks of gestation. By 36 weeks of gestational age, the nasal retina becomes fully vascularized; and by approximately 40 weeks of gestational age, the temporal vessels reach the ora serrata, a junction between the retina and the ciliary body. Therefore, under normal conditions, the human infant is born with fully developed retinal vessels and with a regressed hyaloid vasculature (Saint-Geniez and D'Amore 2004).

There are several development disorders that interfere with the normal growth and development of the retinal vasculature. For example, Norrie's disease, an X-linked recessive disorder, is associated with persistent hyaloid vessels, incomplete development of the retina, and pathological neovascularization, resulting in the blindness (Xu, Wang et al. 2004). Familial exudative vitreoretinopathy (FEVR) is a rare disorder inherited in an autosomal dominant, autosomal recessive or X-linked manner and principally affects the retinal angiogenesis, thereby leading to faulty vascular differentiation and incomplete peripheral retina vascularization (Gilmour 2014). Persistent fetal vasculature (PFV) (formerly known as persistent hyperplastic primary vitreous (PHPV)) is another developmental disorder that results in malformation of the ocular tissues, tractional detachment of the retina, and blindness (Shastry 2009). Von Hippel-Lindau (VHL) disease is a genetic disorder where vascular endothelial tumors or hemangiomas arise in the retina as well as in many other organs. The VHL protein (pVHL) is involved in the regulation of hypoxia inducible factor 1 α (HIF-1 α) (Robinson and Ohh 2014). Under normoxic conditions, pVHL binds HIF-1 α and renders the protein susceptible to proteasomal degradation (Jaakkola, Mole et al. 2001). Under hypoxic conditions, HIF-1 α is spared from prolyl hydroxylation, dimerizes with HIF-1 β , and translocates into the nucleus, where it promotes induction of angiogenic factors. In animals and humans, mutations in pVHL can lead to unrestrained angiogenesis in response to tissue oxygen levels (Rankin, Higgins et al. 2005).

In contrast to humans, rodent retinal development (*e.g.*, mouse, rat) starts during embryogenesis, but continues after birth. The pups have immature retinal vasculature and persistent hyaloid vessels at birth (Gyllenstein and Hellstrom 1954). In mouse, highly organized pruning of the hyaloid vessels occurs in the postnatal eye, which is regulated by Wnt7b, a short-range paracrine signal (Lobov, Rao et al. 2005). Moreover, the putative Wnt-receptor, Frizzled-5 (FZD5), also has been found to be essential for hyaloid regression (Liu and Nathans 2008). In C57Bl/6 mice, the superficial vascular plexus forms by radial outgrowth of the vessels from the optic nerve to the periphery. This process is closely associated with astrocytes, a specific type of macroglial cells located in the inner most layer of the retina (Dorrell, Aguilar et al. 2002). In fact, retinal astrocytes that express glial

fibrillary acidic protein (GFAP) are observed only in mammalian species having a vascularized retina and are distributed in areas where blood vessels are located (Gariano, Sage et al. 1996). During development, glial lineage-restricted precursors migrate from the neural tube into the retina in the final stages of neurogenesis; the timing of astrocyte migration is thought to be critical for the formation of retinal vessels (Stone, Sandercoe et al. 2006). The pre-existing astrocyte network serves as a template and provides guidance cues for blood vessel growth by forming glial fibers as well as secretion of VEGF (reviewed in (Gariano and Gardner 2005, Dorrell and Friedlander 2006)). The new vessel formation also requires active interactions between endothelial tip cells and glial processes mediated by the cell surface adhesion molecule R-cadherin (Dorrell, Aguilar et al. 2002). In turn, newly developed blood vessels provide feedback signals to inhibit astrocyte proliferation and VEGF secretion, but promote their differentiation into mature astrocytes (West, Richardson et al. 2005). In addition, *in vitro* experiments suggest that astrocytes may sense the changes in retinal oxygen concentrations and, accordingly, regulate their proliferation and VEGF production (West, Richardson et al. 2005). Yet, recent studies using astrocyte-specific conditional knockout mouse lines that lack VEGF and its upstream regulators, HIF-1 α and HIF-2 α , or VHL in astrocytes demonstrated that the hypoxia response or VEGF production in astrocytes is not essential for retinal vascular development; however, astrocyte-derived VEGF is required for pathogenic angiogenesis or retinal NV in oxygen-induced retinopathy (OIR) (Weidemann, Krohne et al. 2010). In contrast, conditional knockout of HIF1 α in neuroretinal cells, specifically Pax6+ retinal progenitor cells, resulted in diminished retinal vascular development accompanied by reduced astrocyte density and decreased level of platelet derived growth factor A (PDGF-A), a potent mitogen for astrocytes. (Nakamura-Ishizu, Kurihara et al. 2012). These findings suggest that inner retinal neurons may be important for sensing oxygen to regulate astrocytes and vascular development.

At approximately postnatal day 8 (P8) the vessels reach the retinal edges. The superficial capillaries begin to sprout vertically from P7 onward to form first the deep and then the intermediate vascular plexus. The deep plexus, positioned in the outer plexiform layer (OPL), forms rapidly and radiates towards the periphery at P12 accompanied by the intermediate plexus in the inner plexiform layer (IPL) sometime between P12 and P15. Like astrocytes, Müller cells, another major type of retinal macroglia, were thought to play a guiding role in the vertical extension of retinal blood vessels into the deep layers (Stone J 2006). In addition, the migration of blood vessels to the deep layer of retina is facilitated by the endothelial filopodial processes which contact and respond to the guidance cue molecules, such as R-cadherin (Dorrell, Aguilar et al. 2002). The formation of new blood vessels involves two distinct models: vasculogenesis, where new blood vessels are formed by the *de novo* synthesis of endothelial cells from precursor cells; and angiogenesis, in which the new blood vessels are formed from the pre-existing vessels. For detailed discussion on vasculogenesis and angiogenesis in retinal blood vessel development, see (Gariano 2003, Zhang and Ma 2007). In addition, there is evidence that retinal vascular remodeling requires the participation of immune cells, such as T cells and leukocytes, which induces Fas ligand-mediated apoptosis of endothelial cells to readjust retinal vascular density to an optimal level (Ishida, Yamashiro et al. 2003). By the end of the third postnatal

week, all three (superficial, intermediate and deep) layers of retinal blood vessels are fully developed and mature.

The regulation of retinal vasculature development is complex and involves multiple oxygen-dependent growth factors. Established by Michaelson, the method of dye perfusion allows the researchers to unveil the embryonic and perinatal vasculature (Patz 1984). Using this method, Ashton observed that the growth of retinal capillaries is more prolific when adjacent to the nascent venules than around the arteries, (Ashton 1966). This reinforces the idea that oxygen regulates the growth of the blood vessels in the retina. Vascular endothelial growth factor (VEGF) is a hypoxia-inducible cytokine and is required for retinal angiogenesis. VEGF serves as a survival and anti-apoptotic factor in the newly formed vessels; it was found that, in rat neonates, there was an increase in endothelial apoptosis when the supply of VEGF was blocked, thereby leading to regression of the blood vessels (Alon, Hemo et al. 1995). VEGF and its isoforms tightly regulate angiogenesis in a spatial and temporal manner, with distinct roles of these various isoforms in retinal vascular patterning and arterial development (Stalmans, Ng et al. 2002).

In addition to VEGF, a number of other growth factors have been implicated in retinal blood vessel development. Growth hormone (GH) as well as its target, IGF-1 (insulin-like growth factor 1), has been shown to play a critical role during retinal angiogenesis. In patients with genetic defects in GH/IGF-1 axis displayed impaired retinal vascularization (Hellstrom, Carlsson et al. 2002). However, the reduction in IGF-1 does not directly impact the VEGF level; instead it represses VEGF-mediated MAPK pathway and Akt pathway activation, which is essential for endothelial cell survival and proliferation (Smith, Shen et al. 1999). Another growth factor named connective tissue growth factor (CTGF) has also been implicated in postnatal retinal vessel development. A recent study showed that CTGF expression was increased in retinal endothelial cells, perivascular cells and tips of superior vascular plexus but not in astrocytes underlying nascent vessels during development (Pi, Xia et al. 2011). Inhibition of CTGF by intravitreal injection of CTGF antibody blocked the growth of superior vascular plexus and filopodial burst during vessel development. This suggests a critical role of CTGF in retinal vascularization (Pi, Xia et al. 2011).

Recent studies have provided some exciting evidence that implicates the UPR pathways in retinal blood vessel development. A recent study by Zeng and colleagues demonstrated that conditional knockout of XBP1 in endothelial cells resulted in retarded development of retinal blood vessels at post-natal day 7 and 14, and impaired ischemia-induced angiogenesis in hindlimb (Zeng, Xiao et al. 2013). In cultured endothelial cells, spliced XBP1 interacts with the PI3K/Akt/GSK3 β / β -catenin/E2F2 pathway to regulate cell proliferation (Zeng, Xiao et al. 2013). In addition, in cancer cells XBP1 forms a complex with HIF-1 α and plays a pivotal role in tumorigenicity (Chen et al, 2014). Whether XBP1 interacts with HIF-1 α in vascular cells and contributes to retinal blood vessel development are yet to be tested. It also remains unclear whether the pro-angiogenic effect of XBP1 during retinal development is ER stress-dependent.

In addition to XBP1, ATF4, a transcription factor in the PERK pathway of the UPR, has been shown to function as an oxygen sensor and interacts with HIF-1 α to regulate VEGF

production (Zhong, Li et al. 2012). Global knockout of ATF4 disturbs lens development, resulting in microphthalmia (Masuoka and Townes 2002). Currently it remains unclear if ATF4 deficiency would impact on retinal vascular development. ATF6, an ER stress-inducible transcription factor, has also been shown to regulate VEGF expression in endothelial cells and inhibition of ATF6 resulted in an even greater reduction in retinal NV or choroidal neovascularization (CNV) than anti-VEGF treatment (Liu et al., 2013). Yet, global knockout of ATF6 α or ATF6 β does not result in developmental defects but double knockout of ATF6 α and ATF6 β is embryonically lethal (Yamamoto et al, 2007).

5. Retinal vasodegeneration, vascular remodeling and neovascularization

In the normal adult, retinal vasculature is well-developed and maintained in a quiescent and non-angiogenic state. This is controlled, in part, by a delicate balance between pro-angiogenic, such as VEGF, IGF-1, erythropoietin (Epo), and anti-angiogenic factors, such as PEDF (for review, see (Zhang and Ma 2007)). Mature retinal blood vessels provide oxygen and nutrients to inner retinal neurons and also play a critical role in maintaining the homeostasis of retinal environment by forming the inner blood-retinal barrier. In pathological conditions, such as diabetes, chronic or acute injury of vascular cells, i.e. endothelial cells and pericytes, leads to apoptosis and cell death resulting in loss of retinal blood vessels and reduced blood supply to retinal neurons (ischemia)(Zhang and Ma 2007). In ROP, premature birth halts the normal development of retinal blood vessels resulting in incomplete vascularization of the retina. Lack of vascular coverage induces retinal ischemia and this is further worsened by vaso-obliteration in the immature retina when exposed to excessive oxygen. Severe and extensive retinal ischemia stimulates the production of angiogenic factors, tipping the balance toward a pro-angiogenic state leading to vasoproliferation and retinal NV.

In most neovascular retinal diseases, such as DR and ROP, the development of retinal NV can be divided into two stages: (1) an ischemic phase characterized by extensive vasodegeneration characterized by retinal endothelial cell/pericyte loss, capillary dropout, and vascular dysfunction that lead to insufficient blood supply in retinal tissue; and (2) a proliferative phase featured by new blood vessel formation. An intrinsic link between these two stages is ischemia-driven over-production of angiogenic stimulators, such as VEGF, in retinal cells (e.g. retinal neurons, astrocytes and Müller cells) (Reviewed in (Zhang and Ma 2007)). These factors promote retinal endothelial proliferation and migration leading to new vessel formation, but also cause increased permeability and vascular leakage. These sequential but paradoxical changes in vasodegeneration and vasoproliferation reflect the complexity of the pathogenesis of retinal NV and also result in difficulties in the development of new therapies for ischemic retinal diseases. For example, treatment with angiogenic factors, e.g. erythropoietin (EPO), in the early stages suppresses retinal endothelial cell death and prevents NV development, however, such treatment would likely promote pathological angiogenesis when given at a late stage (Chen, Connor et al. 2008). The critical timing for pro- or anti-angiogenic therapies limits their clinical application in ischemic retinal diseases.

In addition to a critical role of endothelial cells in retinal NV formation, compelling evidence suggests that bone marrow-derived angiogenic cells are actively involved in vascular remodeling and repair of damaged blood vessels in ischemic retinas (reviewed in (Bhatwadekar, Shaw et al. 2010)). These cells, known as endothelial progenitor cells (EPCs), were first discovered in human peripheral blood in 1997 (Asahara et al. 1997) and display a great proclivity to home to the injury sites of blood vessels and participate in vascular repair and remodeling in adult animals (Asahara, Masuda et al. 1999). Defects in EPC function and/or reduction in EPC number have been observed in diabetic patients with vascular complications (Fadini, Miorin et al. 2005, Segal, Shah et al. 2006), suggesting that diminished capacity of the EPC to repair injured blood vessels may contribute to vasodegeneration in the development and progression of vascular complications in diabetes (Tepper, Galiano et al. 2002). Potential implications of the UPR in the regulation of endothelial cell and EPC function and in the processes of vasodegeneration, vascular remodeling and angiogenesis will be discussed in the following sections.

6. ER stress and neovascular retinal diseases

6.1 Diabetic retinopathy, VEGF production and ER stress

Diabetic retinopathy (DR) is a common microvascular complication of diabetes, characterized by vascular dysfunction, increased permeability, inflammation, vascular degeneration, and neovascularization. Up-regulation of VEGF is believed to be the major mediator of blood-retinal barrier dysfunction, which may occur in any stage of DR, and new vessel growth in the advanced stage. VEGF is a major growth factor that promotes angiogenesis through regulation of endothelial cell proliferation, migration, and tube formation (reviewed in (Ferrara 2009, Chung and Ferrara 2011)). It is also known as vascular permeability factor (VPF), which is 50,000 times more potent than histamine in inducing vascular permeability (Senger, Galli et al. 1983, Dvorak, Brown et al. 1995, Senger, Ledbetter et al. 1996). In addition, VEGF is a potent pro-inflammatory factor and up-regulates adhesion molecules in endothelial cells. Inhibition of VEGF expression using VEGF siRNA, or blockade of VEGF activity using VEGF neutralizing antibody or VEGF receptor chimeric proteins, successfully suppresses inflammation, blood retinal barrier breakdown, and vascular leakage in the diabetic retina (reviewed in (Luo, Tang et al. 2011)). Thus, VEGF is considered to be a key mediator of vascular damage and macular edema in DR.

In 2002, a study by Abcouwer and coworkers demonstrated that induction of ER stress by amino acid deprivation or homocysteine increases VEGF expression in human retinal pigment epithelial (RPE) cells (Abcouwer, Marjon et al. 2002, Roybal, Yang et al. 2004). This pioneering work revealed, for the first time, a role of ER stress in VEGF regulation in the eye. Likewise, induction of ER stress up-regulates VEGF in retinal capillary endothelial cells, and the induction was attenuated by overexpression of an ER chaperone named P58^{IPK} (Li, Li et al. 2008). In line with these *in vitro* studies, Li and colleagues first reported that ER stress is increased in the retinas from type 1 diabetic animals (Li, Wang et al. 2009). Induction of ER stress in the retina by intravitreal injection of tunicamycin was sufficient to induce retinal VEGF expression in mice. In parallel, exposure of retinal vascular endothelial

cells or Müller cells to tunicamycin or thapsigargin also induced a significant increase in VEGF expression (Li et al., 2009; Zhong et al., 2012a)(Chen, Wang et al. 2012). Conversely, reduction of ER stress attenuated VEGF production in cultured retinal cells and in the diabetic retina, as well as in the ischemic retina from mice with oxygen-induced retinopathy (OIR) (Li et al., 2009). Taken together, these results suggest that ER stress plays an important role in the regulation of VEGF in retinal cells and in DR. In good agreement with these results, increased ER stress in the retina was observed in type 1 (Oshitari, Yoshida-Hata et al. 2011, Fu, Wu et al. 2012, Yan, Zheng et al. 2012) and type 2 (Tang, Zhang et al. 2011) diabetic models and from diabetic patients, correlating with the severity of retinopathy (Fu, Wu et al. 2012, Du, Wu et al. 2013). Interestingly, these studies also suggest that the UPR pathways are differentially activated in retinal tissue and retinal cells in various diabetes models and correlate with the duration of diabetes. For example, the study from Yan et al. using qPCR analysis identified 89 ER stress-related genes in retinas from streptozotocin (STZ)-induced diabetic rats after 1 and 3 months of diabetes (Yan, Zheng et al. 2012). Compared to non-diabetic controls, a significant difference was observed in the expression levels of 13 genes and 12 genes in the 1st and 3rd months of diabetes, respectively. Among these genes, only two showed changes at both time points: *HERP* (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member) and *Erdj4* (a GRP78 co-chaperone and DnaJ family member involved in the ERAD pathway). Using various approaches, others have shown increased expression and/or phosphorylation of GRP78, (Li, Wang et al. 2009, Chen, Wang et al. 2012, Fu, Wu et al. 2012, Zhong, Li et al. 2012), IRE1 α (Li, Wang et al. 2009), eIF2 α (Li, Wang et al. 2009, Chen, Wang et al. 2012, Zhong, Li et al. 2012), PERK (Li, Wang et al. 2009, Oshitari, Yoshida-Hata et al. 2011, Chen, Wang et al. 2012, Zhong, Li et al. 2012), ATF4 (Li, Wang et al. 2009, Chen, Wang et al. 2012, Zhong, Li et al. 2012), CHOP (Li, Wang et al. 2009, Oshitari, Yoshida-Hata et al. 2011, Chen, Wang et al. 2012), and ATF6 (Tang, Zhang et al. 2011, Fu, Wu et al. 2012) in diabetic retinas, indicating activation of all three UPR pathways in DR. The discrepancies in UPR activation from diabetic retinas in these studies may be related to the difference in diabetes models (type 1 vs. type 2; chemical (STZ)-induced vs. genetic), duration of diabetes, species and strains of animals, and methods used to examine the UPR components. Nevertheless, these studies provide evidence of ER stress and activation of the UPR in DR (also reviewed in (Hu, Liu et al. 2012, Jing, Wang et al. 2012, Ma, Wang et al. 2014)).

A number of studies have attempted to investigate the role of the UPR pathways in the pathogenesis of DR, although most of these studies have concentrated on the IRE1-XBP1 and PERK-eIF2 α -ATF4-CHOP UPR pathways using cultured retinal cells in addition to animal models. In retinal Müller and vascular endothelial cells, high glucose is sufficient to induce ER stress and activate the IRE1 and PERK pathways; however, in retinal pericytes, only fluctuating glucose concentration, but not chronic (constant) high glucose, results in ER stress (Zhong, Li et al. 2012, Zhong, Wang et al. 2012). Reducing ER stress by treatment with chemical chaperones, such as 4-phenylbutyrate or tauroursodeoxycholic acid (TUDCA), suppresses high glucose-induced and glucose fluctuation-induced inflammatory factor expression, suggesting a role of ER stress in high glucose-induced inflammatory factor production from retinal cells. Furthermore, studies identified that activation of ATF4 is

essential for the induction of HIF-1 α and VEGF secretion by ER stress or high glucose in retinal Müller cells (Zhong, Li et al. 2012). Genetic inhibition of ATF4 activity attenuates diabetic-induced retinal inflammation and vascular leakage (Chen, Wang et al. 2012), suggesting that up-regulation of ATF4 contributes to retinal inflammation and endothelial barrier dysfunction in DR. More recently, Fu and associates demonstrated that heavily oxidized and glycated low-density lipoprotein (HOG-LDL) induces CHOP-dependent apoptotic pericyte loss via activation of oxidative stress and ER stress (Fu, Wu et al. 2012). Thus, induction of the ATF4/CHOP pathway may contribute to pericyte loss and vasodegeneration in DR; however, this speculation requires confirmation by further studies using suitable animal models of DR.

Finally, although there is much evidence of ER stress and UPR activation in DR, it remains unclear how the UPR pathways are implicated in retinal neovascularization (NV) formation in late-stage diabetes. This is largely attributed to the limitation of currently available diabetes animal models (reviewed in (Lai and Lo 2013)). While almost all rodent models of diabetes develop retinal neurovascular changes that mimic the early symptoms of non-proliferative DR, none of these models, except the recently reported Akimba mice (Rakoczy, Ali Rahman et al. 2010), develop retinal NV and NV-associated proliferative changes as seen human disease. Generated by crossing the Akita mouse (Ins2Akita), a type 1 diabetes model, with the Kimba mouse (VEGF^{+/+}) that transiently overexpress human VEGF in photoreceptors, Akimba mice develop retinal NV and more severe vaso- and neural degeneration in the retina (Rakoczy, Ali Rahman et al. 2010). Interestingly, this model has not been widely used in the field since its generation in 2010, and is yet to be tested for the molecular basis of diabetes-related retinal pathologies, including NV. A recent study from Kang Zhang's group (Luo, Zhao et al. 2013) examined the genetic association of transcription factor 7-like 2 (TCF7L2) and proliferative diabetic retinopathy (PDR) in human subjects. That study reported that in cells expressing the risk allele (rs7903146-TT), induction of ER stress results in higher expression of TCF7L2 and VEGF. This suggests that retinal cells from rs7903146-TT patients may be more sensitive to ER stress-associated VEGF up-regulation than normal, and therefore may have a greater chance of developing PDR.

6.2. Retinopathy of prematurity

In contrast to DR, which primarily causes vision loss in adults, retinopathy of prematurity (ROP), formerly known as retrolental fibroplasia, is the major ocular disorder of preterm birth infants and is the dominant cause of severe visual impairment in children in the industrialized world (Reynolds 2010, Sapienza, Joyal et al. 2010, Hellstrom, Smith et al. 2013). The disease is initiated with degeneration of the retinal microvasculature (vaso-obliteration) associated with cessation of the progression of vascular growth toward the peripheral retina, and then followed by the abnormal compensatory neovascularization in response to retinal ischemia. Eventually, uncontrolled retinal NV leads to vitreous hemorrhage and tractional retinal detachment, resulting in blindness. Even in the milder form of ROP, the incidence of emmetropias, refractive errors, strabismus, and disorders of color discrimination is increased compared to normal subjects. During the past 30 years, new and exciting mechanisms in the genesis of the ROP have been identified, such as

activation of oxidative stress, dysregulation of pro- and anti-angiogenic factors, induction of pro-inflammation factors, and metabolic disorder (Sapieha, Joyal et al. 2010, Hartnett and Penn 2012, Hellstrom, Smith et al. 2013). These mechanisms, to some extent, have deepened our understanding of retinal angiogenesis as well.

The oxygen-induced retinopathy (OIR) model has been widely used as a valuable tool in the research of retinal NV, which to some extent reproduces the vaso-oblivation and neovascularization stages of ROP (Stahl, Connor et al. 2009). Using this model, several groups have demonstrated that ER stress is induced in the ischemic retina and contributes to retinal NV formation (Li, Wang et al. 2009, Nakamura, Takizawa et al. 2013, Zeng, Tallaksen-Greene et al. 2013). Li et al. showed that both mRNA and protein levels of GRP78/Bip are increased in P15 OIR retinas, prior to the significant increase of VEGF and the peak of hyperpermeability (Zhang, Ma et al. 2005, Zhang, Wang et al. 2006), accompanied by activation of the p-eIF2 α /ATF4 pathway (Li, Wang et al. 2009). Treatment with low doses of ER stress inducers accelerates retinal NV; this effect likely occurs via induction of GRP78 (Nakamura, Takizawa et al. 2013). Consistently, increased levels of p-IRE1 α , p-PERK, GRP78, and ATF4 were observed in neuronal retinas from P14 OIR mice, localized predominantly in the avascular zone, suggesting that the UPR may be activated in retinal neurons by hypoxia/ischemia (Binet, Mawambo et al. 2013). Likewise, ER stress was observed in the OIR retina using an ER stress reporter (ERAI) mouse line (Zeng, Tallaksen-Greene et al. 2013); interestingly, the signal of ER stress, indicated by GFP protein expression driven by XBP1 splicing, intensified around the abnormal neovasculature in retinal whole mounts. These results, together with previous findings that ER stress regulates VEGF expression in various retinal cells (Li, Li et al. 2008, Li, Wang et al. 2009, Chen, Wang et al. 2012, Zhong, Li et al. 2012), indicate that activation of the UPR by ER stress mediates VEGF up-regulation and retinal NV in OIR. Indeed, genetic manipulation of ATF4 using heterozygous ATF4 knockout mice reduces the area of neovascularization with increased avascular zone (Zeng, Tallaksen-Greene et al. 2013). Furthermore, Binet et al. showed that inhibition of IRE1 α by lentiviral vector carrying small hairpin RNAs (Lv. shIRE1 α), but not inhibition of PERK by Lv. shPERK, accelerates retinal revascularization while decreasing both the avascular area and the neovascular area (Binet, Mawambo et al. 2013). This suggests a vital role for IRE1 in regulation of retinal vascular remodeling and angiogenesis. Interestingly, this effect appears to be mediated by the endoribonuclease activity of IRE1, which degrades netrin-1, a protein secreted from retinal ganglion cells (RGCs) that guide neurovascular repair. According to this study, prolonged ER stress in RGCs results in netrin-1 degradation, which fails to activate the reparative pro-angiogenic programs in microglial cells, and thus hinders regeneration of the normal retinal vasculature. Supplementation of netrin-1 as well as inhibition of ER stress thereby may confer beneficial effects to promote vascular regeneration in ischemic retinal disease.

6.3. Inflammation-related angiogenesis and age-related macular degeneration

Ample evidence suggests that inflammation plays a critical role in the development of a wide range of neovascular retinopathies, such as DR (Luo, Tang et al. 2011, Zhang, Sanders et al. 2011), Eales' disease (ED) (Murugeswari, Shukla et al. 2008, Sen, Paine et al. 2011), uveitis (Pan, Kapur et al. 2014), and age-related macular degeneration (AMD) (Cherepanoff,

McMenamin et al. 2010, Parmeggiani, Romano et al. 2012). Increasing levels of a variety of inflammatory mediators, such as tumor necrosis factor (TNF)- α , VEGF, interleukin (IL)-6, IL-8, endothelin (ET)-1, and monocyte chemotactic protein (MCP)-1, have been found in the retinas or vitreous from patients with retinal NV (Yoshimura, Sonoda et al. 2009). Interestingly, anti-VEGF therapy blocks inflammatory cell infiltration and re-entry into the circulation in both retinal (Nakao, Arima et al. 2012) and corneal angiogenesis (Nakao, Zandi et al. 2012). These findings suggest that a vicious cycle is formed by inflammatory mediators and angiogenic factors in retinal diseases.

New observations suggest that the UPR can initiate inflammation, and the coupling of these signaling events in specialized cells and tissues is now thought to be fundamental in the pathogenesis of inflammatory diseases (Zhang and Kaufman 2008, Civelek, Manduchi et al. 2009, Adolph, Niederreiter et al. 2012). For example, during the development of atherosclerosis, dysfunction of the ER and subsequent activation of the UPR can be induced by the accumulation of oxidized lipids in endothelial cells and macrophages, which further activate JNK and NF- κ B signaling, resulting in expression of inflammatory cytokines (including TNF- α and IL-6) (Li, Schwabe et al. 2005, Sanson, Auge et al. 2009). In human aortic endothelial cells, ATF4 and XBP1 are required for the production of the inflammatory cytokine IL-6 and the chemokines IL-8, CC-chemokine 2 (CCL2), and chemokine (C-X-C motif) ligand 3 (CXCL3) (Gargalovic, Gharavi et al. 2006). In cultured retinal endothelial cells (Chen, Wang et al. 2012), Müller cells (Zhong, Li et al. 2012), as well as in mouse retinas (Li, Wang et al. 2009), induction of ER stress is sufficient to trigger inflammation and up-regulation of inflammatory mediators, such as TNF- α , ICAM-1, and VEGF. In contrast, inhibiting ER stress by the use of chemical chaperones ameliorates inflammation induced by hypoxia (Li, Wang et al. 2009), hyperglycemia (Chen, Wang et al. 2012), and 4-hydroxy-*trans*-2-nonenal (HNE) (Vladykovskaya, Sithu et al. 2012) in endothelial cells or blood vessels, suggesting that ER stress is a contributing factor to endothelial and vascular inflammation.

CNV formation resulting from new blood vessel growth from the choroid is a hallmark lesion in wet AMD and is a major cause of vision loss in the elderly. As reviewed previously, CNV formation is closely related with VEGF increase and the imbalance between pro-angiogenic and anti-angiogenic factors (Zhang and Ma 2007). Yet, compelling evidence suggests that dysregulated inflammation plays a critical role in CNV pathogenesis (reviewed in (Ozaki, Campbell et al. 2014). A recent study showed that monocyte chemotactic protein-induced protein (MCPIP) mediates inflammation-associated angiogenesis likely through sequential induction of oxidative stress, ER stress, and autophagy in endothelial cells (Roy and Kolattukudy 2012). Likewise, 7-ketocholesterol (7kCh), one of the major toxic and inflammatory components of oxidized lipoprotein deposits in AMD (Huang, Amaral et al. 2012, Rodriguez, Clark et al. 2014), induces ER stress and enhances the production of pro-inflammatory factors, such as VEGF, IL-6 and IL-8 from the RPE, which in turn promotes CNV formation (Moreira, Larrayoz et al. 2009, Rodriguez and Larrayoz 2010). Oxidized phospholipids (OxPLs), another common class of lipid oxidation products, which accumulate in RPE and photoreceptors with increasing level in older eyes or eyes from AMD patients (Suzuki, Kamei et al. 2007, Suzuki, Tsujikawa et

al. 2012), have been shown to possess pro-inflammatory and pro-angiogenic properties (Bochkov, Oskolkova et al. 2010, Amaral, Lee et al. 2013, Huang, Amaral et al. 2014). These oxidized lipids induce VEGF production in RPE cells likely through the UPR downstream transcription factor ATF4 (Pollreisz, Afonyushkin et al. 2013). More recently, Liu and colleagues reported that the combination of siRNA targeting the IRE1 α /XBP1 and ATF6 pathways with anti-VEGF treatment achieved strong inhibition of CNV formation in a laser-injured mouse model (Liu, Qi et al. 2013). These findings provide exciting evidence that the UPR pathways may regulate VEGF and inflammation contributing to the pathogenesis of CNV.

7. UPR signaling in regulation of angiogenic process

A clear interaction between UPR signaling and the angiogenic process is evident in a variety of otherwise disparate diseases, such as cancer, retinal angiogenesis, atherosclerosis, and ischemic renal disease (McAlpine, Bowes et al. 2010, Salminen, Kauppinen et al. 2010, Zhang, Sanders et al. 2011, Schonthal 2012). Knowledge about the role of the UPR in angiogenesis could lead to the development of new therapeutics that target angiogenesis by inhibiting specific ER stress mediators.

7. 1. IRE1 and angiogenesis

Recently, the IRE1/XBP1 pathway has been shown to contribute to angiogenesis and tumor growth (Romero-Ramirez, Cao et al. 2004, Drogat, Auguste et al. 2007, Romero-Ramirez, Cao et al. 2009), and emerging evidence also suggests a potential role of this branch of the UPR in regulation of retinal NV (Binet, Mawambo et al. 2013, Zeng, Xiao et al. 2013) (**Figure 1**). In 2007, the results of a study by Drogat and coworkers indicated that IRE1 α is required for hypoxia-induced tumor angiogenesis (Drogat, Auguste et al. 2007). Tumor cells expressing a dominant-negative IRE1 α transgene as well as IRE1 α -null mouse embryonic fibroblasts were unable to trigger VEGF up-regulation upon either oxygen or glucose deprivation (modeling hypoxia and hypoglycemia). This finding for the first time established the functional link between IRE1 activity and VEGF up-regulation (Drogat, Auguste et al. 2007). Since then, a large body of evidence has confirmed the role of IRE1 as a key regulator of angiogenesis in tumor survival and metastasis, placental development, and embryonic viability (Iwawaki, Akai et al. 2009, Romero-Ramirez, Cao et al. 2009, Auf, Jabouille et al. 2010). IRE1 α -null mice exhibit low VEGF production and impaired angiogenesis in the placenta, despite greater activation of the PERK/eIF2 α and ATF6 pathways and increased levels of HIF-1 α (Iwawaki, Akai et al. 2009). Moreover, loss of XBP1 produced no effect on the expression levels of VEGF in the placenta, suggesting that the effects of IRE1 α on VEGF regulation are XBP1-independent (Iwawaki, Akai et al. 2009). Using Ire1 α ^{-/-} and Perk^{-/-} mouse embryonic fibroblasts and ATF6 α -knockdown HepG2 cells, Ghosh and associates showed that induction of VEGF-A mRNA by ER stress is attenuated as compared to control cells (Ghosh, Lipson et al. 2010). Rescue of IRE1 α and PERK in Ire1 α ^{-/-} and Perk^{-/-} cells, respectively, also prevented VEGF-A mRNA attenuation. These results indicate that these UPR branches are involved in the regulation of VEGF production (Ghosh, Lipson et al. 2010).

Apart from mediating XBP1 splicing, activated IRE1 α possesses a kinase activity that facilitates its ability to recruit TRAF2 and, in turn, activates stress kinases, such as JNK and I κ B (inhibitor of κ B) kinase (IKK) (**Figure 1**) (reviewed in (Hetz and Glimcher 2009, Hetz, Martinon et al. 2011)). These kinases have been shown to play a pro-angiogenic role, independent of XBP1 splicing. Furthermore, emerging evidence shows that, besides splicing XBP1 mRNA, activated IRE1 α degrades micro-RNA and mRNA from other genes by virtue of its endoribonuclease activity. In addition to VEGF-A, impaired IRE1 activity resulted in a multifold decrease in the expression of several potent pro-angiogenic factors, including IL-1 β , IL-6, and IL-8 (Auf, Jabouille et al. 2010). Furthermore, recent studies have shown that IRE1 α regulates a family of small non-coding RNA molecules that coordinate pro- and anti-angiogenic factor expression to modulate angiogenesis. For example, hyper-activated IRE1 α increases the degradation of miR-17, a microRNA that destabilizes thioredoxin-interacting protein (TXNIP), resulting in stable TXNIP over-expression. In turn, TXNIP promotes pro-angiogenic or inflammatory genes, such as VEGF-A, ICAM-1, and cyclooxygenase (COX)-2, as well as IL-1 β maturation and secretion (Perrone, Devi et al. 2009, Lerner, Upton et al. 2012). In the neural retina, hypoxia-activated IRE1- α degrades netrin-1 mRNA, which modulates the pro-angiogenic properties of macrophage/microglial cells, resulting in disturbed vascular regeneration of the retina and enhanced pathologic neovascularization (Binet, Mawambo et al. 2013). Conversely, siRNA knockdown of IRE1 α remarkably reduces VEGF-stimulated tube formation of retinal endothelial cells and diminishes retinal and choroidal NV in mouse models (Liu, Qi et al. 2013). These data collectively demonstrate a role for IRE1 in the regulation of angiogenesis, involving both its kinase and the endoribonuclease domains. Future studies are needed to provide a better understanding of the potentially distinct roles of the kinase activity of IRE1 α from its endoribonuclease activity to further dissect out the downstream pathways in IRE1 α -mediated pro-angiogenic effects on blood vessels and endothelial cells. Targeting one or the other of these specific IRE1 α activities may provide novel avenues for therapeutic intervention into pathological NV.

7.2. XBP1 and angiogenesis

XBP1 was first discovered as an essential transcriptional factor that regulates the maturation of B lymphocytes (reviewed in (Schebesta, Heavey et al. 2002)). In the past two decades, additional and novel functions of XBP1 have been revealed, and more continue to be disclosed (Glimcher 2010). Recent studies have demonstrated that XBP1 is essential for angiogenesis during early tumor growth. Inhibiting XBP1 expression by shRNA reduces blood vessel formation in tumors from mouse embryonic fibroblast cells and human fibrosarcoma tumor cells (Romero-Ramirez, Cao et al. 2004), while expression of spliced XBP1 (XBP1s) restores angiogenesis in cells lacking functional IRE1 α (Romero-Ramirez, Cao et al. 2009). Pereira and colleagues reported that ER stress-stimulated up-regulation of VEGF is mediated by IRE1 α /XBP1, but not through the ATF6 pathway (Pereira, Liao et al. 2010). Furthermore, they have shown that spliced XBP1 binds to two different sites (~1.9kb and ~5.2kb upstream of start codon) in the promoter of the VEGF-A gene by using a chromatin immunoprecipitation (ChIP) assay. These results illustrate the complex nature of regulating VEGF expression by multiple upstream genes and their co-factors, which may vary in different cells and tissues as well as under different physiological conditions.

Interestingly, although XBP1 may bind to the promoter region of VEGF and up-regulate its transcription in mouse embryonic fibroblasts (Ghosh, Lipson et al. 2010) and RPE cells (Miyagi, Kanemoto et al. 2013), the pro-angiogenic effect of XBP1s seems to be independent of VEGF (Romero-Ramirez, Cao et al. 2009). Yet, exactly how XBP1 regulates endothelial cell activity and the angiogenic process remains poorly understood. To examine the effects of XBP1 on endothelial cells, Zeng and associates generated endothelial cell-specific XBP1 knockout (*XBP1^{ecto}*) mice. These mice exhibited impaired angiogenesis in response to ischemia (Zeng, Xiao et al. 2013). Furthermore, that study showed that XBP1s regulates endothelial cell proliferation in a PI3K/Akt/GSK3 β / β -catenin/E2F2-dependent manner (Zeng, Xiao et al. 2013). In another study, deletion of XBP1 using siRNA delivered by self-complementary adeno-associated virus serotype 2 (scAAV2) vectors resulted in a ~70% inhibition of tubule formation and an induction of apoptosis in mouse microvascular endothelial cells (Ruan, Xi et al. 2013). Collectively, these studies support an important role of XBP1 in endothelial cells and angiogenesis.

In contrast to the evidence that XBP1 has a pro-angiogenic role, Nakamura and colleagues showed that low doses of ER stress inducers, such as tunicamycin or thapsigargin, increased the proliferation and migration of retinal endothelial cells and induced retinal NV *without* activation of XBP1 (Nakamura, Takizawa et al. 2013). Sustained activation of XBP1 results in apoptosis of endothelial cells by activation of a caspase-dependent pathway, inhibition VE-cadherin transcription (Zeng, Zampetaki et al. 2009), and increasing Beclin-1-mediated autophagy (Margariti, Li et al. 2013). Banerjee and coworkers demonstrated that induction of the UPR by tunicamycin inhibits angiogenesis both *in vitro* and *in vivo* via up-regulating thrombospondin-1 (TSP-1, an endogenous angiogenic inhibitor) and by suppressing VEGF-induced activation of VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2) (Banerjee, Lang et al. 2011). These results are consistent with our finding (S.X. Zhang, unpublished results) that over-expression of XBP1 inhibits retinal endothelial cell proliferation, migration, and tube formation, suppresses the VEGF-activated VEGFR2 signaling pathway *in vitro*, and attenuates retinal neovascularization in a mouse model of OIR *in vivo*. This indicates that the variable components in the UPR pathways differentially regulate expression of pro-angiogenic and anti-angiogenic signaling in endothelial cells. Further insights into the regulation of the angiogenic process by UPR genes may identify underlying and heretofore unknown mechanisms that regulate the pathogenesis of retinal ischemia and neovascularization.

7.3. PERK/eIF2 α and angiogenesis

In addition to the IRE1 pathway, the PERK/ATF4-UPR branch also has been implicated in the regulation of angiogenesis (**Figure 2**). For example, PERK has been shown to regulate a subset of pro-angiogenic transcripts in tumor cells under hypoxic stress, such as VEGF and type 1 collagen-inducible protein (VCIP), an adhesion protein that promotes cell adhesion, spreading, and integrin binding within the tumor endothelium (Blais, Addison et al. 2006). Tumors derived from K-Ras-transformed PERK^{-/-} mouse embryonic fibroblasts are smaller and exhibit less angiogenesis than tumors with an intact integrated stress response. These findings implicate PERK in the angiogenic process. During ER stress, activated PERK phosphorylates eIF2 α , which in turn contributes to the activation of nuclear factor κ B (NF-

κ B), a potent transcriptional factor that regulates angiogenesis and inflammation in proliferative retinal diseases (Zhang and Kaufman 2008). In these conditions, increased phosphorylation of eIF2 α reduces protein translation of I κ B, the repressor of NF- κ B, resulting in elevated NF- κ B activation (Deng, Lu et al. 2004). In addition, p-eIF2 α can directly phosphorylate I κ B, thereby promoting its degradation and, consequently, NF- κ B activation (Jiang, Wek et al. 2003). The exact effects of PERK/eIF2 α on endothelial cell activity and retinal angiogenesis are yet to be fully elucidated.

7.4. ATF4 and angiogenesis

Compared to PERK and eIF2 α , ATF4, a downstream effector of the PERK/eIF2 α pathway (**Figure 2**), has been more extensively studied with respect to angiogenesis and VEGF regulation. ATF4 can be activated by hyperglycemia (Chen, Wang et al. 2012), hypoxia (Li, Wang et al. 2009), and oxidized phospholipids (OxPLs) (Oskolkova, Afonyushkin et al. 2008) in endothelial cells. Activation of ATF4 results in the transcriptional up-regulation of VEGF and MCP1, IL-6, IL-8, and CXCL3 (**Figure 2**) (Gargalovic, Gharavi et al. 2006, Afonyushkin, Oskolkova et al. 2010, Ghosh, Lipson et al. 2010). In the retina, reduced ATF4 expression attenuates diabetes-induced retinal inflammation and vascular leakage (Chen, Wang et al. 2012, Zhong, Li et al. 2012) and alleviates retinal NV in OIR (Zeng, Tallaksen-Greene et al. 2013). Mechanistically, ATF4 cross-talks with multiple signaling pathway molecules implicated in angiogenesis, such as STAT3 (Chen, Wang et al. 2012), HIF-1 α , and JNK (Zhong, Li et al. 2012). In RPE cells, OxPLs-stimulated VEGF is largely dependent on ATF4 (Pereira, Frudd et al. 2013), where it directly binds to the AARE site in the VEGF gene and up-regulates VEGF expression (Roybal, Hunsaker et al. 2005). In retinal Müller cells, ATF4 is sufficient to induce JNK activation, and inhibition of JNK attenuates ATF4-mediated VEGF up-regulation (Zhong, Li et al. 2012). Moreover, ATF4 stabilizes HIF-1 α under ER stress and hypoxia, and is essential for HIF-1 α activation and VEGF expression in these conditions (Zhong, Li et al. 2012). Interestingly, ATF4 induced by ER stress may function synergistically with HIF-1, which is mainly activated by hypoxia, in up-regulation of VEGF expression (Pollreisz, Afonyushkin et al. 2013). In addition, several new molecules were recently identified as being involved in ATF4-mediated angiogenesis, including aryl hydrocarbon receptor (AhR) (Terashima, Tachikawa et al. 2013), nuclear factor erythroid-derived 2-related factor 2 (Nrf2) (Afonyushkin, Oskolkova et al. 2010), and general control non-derepressible 2 (GCN2, an AAD sensor) kinase (Wang, Ning et al. 2013). Taken together, these results point to a linkage of ATF4 to a wide range of crosstalk between the UPR and different pro-angiogenic pathways.

In contrast to its pro-angiogenic effect, studies have shown that ATF4 promotes endothelial cell death by inducing CHOP, the major pro-apoptotic gene that mediates ER stress-associated apoptotic cell death (Csordas, Kreutmayer et al. 2011). CHOP, in turn, activates caspase-3 and -4 and interacts with other pro- and anti-apoptotic molecules to modulate the apoptosis program (reviewed in (Jing, Wang et al. 2012)). In these cases, PERK/p-eIF2 α /ATF4 is a very important pathway leading to endothelial apoptosis and vascular degeneration (Kyriakakis, Philippova et al. 2010, Scull and Tabas 2011). In different cell types or upon exposure to different stress inducers, ATF4 may activate distinct downstream signaling pathways, thereby resulting in diverse effects on cell survival, proliferation, and

migration. It is possible that activation of ATF4 participates in both vascular degeneration as well as retinal NV formation in neovascular retinal diseases such as DR, where *in situ* endothelial cell dysfunction and apoptosis are common initial changes and vascular degeneration eventually leads to ischemia and angiogenesis.

7.5. ATF6 and angiogenesis

ATF6 acts differently than the other two major UPR transducers (IRE1 and PERK), as it is packaged and trafficked to the Golgi apparatus instead of remaining in the ER and acting via cytoplasmic effectors (**Figure 3**) (Haze, Yoshida et al. 1999, Schindler and Schekman 2009). Cleaved ATF6, which is a basic leucine zipper (bZIP)-containing transcription factor, moves to the nucleus and binds to the promoter of ER stress response element-related genes encoding ER chaperones, enzymes, and transcription factors, such as GRP78, GRP94, and protein disulfide isomerase (PDI) (Haze, Yoshida et al. 1999, Wang, Shen et al. 2000, Vekich, Belmont et al. 2012), XBP1 (Yoshida, Matsui et al. 2001), and CHOP (Yoshida, Okada et al. 2000). It has been demonstrated that ATF6 can be activated by VEGF, hyperglycemia, hydrogen peroxide (Liu, Qi et al. 2013) and oxidation of lipids (Vladykovskaya, Sithu et al. 2012) in endothelial cells, and is involved in cell proliferation and inflammation (**Figure 3**). ATF6 increases VEGF transcription by binding to its promoter (Ghosh, Lipson et al. 2010). Ruan et al. demonstrated that knockdown of ATF6 causes up to a 50% reduction in tubule formation with an induction of apoptosis in endothelial cells, and effectively inhibits angiogenesis in breast cancer xenograft models (Ruan, Xi et al. 2013). A similar effect of ATF6 knockdown was observed in Liu's study: decreasing ATF6 levels reduced VEGF-induced tubule formation in retinal endothelial cells. Targeting the ATF6 arm in OIR or CNV mouse models by siRNA also was shown to cause approximately a 35% reduction in angiogenesis, and a further 25% to 40% reduction can be achieved when combined with a VEGF-neutralizing antibody (Liu, Qi et al. 2013).

Compared to the other two branches of the UPR pathway, the current state of knowledge regarding exactly how ATF6 regulates angiogenesis is limited, although its downstream target proteins have been confirmed as crucial factors in angiogenesis. For example, GRP78 is considered to be a critical regulator in tumor proliferation, survival, and angiogenesis (Pfaffenbach and Lee 2011). Dong and associates found that under conditions of GRP78 heterozygosity (GRP78^{+/-}), where the GRP78 expression level was reduced by about half, this had no effect on organ development or antibody production, but significantly impeded tumor growth in mice, partially by substantially reducing tumor microvessel density (Dong, Ni et al. 2008). Kern et al. demonstrated that recombinant GRP78 can increase endothelial cell viability and resistance to bortezomib-induced apoptosis by increasing the phosphorylation of extracellular signal-regulated kinase (ERK) and Akt, which are mediators of endothelial cell proliferation and migration, and inhibiting p53-mediated induction of Bcl2 family pro-apoptotic proteins, BOK and NOXA (Kern, Untergasser et al. 2009). Recent research also has shown that, besides ER retention by GRP78, cell-surface GRP78 functions as a receptor, augmenting growth factor signaling and apoptosis signaling (Nakatsuka, Wada et al. 2013). Recruitment of GRP78 from the ER to the cell surface is facilitated by ER stress inducers. GRP78 interacts with plasma membrane-anchored proteins, such as voltage dependent anion channel (VDAC), and localizes on the surface of

endothelial cells. This interaction is thought to be a potential mechanism that promotes endothelial cell proliferation, inhibits apoptosis, and protects against vascular injuries under stress conditions, such as diabetes. GRP78 also facilitates survival signaling by increasing Akt phosphorylation (Nakatsuka, Wada et al. 2013). Masking cell surface GRP78 by treating cells with anti-GRP78 antibody suppresses cell growth by reducing PI3K/Akt activation (Misra and Pizzo 2010).

Another protein downstream of ATF6, α -basic crystallin (CRYAB), has been revealed as a potent pro-angiogenic factor (**Figure 3**) (Kase, He et al. 2010, Ruan, Han et al. 2011, Liu, Qi et al. 2013). CRYAB belongs to the small heat shock protein (sHSP) family, which responds to the UPR and facilitates protein refolding. It has been demonstrated that inhibition of IRE1 or ATF6 leads to attenuated CRYAB expression in endothelial cells (Ruan, Han et al. 2011). Deletion of CRYAB decreases retinal VEGF levels and reduces the extent of retinal and choroidal NV in OIR and in laser-induced CNV, respectively (Kase, He et al. 2010). Conversely, overexpression of CRYAB promotes endothelial cell proliferation and survival, while knockdown of CRYAB results in endothelial cell apoptosis (Ruan, Han et al. 2011). Further experimental results suggest that CRYAB is vital for protecting VEGF from proteolytic degradation (Kase, He et al. 2010, Liu, Qi et al. 2013), and loss of CRYAB also may reduce autocrine VEGF production (Ruan, Han et al. 2011). Taken together, these results indicate that ATF6 regulates a subset of UPR response genes, which have been demonstrated or might be revealed to be functioning as pro- or anti-angiogenic factors. Additional functions of ATF6 remain to be identified.

8. ER stress, angiogenic progenitor cells and vascular repair

In recent years, the role of EPCs in the pathogenesis and treatment of neovascular retinal diseases has attracted considerable attention (Caballero, Hazra et al. 2013). Although many elegant studies have revealed novel and insightful mechanisms underlying EPC dysfunction (Caballero, Sengupta et al. 2007, Bhatwadekar, Shaw et al. 2010), we are still far from understanding exactly how EPCs are regulated under normal and diseased conditions. Emerging evidence suggests that the UPR and ER chaperones are involved in regulation of hematopoietic stem and progenitor cell (HSPC) survival (Luo, Lam et al. 2011) and differentiation (van Galen, Kreso et al. 2014). These exciting findings have shed some light on a potential role of the UPR in the regulation of EPC function and vascular repair in ischemic retinal diseases (**Figure 4**).

As mentioned briefly in Section 5, EPCs are bone marrow-derived precursor cells that can differentiate into endothelial cells during vasculogenesis and vascular remodeling (Khakoo and Finkel 2005). However, currently there is no unique cell surface marker(s) that can clearly define EPCs in humans and mice. In some studies, putative EPCs were identified as one specific subset of CD34⁺ cells (CD34⁺/VEGFR2⁺/KDR⁺/CD133⁺) derived from hemangioblasts, thereby sharing similar characteristics with embryonic angioblasts (Schattenman and Awad 2004); however, recent evidence suggests that these cells represent an enriched population of hematopoietic precursors and do not differentiate into endothelial cells (Case, Mead et al. 2007). In other studies, EPCs are considered as CD34⁻/CD133⁺/VEGFR2⁺ cells derived from bone marrow multipotent progenitor cells (Capiod, Tournois

et al. 2009). These cells differentiate into endothelial cells when grown in serum free condition in the presence of VEGF (Reyes, Dudek et al. 2002). Last, but not the least, EPCs can be derived from bone marrow myelo/monocytic CD14⁺ cells, which differentiate into endothelial cells expressing VEGFR2, CD45 (leucocyte common antigen) and vWF in culture (Khakoo and Finkel 2005). Nevertheless, nearly all EPCs, regardless of their origins, exhibit the ability to bind to *Ulex europaeus* agglutinin 1 (UEA1) and assimilate acetylated LDL (ac-LDL) (Rehman, Li et al. 2003). All three groups of progenitors—hemangioblasts, multipotent adult progenitor cells, and myelo/monocytic cells—can form mature endothelial cells *in vivo*. Furthermore, two subtypes of EPCs have been characterized in *in vitro* models: the early EPCs that express monocytic surface markers (CD45, CD11c, CD11b and CD14) and the late EPCs that express CD34 and VEGFR2 but lack CD14 and CD45 (reviewed in (Khakoo and Finkel 2005, George, Bangalore-Prakash et al. 2011)).

HSPCs are multipotent stem cells that give rise to all the lineages of blood cells and EPCs, and possess the capacity of self-renewal (Wey, Luo et al. 2012). In the adult hematopoietic system, regulation of HSPC survival, renewal and differentiation is governed by both intrinsic genes and the HSPC niche, which refers to the specialized local environment that maintains the HSPCs in the dormant state and thereby preserves their self-renewal capacity (Wey, Luo et al. 2012) (**Figure 4**). Like other stem cells (mesenchymal and neural stem cells), HSPCs require a hypoxic niche for their maintenance in an undifferentiated state as well as for proliferation and lineage commitments (Orkin and Zon 2008, Simon and Keith 2008, Mohyeldin, Garzon-Muvdi et al. 2010). In spite of the hyper-vascularity of the bone marrow cavity, the oxygen tension is as low as in venous blood, owing to high levels of oxygen consumption by the proliferating HSPCs (Nombela-Arrieta, Pivarnik et al. 2013). Conditional knock-out of the *Hif1 α* gene leads to increased levels of reactive oxygen species (ROS) in HSPCs and depletion of the cells, which suggests that HIF1 α is indispensable for stem cell function in the hematopoietic system (Imanirad, Solaimani Kartalaei et al. 2014). In addition, studies have shown that HSPCs are generally quiescent cells that are sensitive to intracellular ROS (Orkin and Zon 2008). These cells possess higher levels of anti-oxidant enzymes than differentiated endothelial cells in order to ensure self-renewable capacity (Yang, von Ballmoos et al. 2010).

HSPC/EPC mobilization and homing relies on a gradient of proteins and cytokines, such as VEGF, CXCL12 (stromal cell derived factor-1 α (SDF-1 α)), CXCL-8 (IL-8), and granulocyte monocyte-colony stimulating factor (GM-CSF) (**Figure 4**). Nitric oxide (NO) and the NO synthase enzyme eNOS are also required for the migration of bone marrow-derived cells. NO activates matrix metalloproteinase (MMP)-9 (also known as gelatinase B, 92 kDa gelatinase, and 92 kDa type IV collagenase), which cleaves the soluble c-Kit ligand and switches the resident bone marrow cells from dormant to proliferative niches and further facilitates the migration of the cells into the circulation (Shintani, Murohara et al. 2001, Heissig, Hattori et al. 2002). GM-CSF has been shown to stimulate proliferation, migration, and tube formation capacity of HSPCs and EPCs mediated in part by up regulation of VEGF and integrin β 2 and ERK signaling (reviewed in (Kawamoto and Losordo 2008))(Qiu, Xie et al. 2014). Several other factors, such as CXCL-8 (Schomig, Busch et al. 2006), CXCL1 and CXCL7 (Hristov, Zernecke et al. 2007), also modulate EPC mobilization, proliferation, and

homing to the site of vascular injury. In addition, CXCL12 promotes HSPC and EPC mobilization and recruitment, and thereby has been implicated in peripheral vasculogenesis and tissue repair in diabetes (Peled, Grabovsky et al. 1999).

Several studies have examined the mechanisms of HSPC/EPC dysfunction in mouse models of type 1 and type 2 diabetes, and the results suggest that diabetes-induced defects in angiogenic progenitors are causally associated with increased oxidative stress, apoptosis, and DNA damage (Reviewed in Oikawa et al. (Oikawa, Siragusa et al. 2010) and Bhatwadekar et al. (Bhatwadekar, Shaw et al. 2010)) (**Figure 4**). Diabetes reduces the level eNOS, resulting in diminished eNOS activity and decreased NO production, which leads to disturbance in EPC mobilization (Jarajapu, Caballero et al. 2011). In addition, diabetes increases eNOS uncoupling and NADPH oxidase expression, resulting in increased ROS generation. Superoxide, in turn, interacts with NO to form peroxynitrite (ONOO^-); this leads to the loss of bioavailable NO and exacerbated oxidative stress (Szabo, Mabley et al. 2002, Camici, Schiavoni et al. 2007). In addition, there is evidence suggesting that reduced release of VEGF and CXCL12 from ischemic tissues contributes to defective EPC migration in diabetic condition (Fadini, Sartore et al. 2006). In the bone marrow, diabetes increases the level of ROS, which leads to decreased hypoxia, thereby disrupting the stem cell niche. Blocking ROS generation by inhibiting NADPH oxidase reduces the signs of diabetic EPC dysfunction (Jarajapu, Caballero et al. 2011). Given the close interrelations between ER stress and oxidative stress, we speculate that increased ROS generation by diabetes may, in turn, induce ER stress resulting in cell apoptosis and/or disturbed equilibrium between the self-renewal and differentiation of bone marrow progenitor cells (**Figure 4**). Future studies will test this hypothesis.

Although currently there is no experimental evidence supporting a role of ER stress in diabetes-induced EPC defects, recent studies in the stem cell field suggest that ER stress and the UPR are implicated in the process of stem cell differentiation. For instance, ER stress was induced in hematopoietic CD34^+ cells isolated from cord blood when treated with thrombopoietin to promote megakaryocyte differentiation and maturation (Lopez, Palazzo et al. 2013). Moreover, induction of ER stress was observed in embryonic stem cells and in bone marrow stromal cells when these cells were differentiated into neurons (Cho, Jang et al. 2009). Additionally, increased ER stress is potentially correlated with apoptosis and loss of the “stemness” (*i.e.*, stem cell-like properties) of stem/progenitor cells. As discussed above, GRP78 is a molecular chaperone that acts as an inhibitor of ER stress and a repressor of the UPR. It is also a major transcriptional target of the UPR; thus, increased GRP78 level has been used widely as an indicator of ER stress signalling. Immunohistochemical analysis has shown decreased GRP78 expression in intestinal stem cells as compared to other differentiated cells, and conditional knockout of GRP78 in the intestinal epithelium results in a loss of stemness due to increased UPR in a PERK-eIF2 α -dependent manner (Heijmans, van Lidth de Jeude et al. 2013). In the hematopoietic system, conditional deletion of GRP78 leads to a decrease in the bone marrow pool of hematopoietic stem cells (HSCs) (Wey, Luo et al. 2012). Also, GRP78-deficient bone marrow cells exhibited constitutive activation of all the major UPR signaling pathways, including eIF2 α , ATF6, and XBP1 splicing accompanied by caspase activation and apoptosis (Wey, Luo et al. 2012). In general, this

study revealed the pleiotropic roles of GRP78 in the bone marrow by regulating HSC survival. In contrast, conditional knockout of GRP94 in HSCs led to increased quiescent bone marrow cells compared to wildtype controls (Luo, Lam et al. 2011), suggesting that different ER chaperones may have very distinct roles in HSPCs.

HSPCs are long lived due to their capacity to undergo self-renewal, which in turn increases the propensity of HSPCs to become exposed to a variety of stress stimuli, especially ROS, nutrient imbalance, and DNA damage (Geiger, de Haan et al. 2013). Therefore, the stressed HSPCs must be tightly regulated in order to prevent their clonogenic expansion and loss of function. A recent study demonstrated for the first time the importance of ER stress in maintaining the cellular integrity of HSPCs throughout their lifetime (van Galen, Kreso et al. 2014). Gene expression analysis of lineage-depleted human cord blood cells revealed the up-regulation of XBP1, ATF4, CHOP, and PERK in HSPCs, indicating an enrichment of UPR components in HSPCs as compared to the downstream progenitors (common myeloid progenitors, multilymphoid progenitors, granulocyte/macrophage progenitors, and megakaryocyte and erythrocyte progenitors). Furthermore, over-expression of co-chaperones, such as ERDJ4 (also known as DNAJB9), resulted in enhanced protein folding through increase in activity of GRP78, and protected the HSCs against UPR-induced apoptosis. The over-expression also ameliorated the repopulation capacity of HSCs in xenograft assays. These findings suggest that the HSPC pool may maintain its clonal integrity by activation of stress responses, including the UPR, and clearance of individual HSPCs to prevent propagation of damaged stem cells (van Galen, Kreso et al. 2014). In agreement with these results, recent work by Kenichi Miharada et al. demonstrated that developmental pluripotency-associated 5 protein (Dppa5), an RNA binding protein highly expressed in pluripotent stem cells, regulates HSC activity by diminishing ER stress. Reconstitution capacity of HSCs was robustly increased after over-expression of Dppa5 *in vitro*, and ectopic expression of this protein also resulted in significant reduction in ER stress and subsequent apoptosis. In contrast, knockdown of Dppa5, significantly increased apoptosis and ER stress, and abrogated the reconstitution capacity of HSCs. These findings implicate ER stress in the regulation of HSC survival. More excitingly, they revealed that inhibition of ER stress by a chemical chaperone markedly enhanced the engraftment capacity of HSC (Miharada, Sigurdsson et al. 2014). Collectively, these encouraging new findings accentuate an important role of ER stress and the UPR in governing the longevity and function of hematopoietic progenitor/stem cells. Whether these pathways are also implicated critically in EPC regulation, retinal vascular development, remodeling, and repair are yet to be clarified.

9. Perspectives and future directions

The ER plays a vital role in cellular physiology by ensuring the correct folding of secretory and transmembrane proteins; this important function is monitored and dynamically controlled by the UPR (Minchenko, Kubaichuk et al. 2013). Over the past two decades, new research has provided exciting evidence suggesting that activation of the UPR not only maintains a homeostatic environment in the ER, which in a broader context is crucial for cellular proteostasis, but also is involved in regulation of a wide variety of other cellular processes, including cellular proliferation and differentiation, inflammation, apoptosis, and

angiogenesis. Accordingly, increased ER stress and UPR activation have been reported in many human diseases, including cancer, neurodegenerative diseases, diabetes and metabolic disorders. While these findings indicate the importance of the UPR and ER function in both normal as well as disease-related cellular activities, they also reflect the complexity and challenges in fully understanding the mechanisms and physiological significance of these pathways. Moreover, as demonstrated in many studies, activation of the UPR appears to be a dynamic process closely correlated with the duration and extent of ER stress (reviewed in (Ron and Walter 2007)). The three UPR pathways and their downstream effectors can be activated differentially in the presence of ER stress, resulting in distinct biological effects in different cell types. Thus, different experimental conditions, *e.g.*, as applied to cells *in vitro*, may significantly impact the results and the conclusions derived therefrom, which should be taken into consideration when designing and executing such studies. Furthermore, regarding the complexity of UPR regulation, a recent study has confirmed that UPR genes (GRP78, IRE1, XBP1, PERK, ATF4, CHOP and ATF6) reciprocally regulate each other in that GRP78 and CHOP are transcriptionally up-regulated by all UPR genes, whereas XBP1 and ATF6 are regulated by some, but not all, UPR genes (Takayanagi, Fukuda et al, 2013). In addition, GRP78 is regulated at the post-translational level when there is DNA damage, further indicating the importance of the UPR in cellular stress response. Apart from the regulatory network within UPR branches, the UPR genes have been shown to interact with other transcription factors/signaling molecules in many diverse cellular pathways, such as autophagy, redox signaling, mitochondrial function, and Ca²⁺ regulation (reviewed in (Jing, Wang et al. 2012)). These interactions, again, can be cell- and condition-specific; nevertheless, understanding how the UPR interacts with these pathways may provide important insight for understanding cell behavior in normal tissue and disease development, including angiogenesis.

As a part of the central nervous system, the retina is composed of hundreds of millions of neurons and functions to generate visual signals and transmit that information to the brain. These processes require constant blood flow through the vasculature of the retina and choroid to ensure the availability of sufficient oxygen as well as glucose (review in (Caprara and Grimm 2012)). Therefore, maintaining a homeostatic and functional retinal vascular system is extremely important for retinal cell survival and function. This is achieved by the coordinated and integrated regulation of multiple processes, including vessel formation, remodeling and maturation during development, as well as maintaining endothelial cell and pericyte survival and function in adult retina. The results of research performed to date, as summarized above, provide initial but exciting evidence that collectively support a role for the UPR in retinal vascular development as well as in vasodegeneration and pathological angiogenesis (Ozawa, Tsukamoto et al. 2001, Zhang, Sanders et al. 2011, Nakamura, Takizawa et al. 2013, Zeng, Tallaksen-Greene et al. 2013, Zeng, Xiao et al. 2013). However, most of the mechanistic studies to date have focused on the UPR with respect to its involvement in VEGF regulation (Ghosh, Lipson et al. 2010, Luo and Lee 2013). For example, the UPR has been shown to regulate VEGF expression in various types of retinal cells, including RPE cells (Roybal, Yang et al. 2004, Pollreis, Afonyushkin et al. 2013), pericytes (Zhong, Wang et al. 2012), endothelial cells (Chen, Wang et al. 2012), and Müller glial cells (Zhong, Li et al. 2012). This regulation involves the transcription factors XBP1,

ATF4, and ATF6 in all three UPR branches. In addition to direct regulation VEGF transcription, these UPR-target transcription factors also interact with other signaling molecules, such as STAT3, JNK, and HIF-1, to modulate VEGF expression (Harding, Zhang et al. 2003, Drogat, Auguste et al. 2007, Iwawaki, Akai et al. 2009, Ghosh, Lipson et al. 2010, Li, Wang et al. 2011, Chen, Wang et al. 2012, Pereira, Frudd et al. 2013, Zeng, Xiao et al. 2013). In contrast to the fairly extensive investigation of VEGF, little is known about how the UPR regulates other angiogenic factors, such as IGF-1, Epo, FGF, platelet-derived growth factor (PDGF), transforming growth factor (TGF), placental endothelial cell growth factor (PIGF), as well as angiogenic inhibitors, such as pigment epithelium-derived factor (PEDF). In addition, there is very limited information available with respect to how individual UPR genes are implicated in controlling the angiogenic process. Since the UPR is central to cell survival, global knockout of several key UPR genes leads to embryonic lethality; cell-specific deletion of one or more UPR genes would be necessary to understand further the involvement of UPR signaling in retinal angiogenesis. Furthermore, a comprehensive characterization of ER stress and UPR activation in retinal blood vessels during the course of retinal development and postnatal remodeling is desperately needed. Likewise, a better characterization of ER stress and UPR activation in the retina in animal models and human subjects with retinal NV will help delineate the role of UPR pathways in pathogenic angiogenesis in retinal diseases.

Degeneration of blood vessels is a hallmark pathological change in ischemic retinal diseases such as DR and ROP prior to when proliferative retinopathy occurs. This phenomenon highlights the importance of understanding the causes of vascular cell loss in neovascular diseases. Despite strong evidence that supports an essential role for the UPR in mediating angiogenesis, prolonged ER stress has been observed in various pathological conditions, which eventually leads to activation of pro-apoptotic UPR signaling, ultimately resulting in cell death (Yamamoto, Sato et al. 2007, Lin, Walter et al. 2008, Zhang and Kaufman 2008). Major pro-apoptotic pathway molecules activated by ER stress include JNK, CHOP, and B-cell lymphoma-2 (Bcl-2) family proteins as downstream effectors of the IRE1 α and PERK branches (Szegezdi, Logue et al. 2006). ER stress-related disturbance in calcium homeostasis and mitochondrial dysfunction also contribute to apoptosis (reviewed in (Jing, Wang et al. 2012)). Thus, prolonged and uncompensated ER stress could play an important role in endothelial cell and pericyte loss in neovascular retinal diseases such as DR. In addition, the UPR is closely associated with, and actively modulates, the inflammatory response and redox status of endothelial cells (Gargalovic, Gharavi et al. 2006, Li, Wang et al. 2009, Afonyushkin, Oskolkova et al. 2010, Auf, Jabouille et al. 2010, Ghosh, Lipson et al. 2010, Chen, Wang et al. 2012, Roy and Kolattukudy 2012, Vladykovskaya, Sithu et al. 2012). These effects will likely contribute to retinal NV formation in pathological conditions. Given the predominant role of the UPR in cell adaption to stress, it is not surprising that multiple signaling pathways are activated and coordinated by the UPR as a complex network in vascular cells during the processes of vasodeneration, vascular remodeling, and retinal NV. In fact, some signaling molecules are not only regulated by the UPR, but also play a role in the *activation* of the UPR under certain conditions. For example, signal transducer and activator of transcription 3 (STAT3), an important signaling molecule that regulates angiogenesis, has been shown to mediate high glucose-elicited ER

stress and ATF4 activation, which, in turn, exacerbates STAT3 activity resulting in up-regulation of inflammatory cytokines, including VEGF (Chen et al., 2012). Such interactions also have been observed in other transcription factors and signaling pathways, *e.g.*, HIF-1 α , JNK and NF- κ B, contributing to the fine tuning and regulation of cellular responses during vascular development and retinal NV.

Finally, there is emerging evidence that the UPR plays an important role in controlling stem cell survival as well as self-renewal, maturation and differentiation. Clearly, this represents a new opportunity, and possibly a paradigm shift, in the research of stem and progenitor cell biology with relation to vascular repair and regeneration in chronic ischemic diseases, such as DR and ROP, as well as understanding the process of vascular development. In ischemic retinopathy, dysfunction of the EPCs resulting in a reduced capacity for vascular repair leads to sustained and irreversible blood vessel damage and loss. Exactly how ER stress and the UPR are implicated in the regulation of EPC survival and function under diabetic and ischemic conditions remains to be elucidated. Future investigation of UPR target genes and ER chaperones, such as GRP78, XBP1, ATF4, CHOP, ATF6, with regard to their involvement in EPC generation, differentiation, and mobilization could provide new insights for better understanding and manipulating angiogenic cells for therapeutic use. Furthermore, providing a better understanding of how the UPR regulates the processes of EPC integration into injured vascular sites and promoting new (physiologically and structurally intact) blood vessel formation could be of great significance in identifying new targets for the treatment of neovascular blinding diseases. In addition, there is a growing interest in pursuing such strategies for treating a wide variety of significant and wide-spread human diseases, many of which involve vascular elements, including age-related macular degeneration (AMD) and other retinal degenerations (reviewed by (Haeri and Knox, 2012)), diabetes and related metabolic diseases (reviewed by (Engin and Hotamisligil, 2010)), cancer (reviewed by (Boelens et al., 2007)), and cardiovascular disease (reviewed by (Minamino et al., 2010)). Taken together, current research suggests that investigation of UPR signaling in the vascular system and in angiogenic cells represents a new and exciting area to explore in the field of retinal angiogenesis and vascular diseases.

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Abbreviations

AhR	aryl hydrocarbon receptor
AMD	age-related macular degeneration
ATF	activating transcription factor
Bip	immunoglobulin heavy chain-binding protein

BRVO	branch retinal vein occlusion
CaMK	calcium/calmodulin-dependent protein kinase
CHOP	C/EBP homologous protein-10
CNV	choroidal neovascularization
CRVO	central retinal vein occlusion
CRYAB	α -basic crystallin
CTGF	connective tissue growth factor
DR	diabetic retinopathy
eIF2α	eukaryotic initiation factor 2 α
EPCs	endothelial progenitor cells
Epo	erythropoietin
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FZD5	Frizzled-5
GCN2	general control of amino-acid synthesis 2
GH	growth hormone
GRP78	glucose-regulated protein 78
HDI	heme-regulated inhibitor kinase
HIF-1α	hypoxia inducible factor 1 α
HSCs	hematopoietic stem cells
HSPCs	hematopoietic stem and progenitor cells
IGF-1	insulin-like growth factor 1
IRE1	inositol-requiring enzyme 1
MSCs	mesenchymal stem cells
NV	neovascularization
OIR	oxygen-induced retinopathy
ORP150	oxygen-regulated protein 150
OxPLs	oxidized phospholipids
PBA	4-phenylbutyric acid
PDI	protein disulfide isomerase
PEDF	pigment epithelium-derived factor
PERK	PKR-like endoplasmic reticulum kinase

PKR	dsRNA-dependent protein kinase
RGCs	retinal ganglion cells
RIDD	regulated Ire1-dependent decay
ROP	retinopathy of prematurity
ROS	reactive oxygen species
RPE	retinal pigment epithelium
STAT1	signal transducer and activator of transcription-1
TMAO	trimethylamine-N-oxide
TRAF2	tumor necrosis factor-associated factor 2
TUDCA	tauroursodeoxycholic acid
UPR	unfolded protein response
VEGF	vascular endothelial growth factor
XPB1	X-box binding protein 1
XPB1s	spliced XPB1 isoform

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Figure 1. Implications of the IRE1/XBP1 pathway in angiogenesis

Upon ER stress, IRE1 is activated by autophosphorylation and oligomerization. Activated IRE1 splices the mRNA encoding XBP1 to generate spliced XBP1 (XBP1s). XBP1s induces UPR target genes, thereby restoring ER homeostasis and promoting cell survival. XBP1s can bind to the VEGF promoter to regulate VEGF transcription. In addition, activated IRE1 recruits TRAF2, which, in turn, results in the activation of stress kinases, such as JNK and IKK; this leads to induction of inflammatory cytokines. Hyperactivated IRE1 degrades mRNA, such as micR-17 and netrin-1, and leads to increased pro-angiogenic factor expression.

Figure 2. Signaling pathways of the PERK branch of the UPR in angiogenesis

The PERK branch of the UPR regulates angiogenesis mainly through induction of ATF4. During ER stress, PERK is activated by autophosphorylation and oligomerization. Activated PERK phosphorylates the translational initiation factor eIF2α, resulting in the global arrest of protein synthesis. Enhanced eIF2α phosphorylation preferentially increases the translation of the transcription factor ATF4; in turn, ATF4 induces the expression of inflammatory factors (MCP-1, IL-6, IL-8 and CXCL3) and VEGF, thereby causing increased inflammation and promoting angiogenesis. Additionally, ATF4 is a major inducer of CHOP, whose activation modulates pro- and anti-apoptotic pathways, resulting in apoptosis and cell death, and also contributes to increased oxidative stress and dysregulated calcium homeostasis.

Figure 3. Activation of the ATF6 pathway and angiogenesis

Upon ER stress, ATF6 disassociates from GRP78 and translocates to the Golgi apparatus, where it is cleaved by proteases. Cleaved ATF6 is an active bZIP transcription factor: it translocates to the nucleus and binds to the promoters of UPR target genes, such as BIP/GRP78, CRYAB, and XBP1, and VEGF. These genes directly and indirectly regulate the angiogenic process in various ways.

Figure 4. Potential role of ER stress in hematopoietic stem cell and progenitor cell dysfunction in diabetic vascular degeneration

In normal physiological conditions HSPCs reside in the stem cell niche of the bone marrow. The hypoxic environment in the niche maintains the quiescent state of HSPCs and sustains their self-renewal capacity through induction of HIF-1 α . Release of HSPCs from the niche is mediated by MMP-9. Once in the bone marrow, HSPCs proliferate and differentiate into multipotent progenitor (MPP) cells, and mobilize into the circulation. The mobilization process is facilitated by cytokines and proteins, including CXCL-12, VEGF, NO, GM-CSF, and CXCL-8. In diabetes mellitus, increased levels of reactive oxygen species (ROS) in the bone marrow leads to decreased hypoxia, thereby disrupting the stem cell niche. This, in turn, leads to ER stress, resulting in apoptosis of HPSCs, hence disturbing the equilibrium between self-renewal and differentiation. Decreased levels of CXCL-12, VEGF, NO, GM-CSF, and CXCL-8 impair mobilization of cells from the bone marrow into the circulation.