Vitreous Cytokines and Regression of the Fetal Hyaloid Vasculature

I.D.

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Outline

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Key Concepts

1. Regression of the fetal hyaloid vasculature is a vital process required for vision that can impact the proper development of vitreous, retina, and other ocular structures.
2. Although the timing of hyaloid vasculature regression is well defined, little is known about the molecular mechanisms involved. Recent proteomic studies in mice identified 770 proteins, while studies of human embryonic vitreous identified 896 unique proteins. Changes in the human fetal proteome during the second trimester and differences in comparison to young adult human vitreous are being studied to identify molecules and pathways that may be involved in the regression of the hyaloid vasculature.
3. Many complex mechanisms must occur sequentially and concurrently for proper and complete hyaloid vasculature regression. Apoptosis, increased expression of antiangiogenic factors, reduced expression of endothelial survival factors, macrophagy, and perhaps external stimuli are all involved in regression of the hyaloid vasculature.

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I. Introduction

The fetal hyaloid vasculature plays a critical role in many aspects of proper ocular development. The hyaloid artery (HA) is a large vessel that extends from the optic disc through the vitreous body where it branches and extends to the posterior surface of the lens to anastamose with the tunica vasculosa lentis (TVL) which supplies nutrients to the developing lens in the fetus (Figure I.D-1). In humans, the entire embryonic vascular system regresses by birth, which is necessary for media clarity and normal vision. Regression of the hyaloid vasculature is a very complex process likely requiring the participation of many events. The mechanisms of hyaloid vessel regression are not known, but insight could be obtained by understanding how this fetal vasculature forms. One study of human embryonic vitreous revealed the presence of blood islands composed of aggregates of primitive erythroblasts and hemangioblasts, as early as the seventh week of gestation (WG) [1]. These cell aggregates express hematopoietic stem cell markers, and the ligands for these markers are expressed in high concentrations in the lens and retina, probably functioning to guide the cells into the vitreous body assembling the fetal vitreous vasculature by hemo-vasculogenesis [2].

A better understanding of the mechanisms of fetal hyaloid vasculature formation and regression would help in understanding conditions that impair vision due to hyaloid vessel persistence in youth, since it is necessary that fetal vitreous vessels regress to establish a clear medium through which light can traverse to reach the retina. The mechanisms of this process may also provide insight into how nature induces blood vessel regression. Unraveling these secrets could enable new avenues of research to develop ever more-effective therapies for pathological neovascularization in diseases such as exudative age-related macular degeneration, ischemic proliferative (diabetic and other) retinopathies, and metastatic carcinomas.

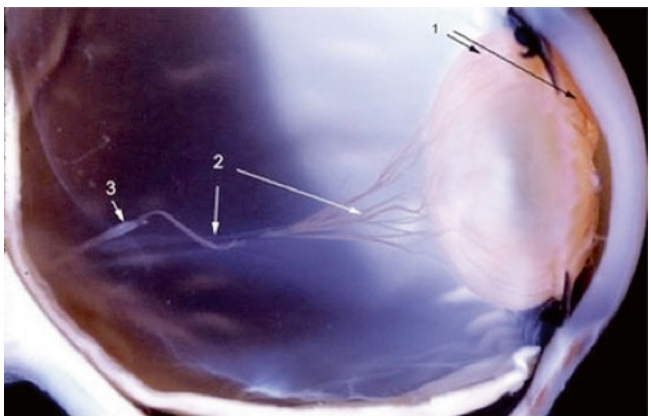

Figure I.D-1 The tunica vasculosa lentis (TVL) surrounds and feeds the developing lens (*1*). The vasa hyaloidea propria (*2*) arises from the hyaloid artery (*3*) and forms anastomoses with the TVL

II. Structure of the Hyaloid Vasculature

The anatomy of the hyaloid vasculature was first described by Ida Mann in 1928 [2]. During this time, studying the hyaloid vasculature was difficult because fixation methods would destroyed hyaloid structures and because freshly excised eyes were difficult to dissect while preserving fetal vitreous structures. Slit-lamp biomicroscopy made it possible to observe Cloquet's canal, a clear central space through the central vitreous that was once occupied by the hyaloid artery *in utero*. More thorough examination of the hyaloid vasculature has been made possible by advances of *in vivo* and fixed tissue imaging.

The hyaloid vasculature is first seen in humans at approximately the fourth week of fetal development [see chapter II.A. Development and developmental disorders of vitreous]. It is comprised of the main trunk of the *hyaloid artery* (HA) which divides into the *vasa hyaloidea propria* (VHP), a vascular network which divides further to form a dense capillary network called the *tunica vasculosa lentis* (TVL) that approaches and attaches to the posterior lens [2]. The *pupillary membrane* (PM) is an extension of the TVL that covers the anterior lens (Figure I.D-1). The well-developed primate hyaloid artery has the ultrastructural characteristics of an arteriole with a non-fenestrated intima, a multilayered smooth muscular media, a connective tissue adventitia, and a perivascular sheath. The branching networks of the HA which include the VHP, the TVL, and the pupillary membrane appear to be type A-1 alpha capillaries with non-fenestrated endothelium, incomplete pericyte layer, and a basement membrane surrounding each layer [3].

The hyaloid vasculature contains junctional complexes including zonulae and macula adherens and possible zonulae occludens between adjacent endothelial cells [4]. The hyaloid artery and its branch systems do not exhibit leakage of fluorescein dye, consistent with the presence of endothelial tight junctions [3]. A heterogeneous population of leukocyte-derived hyalocytes associated with the human hyaloid vasculature has also been demonstrated [4]. The hyaloid vasculature is further characterized by the absence of veins. All vessels of the hyaloid system are arteries, and venous drainage is accomplished through the choroidal vessels [5].

III. Timeline of Fetal Hyaloid Vasculature Regression

A. Lower Mammals

The pattern of normal hyaloid vasculature development is basically the same in mice as in rats and rabbits. In contrast to humans, the hyaloid system in these species regresses after birth. In mice the hyaloid vascular system forms at

embryonic day (ED) 10.5 and is fully developed by ED 13.5. Apoptosis of the endothelial cells that constitute the TVL occurs as early as ED 17.5, but the TVL persists until post-gestational day (PGD) 16 and undergoes gradual regression from PGD 14–30 [6, 7]. The VHP and PM disappear between PGD 12 and 16 in rodents and between PGD 10 and 12 in rabbits [8]. During vascular regression in rats, apoptosis occurs in pericytes as well as in endothelial cells. Capillary apoptosis occurs mostly from day 10 to 20 after birth [9]. The earliest detectable regression-related changes are apparent around day 3 and initially involve the capillaries surrounding the posterior lens, then proceed posteriorly [10].

B. Humans

At about the 48 mm stage of embryologic development (ninth week of gestation, WG), the fetal intraocular blood system has reached maximal development, and thereafter every subsequent stage reveals signs of vessel regression [2, 4]. Regression of the hyaloid vasculature begins at 12–13 WG, and complete involution is achieved by 35–36 WG [1, 6, 11, 12]. Variability in the timeline of regression may exist, as another human study showed that regression is not evident until 18 WG and is complete by 29 WG [13]. *In vivo* ultrasonography has suggested that the hyaloid vessels are detectable at 20 WG and that regression begins in the early third trimester. By 30 WG, the hyaloid artery is normally not detectable [14]. What is likely, however, is that all cytokine stimuli for hyaloid vessel regression predate the aforementioned cellular events.

After the 65 mm stage, atrophy of the fetal vascular system becomes apparent beginning with the elongation and thinning of the vessels of the VHP then the TVL, followed by the PM. Complete involution of the VHP, TVL, and PM occurs by about 8.5 months. During the eighth month of gestation, the main trunk of the hyaloid artery gradually atrophies and eventually disappears. Cellular debris and macrophages fill the lumen forming a plug that occludes blood flow and promotes necrosis and phagocytosis of the vessel [15]. Remnants of the atrophied vessels can sometimes form corkscrew configurations after they lose connection with the main trunk due to the elastic properties of endothelial cells and/or their basal laminae [2].

Before birth and up to the fourth week after birth, the vessel walls appear atrophic as well as acellular and the lumen is occluded by a thrombus [3]. Although the timeline for hyaloid development and regression is well defined, the exact molecular mechanisms are not well understood, especially in humans. Insight into this aspect of hyaloid regression may be gained by study of cytokine expression and the key signals and mechanisms underlying the fetal vessel regression.

IV. Vitreous Cytokine Expression

Recent proteomic studies of embryonic human vitreous, aged 14 to 20 WG (during the early period of hyaloid regression), identified the presence of 896 unique protein peptides [16]. Studies are ongoing to ascertain how the human fetal vitreous proteome compares to the young adult and to determine whether any changes in the levels of protein expression can be identified that may have relevance to the phenomenon of fetal hyaloid vasculature regression.

Bioinformatic evaluation of some of this data with Ingenuity Pathway Analysis (IPA) determined that there was generalized decrease in protein synthesis pathways [16], consistent with a decrease in cell metabolism during apoptosis and cell death as the vessels regress. Figure I.D-2a demonstrates that many of the detected proteins with a significant decrease in expression in vitreous during the period of regressing hyaloid vessels are involved with protein synthesis pathways and most of these are known to be cytoplasmic.

Since the cellular *primary* vitreous is replaced by an acellular collagenous *secondary* vitreous, an increase in proteins involved in connective tissue pathways is to be expected. Figure I.D-2b demonstrates that many of the proteins with significantly increased expression during the period of hyaloid vascular regression are involved in collagen synthesis and connective tissue formation [16]. The connective tissue pathway proteins that increased in expression were mostly extracellular.

During this period, there was also a marked decrease in the free radical scavenging pathway [16]. Reactive oxygen species (ROS) have been shown to induce apoptosis in many tissue types. Free radicals can start chain reactions of oxidation, which cause cell damage or death. Antioxidant binding of free radical intermediates halts these processes. Thus, a reduction in free radical scavenging during hyaloid regression would promote the induction of apoptosis via ROS during this period. Consistent with this, IPA bioinformatic analysis revealed that many of the proteins with decreased expression during hyaloid regression are involved in free radical scavenging.

Proteins involved in small molecule biochemistry were also markedly decreased in vitreous from 14 to 20 WG [16]. Small molecules (<900 Da) typically regulate biological processes and can rapidly diffuse across membranes, a characteristic necessary for bioavailability, allowing these regulatory molecules to reach and interact with intracellular action sites [19, 20]. As the cellular primary vitreous is replaced by an acellular secondary vitreous during development, the biological requirement for small molecule-dependent regulation of cellular processes likely diminishes, consistent with the observed decreased expression.

According to the Science Signaling Journal (http://stke.sciencemag.org/about/help/cm.dtl), canonical pathways are "idealized or generalized pathways that represent common

properties of a particular (specific to tissues, cell lines, etc.) signaling module or pathway." In human embryonic vitreous, the two most relevant canonical pathways determined by IPA bioinformatic analysis were EIF2 signaling and protein ubiquitination [16]. Molecules involved in these canonical pathways decreased in detection from 14 to 20 WG. EIF2 activity regulates mRNA translation and regulates protein synthesis in response to various stimuli. Decreased EIF2 activity concurrent with hyaloid vasculature regression may be explained by a reduced need for cellular protein synthesis as a mostly acellular vitreous is formed. The protein ubiquitination pathway plays a major role in the degradation of short-lived regulatory proteins involved in many cellular processes including apoptosis, cell proliferation, and transcription regulation [21]. Decreased detection of molecules involved in protein ubiquitination pathways may be due to reduced need for signals involved in endothelial cell proliferation, pro-apoptotic factors, or complex proteins in general, as the acellular secondary vitreous is formed.

A. Comparative Analysis to a Murine Model of Vitreous Embryogenesis

A previous histologic and proteomic study was performed on vitreous of developing postnatal mice [22]. In this model, the hyaloid network is still prominent at post-gestation day 1 (PGD 1) but is mostly regressed and nonfunctional by PGD 16. Two-dimensional electrophoresis was utilized to investigate the protein profile of the mouse vitreous spanning the period of hyaloid regression (PGD 1–16) and found 770 protein spots [21]. Both the number of detected protein spots and the volume of the spots decreased from PGD 1–16. These findings are similar to the human embryonic vitreous study where three times as many proteins decreased as increased during hyaloid vessel regression [16]. In the mouse vitreous, the number of proteins that had an increased spot volume compared to PGD 1 decreased at PGD 16. The active protein expression and existence in the earlier post-gestation days may indicate that these particular proteins are involved in the actual process of vessel degradation. This study also found that proteins with decreased spot volume relative to PGD 1 increased in number at PGD 16 [21].

A decrease in the number of different proteins and the levels of protein expression during hyaloid vessel regression is consistent with the replacement of a highly vascularized primary vitreous with a largely acellular secondary vitreous during this period. Although the overall changes in protein profiles are similar in human and mice, there were no proteins identified that changed expression significantly in both humans and mice, perhaps limiting the applicability of animal studies to humans. In mice, proteins that were found to be upregulated in PGD 1 were involved in energy metabolism as well as cell proliferation and development. Proteins upregulated in PGD 16 were cancer- and apoptosis-related or inflammatory proteins.

V. Mechanisms of Fetal Vessel Regression

Many processes have been implicated in normal hyaloid vascular regression. Vitreous itself has been shown to be an inhibitor of angiogenesis and inhibits tumor-induced neovascularization in the rabbit cornea by as much as 37 % [23]. Apoptosis, autophagy, macrophagy, antiangiogenesis, reduced expression of survival factors, and some external environmental factors have all been associated with the regression of the hyaloid vasculature. All these biological processes are likely initiated and promoted by cytokines under the influence of protein synthesis regulation, both up and down, as defined above.

A. Apoptosis

Apoptosis has been definitively proven to contribute to the regression of the fetal intraocular vasculature system. *Pro-apoptotic proteins*, *Bax and Bak*, of the B-cell lymphoma 2 (bcl-2) family may serve overlapping functions in the promotion of hyaloid vessel regression. Complete involution of the hyaloid vessels was not observed in the eyes of bax(−/−) bak(−/−) mice, indicating that only deficiencies in both proteins would lead to pathological persistence of the hyaloid vasculature [24]. In the aforementioned study of human embryonic vitreous, isoform alpha of Bax protein decreased during the period of maximal hyaloid regression [16]. As hyaloid regression progresses, there are fewer endothelial cells remaining to undergo apoptosis, and this may explain the decreased detection of pro-apoptotic Bax at the end phase of hyaloid regression [21]. The presence of either of these pro-apoptotic bcl-2 apoptosis promoters is sufficient for complete hyaloid regression [23].

Bim is another member of the bcl-2 family that may influence apoptosis of the hyaloid vessels. In mice that do not express Bim, there is significant attenuation of hyaloid vessel regression [25]. Interestingly, hyaloid vessel regression is not affected in the absence of bcl-2 in mice [26].

Vitreous hyalocytes are the only cell type in the eye that express all four forms of *transforming growth factor-β* (TGF-β) [27]. Hyalocyte production of TGF-β may contribute to the apoptotic process of hyaloid regression [see chapter II.D. Hyalocytes]. TGF-β can induce apoptosis through the SMAD or DAXX pathways and can inhibit vascular endothelial cell proliferation, although TGF-β has also been shown to be pro-angiogenic [28]. TGF-β is also required for *Arf* promoter activation that is induced before hyaloid vessel regression [29]. Arf regulates vascular regression in a tumor protein 53

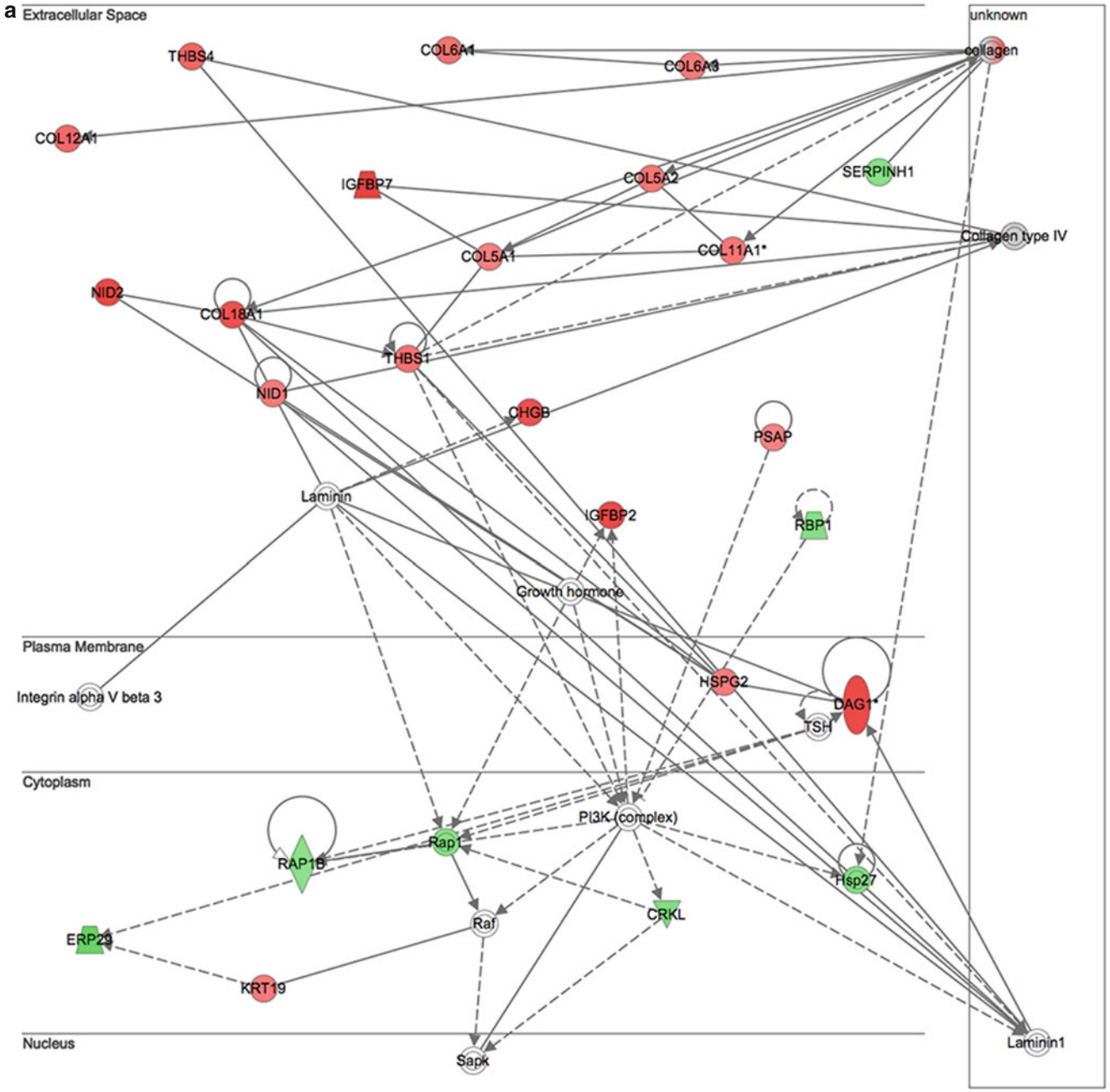

Figure I.D-2 (**a**) The connective tissue pathway was upregulated consistent with the formation of a collagenous secondary vitreous (Bonferroni adjusted *P*-value < 0.00005). The overwhelming majority of this activity occurs in the extracellular compartment [*red* = upregulated]. Of note is the localization in the extracellular space

(p53) independent manner [30]. *Activin receptor-like kinase-1* (ALK-1), a type 1 receptor for TGF-β, may also play a role in the regression of the hyaloid vasculature as ALK-1 overexpression has been shown to inhibit basic fibroblast growth factor-induced corneal neovascularization [31]. TGF-β2 has been detected in human vitreous during early development, and its localization has been associated with the development and regression of the hyaloid vascular network [32]. In mice with *p53* deficiency, there is persistence of parts of the hyaloid vasculature, and these parts eventually develop into fibrovascular plaques that are analogous to persistent hyperplastic primary vitreous. This indicates a role for p53-dependent apoptosis in hyaloid vascular regression [33].

Prolactin (PRL) is proteolytically processed to 16 K-PRL fragments with potent pro-apoptotic and antiangiogenic properties. Apoptosis of the hyaloid vessels in neonatal rats is inhibited by intravitreal injections of neutralizing anti-PRL antibodies, suggesting a role for PRL in hyaloid regression [34].

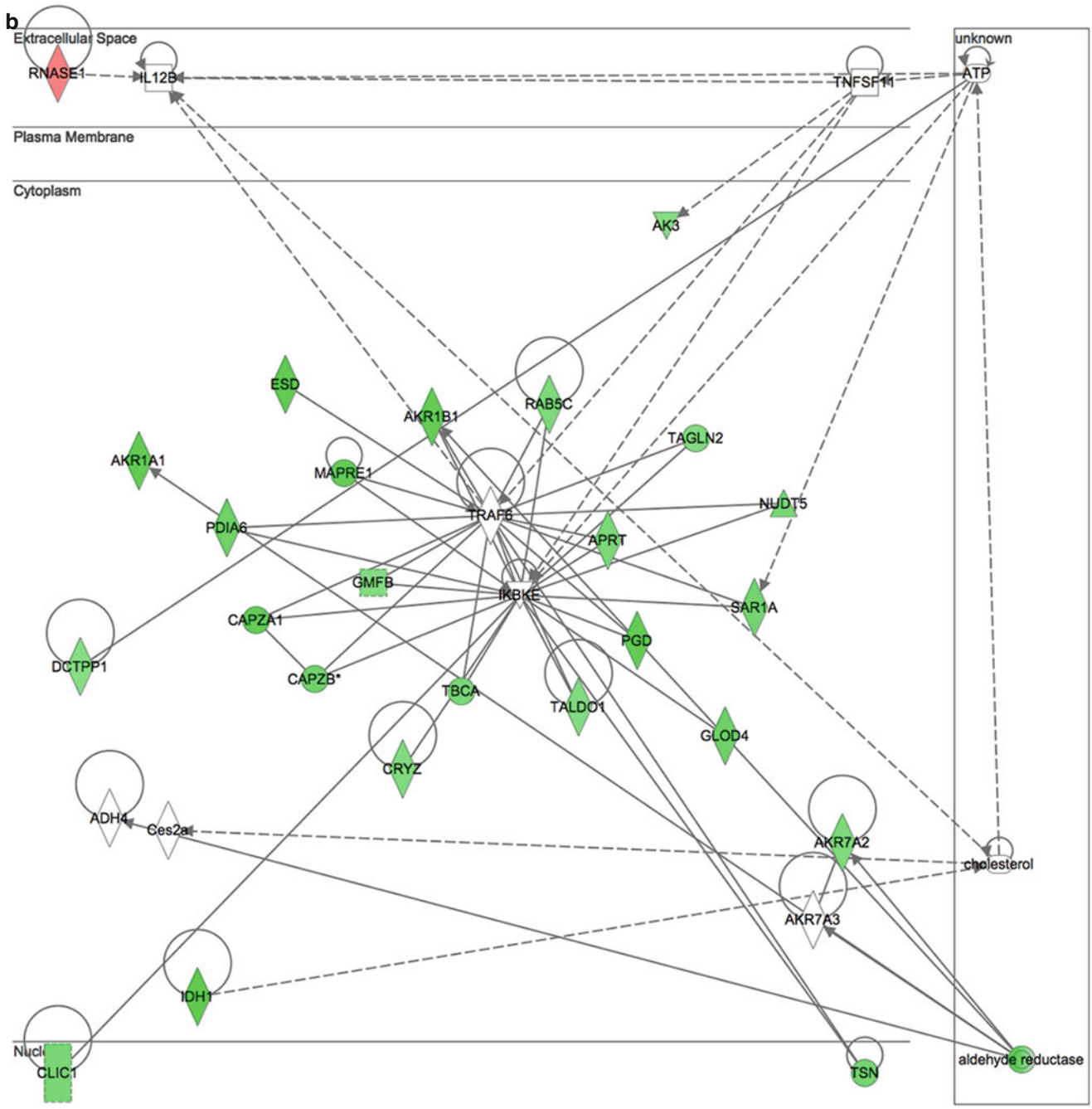

Figure I.D-2b (continued) (**b**) The small molecule biochemistry pathway showed reduced activity during the same period, suggesting a role for reactive oxygen species-driven apoptosis and a reduction in cellular processes as the acellular secondary vitreous is formed and the hyaloid vasculature regresses (Bonferroni adjusted *P*-value < 0.00005) [*green* = downregulated]. Of note is the localization in the cytoplasm

Bovine endothelial cells have decreased viability when cultured in the presence of vitreous, and ascorbic acid from the vitreous has been identified as an apoptosis-inducing factor [35]. Concentrations of ascorbic acid similar to vitreous levels can reduce endothelial cell viability and may function as an inhibitor of neovascularization, which can be completely inhibited by antioxidants [36].

B. Macrophagy

Macrophages induce ocular tissue remodeling and hyaloid vessel regression by phagocytosis of dead cells and by inducing vascular endothelial cell apoptosis [35]. Disruption of mature macrophages in mice leads to persistence of the hyaloid vasculature with the PM retained for up to 14 days

after normal regression [37]. This suggests that macrophages are actively involved in targeted cell death of the hyaloid vessels. Human vitreous and hyalocyte-conditioned culture medium were shown to inhibit human vascular endothelial cell growth *in vitro* [38, 39] in a dose- and time-dependent manner [40]. Macrophages and hyalocytes (macrophage-like cells) have been found adjacent to hyaloid vessels by electron microscopy and immunostaining and are believed to be essential for regression of the hyaloid vascular system [8, 36, 41]. Morphological and immunophenotypic characteristics of the macrophages surrounding the PM are similar to hyalocytes in adult rat vitreous [42]. Macrophage-vascular endothelial cell interactions allow for cooperation between the Wnt and angiopoietin (Ang) pathways, although it remains unclear how macrophages are activated or the exact mechanism of their interaction with vascular endothelial cells.

1. Macrophage Adhesion to Hyaloid Vessels

Ninjurin 1 (nerve injury-induced protein; Ninj1) has been shown to be temporarily upregulated in macrophages, which enhances cell-cell and cell-matrix adhesion of macrophages and stimulates the expression of Wnt7b in macrophages. Ninj1 overexpressing macrophages decrease Ang1 and increase Ang2 in pericytes, triggering apoptosis of hyaloid vascular endothelial cells [35]. Macrophages may express Ninj1 to increase apoptotic signals through cell-cell adhesion.

Periostin (POSTN) also supports cell adhesion and migration. Periostin is secreted by intraocular macrophages and seems to enhance regression of the hyaloid system by intensifying the adhesion of macrophages to hyaloid vessels [43]. Periostin was detected in human embryonic vitreous with no appreciable change in the level of expression over the 2nd trimester [16].

Overexpression of *Aquaporin 4* (AQP4) in the astrocytes of rats delays hyaloid regression [44]. In persistent fetal vasculature conditions, astrocytes abnormally migrate into the vitreous, ensheath the hyaloid vessels and impede the normal macrophage-endothelial cell adhesion and the process of macrophage-dependent hyaloid regression [44].

2. Macrophage-Induced Apoptosis

Impeding macrophage-vascular endothelial cell adhesion delays hyaloid vasculature regression by preventing phagocytosis. Another role of macrophages is the direct induction of programmed cell death in capillary regression. Elimination of macrophages results in persistence of the normally transient endothelial cells; these cells lack apoptotic morphology, express intracellular esterases, and continue to synthesize DNA [45]. Macrophages elicit targeted cell death by inducing apoptosis of PM endothelial cells. During regression of the PM, cells have characteristics of apoptosis, specifically apoptotic bodies with condensed chromatin and nucleosomal fragmented DNA. Apoptosis can occur in single cells of healthy vessels or along the entire length of a capillary segment [41]. In mice, lack of *norrin*, a secreted regulatory protein normally required for ocular angiogenesis in mice impairs macrophage-induced apoptosis of hyaloid endothelial cells delaying vessel regression [46, 47]. Apoptosis and macrophage-dependent apoptosis are key to hyaloid regression although a two-step process has been proposed in previous studies. Regression of the mouse PM begins with macrophage-initiated apoptosis followed by the synchronous death of endothelial cells as a consequence of the cessation of blood flow and/or the loss of survival factors such as VEGF [48].

C. Antiangiogenesis

Many mechanisms of vascular inhibition and antiangiogenesis play significant roles in hyaloid regression. Hyaloid regression can be influenced by the downregulation and neutralization of angiogenic promoters, and/or upregulation and enhancement of angiogenic inhibitors.

1. Angiogenesis Promoters

Regression of the hyaloid vessels may be initiated by the reduction of endogenous survival factors below a critical threshold [2]. Platelet-derived growth factor (PDGF) plays a significant role in angiogenesis [49], and overexpression of PDGF-B under control of the *nestin enhancer* causes delayed regression of the hyaloid vasculature in mice [50].

Vascular endothelial growth factors (VEGFs) are another large family of signaling proteins involved in vasculogenesis (formation of new blood vessels where none exist) and angiogenesis (formation of new blood vessels from existing ones). VEGF-A is necessary for the formation of the normal hyaloid vascular system. Absence of VEGF-A in the mouse lens prevents the formation of the capillary vessels of the pupillary membrane, but does not alter the nearby hyaloid vessels at the surface of the retina [51]. Neutralization of VEGF-A causes a significant reduction in the hyaloid and retinal vasculature [52]. During the regression phase of the hyaloid vascular system, VEGF expression increases in the lens and also persists in adults. VEGF-A secreted by the lens may promote the formation of fetal vasculature, but reduction of VEGF-A does not likely cause regression of these vessels [53].

Angiopoietin-2 (Ang2) is a growth factor that is critical in physiological and pathologic angiogenesis, and physiological but not oxygen-induced vascular regression. Ang2-deficient mice show a lack of regression of the hyaloid vasculature [54].

Fibroblast growth factor (*FGF*) proteins are potent mitogens for endothelial cells and induce the assembly of these cells into vascular-like structures. FGF inhibition in transgenic mice causes failed regression of the hyaloid vessels and eventual massive intravitreal neovascularization [55].

2. Angiogenesis Inhibitors

Vitreous has been shown to be an effective inhibitor of neovascularization and many molecules have been identified [56]. Lutty et al. showed that the anti-neovascular activity of vitreous is dose dependent and effective upon capillary endothelial cells and aortic endothelium. They also found that common glycosaminoglycans such as keratan sulfate, chondroitin sulfate-C, and hyaluronan, all found in vitreous, apparently did not inhibit angiogenesis [57]. On the other hand, a partially purified protein of MW = 6.2 K was found to inhibit vascular endothelial cell proliferation *in vitro* [58]. In another study, a 5.7 K glycoprotein has been derived from bovine vitreous, which inhibits angiogenesis possibly by disrupting collagenase activity [59].

Thrombospondins (TSP or THBS) are potent angiogenic inhibitors that directly affect endothelial cell migration, proliferation, survival, and apoptosis and antagonize VEGF activity [60]. In mice lacking thrombospondin 1 (TSP-1−/−), there is a delay in the regression of hyaloid vessels. In the aforementioned proteomic studies of embryonic human vitreous during hyaloid regression, precursors of thrombospondins 1 and 4 were found to have increased expression, respectively. Increased expression of the VEGF antagonizing thrombospondins is consistent with a regressing vascular system [16].

Collagen type XVIII, alpha 1 (Col18A), is one of the multiplex in extracellular matrix proteins [61]. Proteolytic cleavage of Col18A produces endostatin, an endogenous angiogenesis inhibitor found in mice and humans [62, 63]. *Endostatin* affects many pathways that involve cell mobility or viability, thus suppressing angiogenesis. Endostatin can repress cell cycle control and anti-apoptotic mechanisms in proliferating endothelial cells and also potently inhibits endothelial cell migration [64]. Endostatin inhibits endothelial cell migration by altering FGF signal transduction disrupting cell-to-cell adhesion, cell-to-matrix adhesion, and reorganization [65]. Col18A knockout mice show delayed regression of the hyaloid vascular system, most likely due to the absence of endostatin [66]. The aforementioned proteomic study of embryonic human vitreous discovered that Col18A has increased expression during the period of hyaloid vessel regression, consistent with its potent antiangiogenic properties and a putative role in regression of the hyaloid vasculature [16].

Pigment epithelium-derived factor (PEDF), also known as serpin F1 (SERPINF1), is a multifunctional secretory protein that has antiangiogenic functions [67–69]. PEDF has been shown to affect malignant peripheral nerve sheath tumors, prevents progression of liver metastasis in a mouse model of uveal melanoma, and has been used for therapy in multiple cancer types [70–72]. In the eye, PEDF suppresses retinal neovascularization and endothelial cell proliferation [73, 74]. In mice, PEDF knockout has a minimal effect on the regression of hyaloid vasculature; [75] however, in the aforementioned proteomic studies of embryonic human vitreous, there was an increase in expression of PEDF [16]. The lack of congruity with the mouse models casts further doubt upon the value of these models for studying human hyaloid vessel regression (see above).

It is also possible that the responsiveness to endogenous factors that induce regression can be modified, thus effecting vessel growth or regression. Such a mechanism is present in the vitreous of patients with proliferative diabetic retinopathy (PDR) where lysophosphatidic acid (LPA) has been recently shown to promote regression of blood vessels. However, PDR-vitreous mediates an insensitivity to LPA, which can be overcome pharmacologically. Thus, a decline in the responsiveness to regression factors such as LPA, which are naturally present in the vitreous, may contribute to the pathophysiology of PDR [76]. Whether this concept applies to regression of the hyaloid vasculature is presently unknown.

D. Autophagy

Autophagy is morphologically unique from apoptosis because cell death occurs in the absence of chromatin condensation with concurrent cytoplasmic vacuolization [77]. Also, phagocytes are not associated with cells that die with autophagic morphology [78, 79]. In a murine model, Kim et al. found LC3 and cleaved caspase-3 expression on regressing hyaloid vessels; these are considered respective markers of autophagy and apoptosis. Transmission EM also demonstrated cytoplasmic segregation into autophagosomes, characteristic of autophagy. Autophagic LC3-positive cells progressively decreased in a time-dependent manner. A hypoxia model revealed that LC3-II increased in a treatment time-dependent manner and that autophagy can be induced by a lack of oxygen. Activation of the autophagy pathway by >100 ng/ml of rapamycin decreased the viability of vascular endothelial cells and enhanced hyaloid regression [79]. 3-methyladenine, an autophagy blocker, however, did not completely inhibit hyaloid regression, but significantly attenuated it. Kim et al. demonstrated, for the first time, that hyaloid regression is induced by apoptosis as well as autophagy and that autophagy activation could further enhance regression of hyaloid vessels [80].

E. External Stimuli

It has been proposed that regression of the hyaloid vasculature and the PM occurs in a two-step process beginning with macrophage-dependent apoptosis, followed by the synchronous death of endothelial cells as a direct result of the cessation of blood flow and/or reduced survival factors [8, 36, 81]. Hyaloid vessel regression coincides with a progressive decrease in blood flow and velocity that is thought to be a major trigger in the regression of hyaloid vessels [82]. Arterial vasoconstriction precedes regression of the hyaloid vasculature, which has been reported to be dependent on proximal arterial vasoconstriction [83].

A scanning electron microscopy study showed that exposure to various concentrated gas mixtures of carbon dioxide, oxygen, and nitrogen created marked differences in hyaloid regression of mice when compared to control mice in air; neovascularization was not evident in these mice [84].

Ocular blood vessel development also critically depends on a light-response pathway in mice, where dark-reared mice have been observed to display persistent hyaloid structures as much as eight days postpartum. By post-gestational day (PD) 15 the persistent vessels had regressed, indicating that dark rearing results in a delayed regression. Also quantification of apoptosis at PD5 showed a reduced level, similar to that previously characterized in conditions with persistent hyaloid vasculature [85]. Melanopsin has been implicated as a candidate to mediate light-dependent vascular development because it is expressed early in both humans and mice and is known to be functional from as early as PD10 in mice and because it is expressed in retinal cells adjacent to both the retinal and hyaloid vasculatures [86]. Mice mutated in the Opn4 melanopsin-encoding gene (*Opn4*$^{-/-}$) showed normal hyaloid vessels at PD1 but persistence at PD8 and eventual complete regression by PD15, indicating that the hyaloid persistence was not long term. Dark-reared mice and *Opn4*$^{-/-}$ mutants had VEGFA levels that were seven-fold higher than in controls, which may explain the delay in hyaloid regression. Also, dark rearing from late gestation or in the presence of an *Opn4* mutation produced the same disruption of vascular development, indicating involvement of a melanopsin-dependent light-response pathway [85]. Surprisingly, it was determined that the critical light-response period that stimulates hyaloid regression occurs *in utero* and not at birth.

VI. Miscellaneous Proteins

Dystroglycan is a lamin receptor dystrophin-associated glycoprotein which consists of two subunits, α and β. It has an important role in the formation of glio-vascular connections, cerebral vascularization, and formation of the blood–brain barrier, and thus is involved in many basic processes such as basement membrane assembly, cell survival, and cell migration [87–89]. Dystroglycan also functions as a transmembrane scaffold protein involved in adhesion and adhesion-mediated signaling. Dystroglycan can be a signal transducer from the outside of a cell to the inside or serve as a physical connection between the extracellular matrix and cytoskeleton [90].

Immunostaining (Figure I.D-3) confirmed the presence of dystroglycan on cell membranes and cell junctions of the endothelial cells in the hyaloid vessels of a 14 WG eye, consistent with dystroglycan being a transmembrane protein [16]. The role of dystroglycan in hyaloid vessel regression remains unknown, although loss of dystroglycan was shown

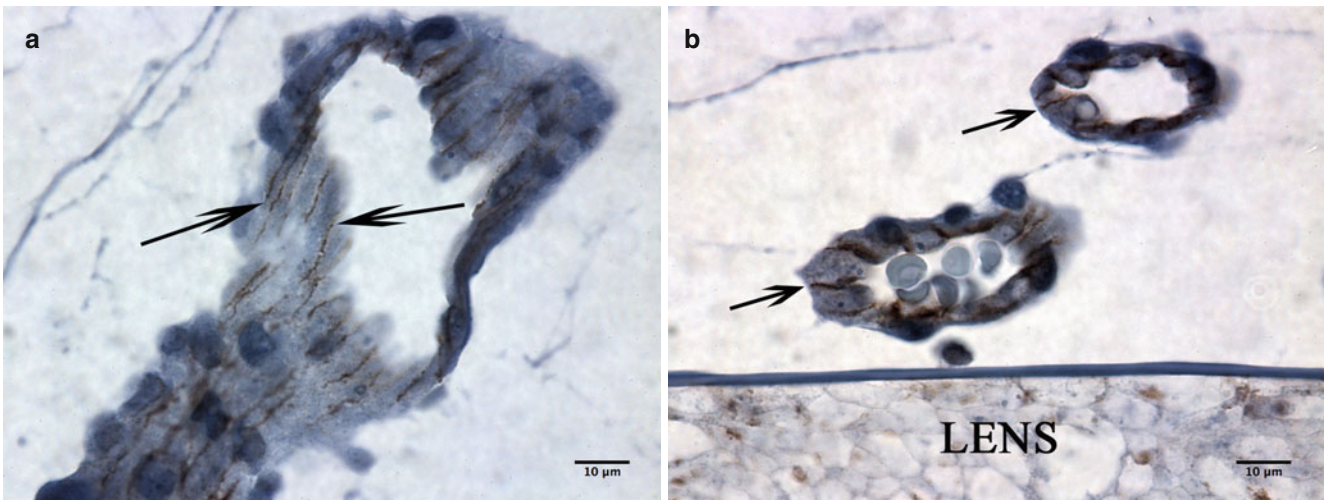

Figure I.D-3 Immunostaining for dystroglycan showed the protein localized to the cell membranes of endothelial cells at the cell junctions of hyaloid vascular endothelium (*arrows*). In the vitreous of a 14 WG human, dystroglycan is seen at cell junctions of the endothelium in the (**a**) hyaloid trunk and the (**b**) tunica vasculosa lentis

to increase tumor formation in prostate cancer [91, 92], and thus further study in the eye is warranted.

Profilin-*1*, a cytoskeletal and cytoplasmic protein, was confirmed by immunostaining to localize to hyalocytes of the vitreous and the endothelial cells of the hyaloid artery (Figure I.D-4a) and tunica vasculosa lentis (Figure I.D-4b). However, there was no appreciable difference in immunostaining intensity throughout the 2nd trimester of development [16]. Profilin-1 has been shown to inhibit endothelial cell migration and proliferation [93]. Interruption of endothelial cell migration and proliferation is necessary for hyaloid vessel regression.

Clusterin (Apolipoprotein J) is a secreted chaperone protein involved in cell death, tumor growth, and neurodegenerative disorders such as Alzheimer's. Clusterin inhibits the movement of (pro-apoptotic) Bax into the mitochondria and thus can prevent the induction of apoptosis [94]. It has also been suggested that Clusterin may be involved in atherosclerosis although its role is controversial. Some studies suggest an anti-inflammatory effect, while others indicate Clusterin can either inhibit or induce vascular smooth muscle cell hyperplasia, as well as protect endothelial cells [95].

Immunohistochemistry in human fetal eyes found positive staining for clusterin in the hyaloid vasculature. Although the study was constrained by a limited number of eyes, the results suggested that at 14 WG (Figure I.D-5a), clusterin was not detected on the structures of the hyaloid vasculature, but at 18 WG (Figure I.D-5b), clusterin expression was observed on hyaloid vessels [16].

Neural cadherin (N-Cadherin, Cadherin-2), part of the cadherin superfamily, is a calcium-dependent cell-to-cell adhesion glycoprotein. N-cadherin is critical for cancer metastasis because it disrupts endothelial cell-cell junctions speeding up the process of transendothelial migration [96]. Disruption of endothelial cell junctions may also be important in the process of regression of the hyaloid vasculature. By immunostaining, N-cadherin was not found in endothelial cells of the hyaloid vasculature in a 14 WG human (Figure I.D-6a), but was observed in the endothelium of 18 WG eyes (Figure I.D-6b) [16]. Given the limited number of specimens studied, this finding needs corroboration by future research.

Platelet endothelial cell adhesion molecule 1 (PECAM-1), also known as cluster of differentiation 31 (CD31), is involved in leukocyte migration, angiogenesis, and integrin activation [97]. In a mouse model of retinal neovascularization during oxygen-induced ischemic retinopathy (OIR), PECAM-1 deficiency (PECAM-1−/−) was shown to decrease retinal vasculature and increase apoptosis of retinal vessels. PECAM-1−/− does not appear to affect the development or regression of the nearby hyaloid vasculature [98].

Opticin is an extracellular matrix glycoprotein that is present throughout the vitreous body and is associated with vitreous collagen fibrils [99, 100]. Opticin is an antiangiogenic protein that regulates extracellular matrix adhesion characteristics by competitively inhibiting endothelial cell interactions with collagen preventing the strong adhesion that is necessary for pro-angiogenic signaling [101]. Opticin production is reduced by hypoxic conditions and by secreted VEGF in human retinal pigment epithelium (RPE) cells [102]. Although opticin is antiangiogenic and inhibits preretinal neovascularization, it does not appear to influence hyaloid vascular regression or retinal vasculature development [102, 103]. This is consistent with the finding that although Opticin precursors and fragments were detected in embryonic human vitreous, there was no significant increase or decrease in their levels during the 2nd trimester [16].

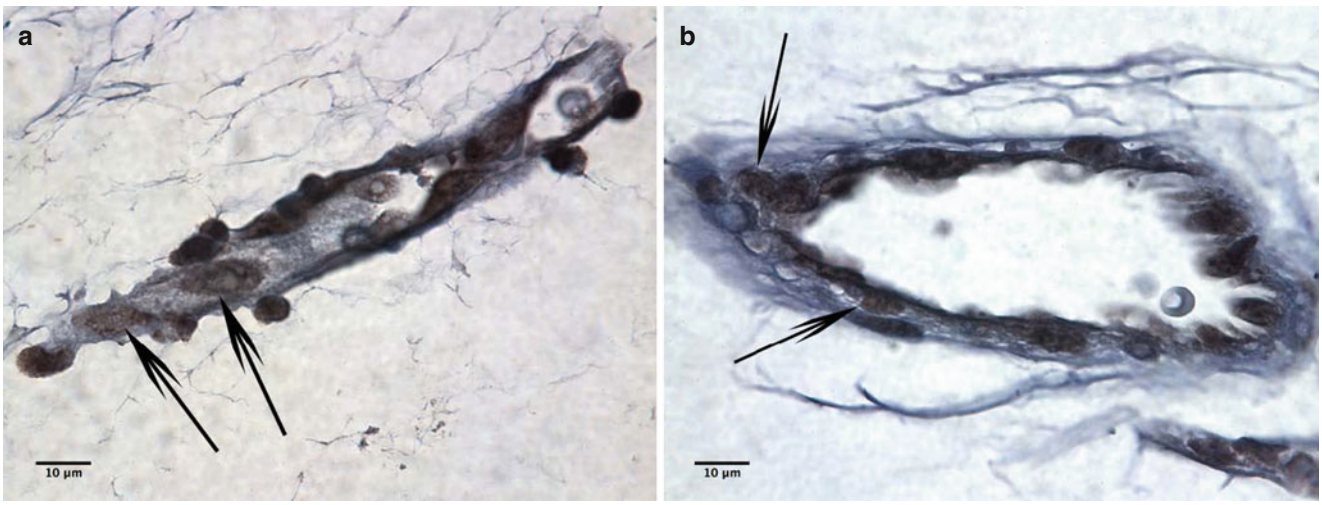

Figure I.D-4 Profilin-1. Profilin-1 was found in endothelial cells (*arrows*) of the hyaloid artery (**a**) and of the vasa hyaloidea propria (**b**) in the vitreous of a 14 WG human

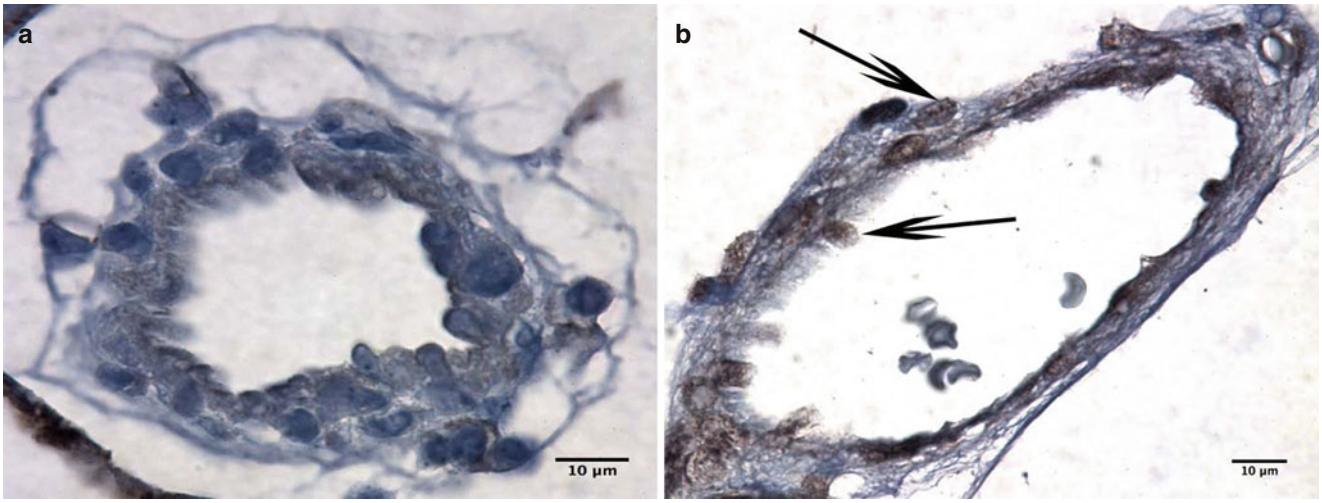

Figure I.D-5 Clusterin. Clusterin was not detected in the hyaloid vessels of 14 WG human vitreous (**a**). In 18 WG eyes, clusterin was found in the endothelial cells (*arrows*) of the tunica vasculosa lentis (**b**)

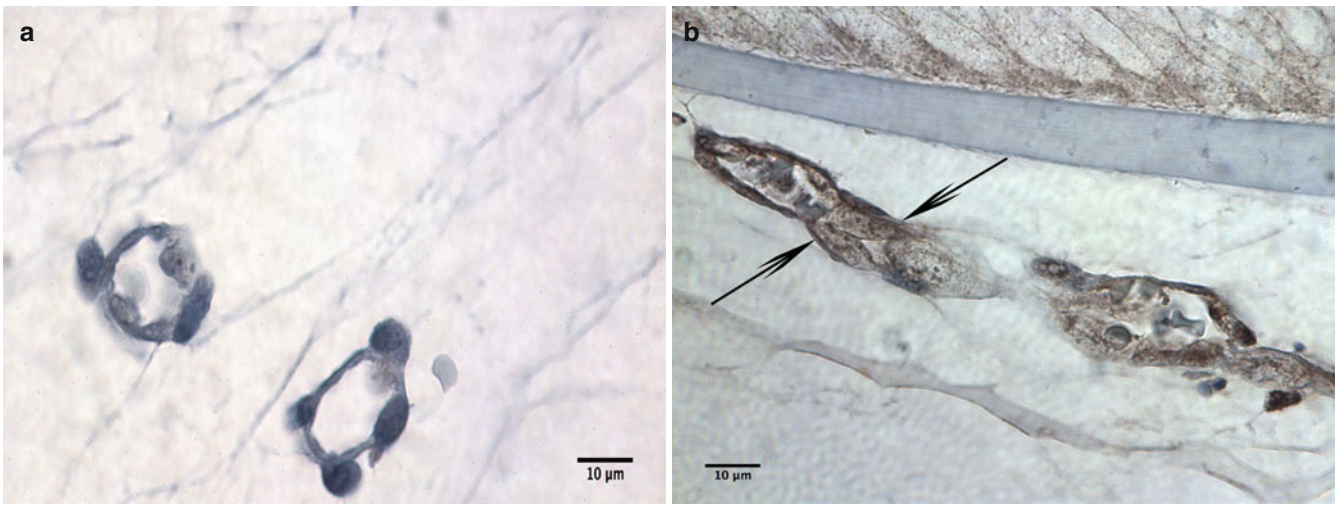

Figure I.D-6 N-Cadherin. In 14 GW eyes (**a**), N-Cadherin was not detected in endothelial cells of the hyaloid vessels. (**b**) However, in 18 WG eyes, N-Cadherin was found in the cytoplasm of endothelial cells that make up the endothelium of the TVL (*arrows*)

VII. Genetic Influences

While many proteins have been shown to have an influence on the regression of the fetal hyaloid vasculature, less is known about the upstream, genetic influences on normal or pathological vitreous development. Several studies have identified specific genes that can influence hyaloid vessel regression.

Endothelial cell-specific gene ablation of either the *transcription factor serum response factor* (*SRF*) or its cofactors *myocardin-related transcription factors A and B* (but not SRF cofactors Eph-like kinase 1 and 4) impairs protrusion of endothelial tip cell filopodia, resulting in the persistence of hyaloid vessels in mice [104].

A spontaneous mouse mutation named *fierce* (frc) involves a deletion for the nuclear receptor Nr2e1 gene (also known as Tix, mouse homologue of Drosophila tailless) that is important for normal development. The impaired regression of the hyaloid system has been observed in frc mouse on three defined genetic backgrounds (C57BL/6 J, 129P3/JEms, and B6129F1) [105]. In these mice, both large and small vessels were observed in the vitreous body, close to the optic nerve and the peripheral retina, suggesting the hyaloid vessels had failed to undergo apoptosis and regress [104]. The murine INK4a locus encodes *tumor suppressor proteins p16* (INK4a) and *p19* (ARF). INK4a–/– mice showed defects in the regression of the hyaloid vasculature [106].

Mice that are mutated in the atypical *opsin melanopsin* gene (Opn4) or are dark reared were recently reported to have persistent hyaloid vessels at 8 days postpartum and an overgrown retinal vasculature that may be explained by the fact that a light-response pathway is critical to ocular blood vessel patterning [85]. This study found that the melanopsin light-response pathway normally suppresses the number of retinal neurons, limiting hypoxia and, as a consequence, local expression of VEGF-A by retinal neurons [84].

Conclusions

Regression of the human fetal hyaloid vasculature is critical for development of vitreous, retina, and surrounding ocular structures. Although the timing of hyaloid vessel regression is established, especially in lower mammals, little is known about the exact processes and molecular mechanisms involved in the human. Recent proteomic studies of embryonic mouse and human vitreous have suggested various protein and pathways that may be involved in the regression of the hyaloid vasculature. These proteomic studies suggest that complex and highly orchestrated mechanisms are required for proper and complete hyaloid vasculature regression. Apoptosis, increased expression of antiangiogenic factors, reduced expression of endothelial survival factors, macrophagy, and perhaps external stimuli are all implicated in the regression of hyaloid vasculature. Understanding these processes will further our knowledge about pathological neovascularization in the eye and perhaps other parts of the body and might guide our approaches to new therapies.

Abbreviations

Alk-1	Activin receptor-like kinase
Ang	Angiopoietin
AQP	Aquaporin
CD	Cluster of differentiation
CO_2	Carbon dioxide
Col18	Collagen type XVIII
ED	Embryonic day
EM	Electron microscopy
FGF	Fibroblast growth factor
HA	Hyaloid artery
IPA	Ingenuity Pathway Analysis
LPA	Lysophosphatidic acid
MPNST	Malignant peripheral nerve sheath
N_2	Nitrogen
Ninj1	Nerve injury-induced protein
O_2	Oxygen
OIR	Oxygen-induced ischemic retinopathy
PDGF	Platelet-derived growth factor
PDR	Proliferative diabetic retinopathy
PECAM	Platelet endothelial cell adhesion molecule
PEDF	Pigment epithelium derived factor
PGD	Post gestational day
PM	Pupillary membrane
POSTN	Periostin
PRL	Prolactin
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor-β
TSP, THBS	Thrombospondin
TVL	Tunica vasculosa lentis
VEGF	Vascular endothelial growth factor
VHP	Vasa hyaloidea propria
WG	Weeks of gestation

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