In vivo analysis of hyaloid vasculature morphogenesis in zebrafish: A role for the lens in maturation and maintenance of the hyaloid

Andrea Hartsock, Chanjae Lee, Victoria Arnold, Jeffrey M. Gross

Department of Molecular Biosciences, Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, United States

A R T I C L E   I N F O

Article info
Article history:Received 6 June 2014Received in revised form 28 July 2014Accepted 30 July 2014

Keywords:
Zebrafish
Eye development
Hyaloid vasculature
Lens

A B S T R A C T

Two vascular networks nourish the embryonic eye as it develops – the hyaloid vasculature, located at the anterior of the eye between the retina and lens, and the choroidal vasculature, located at the posterior of the eye, surrounding the optic cup. Little is known about hyaloid development and morphogenesis, however. To begin to identify the morphogenetic underpinnings of hyaloid formation, we utilized in vivo time-lapse confocal imaging to characterize morphogenesis of the zebrafish hyaloid through 5 days post fertilization (dpf). Our data segregate hyaloid formation into three distinct morphogenetic stages: Stage I: arrival of hyaloid cells at the lens and formation of the hyaloid loop; Stage II: formation of a branched hyaloid network; Stage III: refinement of the hyaloid network. Utilizing fixed and dissected tissues, distinct Stage II and Stage III aspects of hyaloid formation were quantified over time. Combining in vivo imaging with microangiography, we demonstrate that the hyaloid system becomes fully enclosed by 5 dpf. To begin to identify the molecular and cellular mechanisms underlying hyaloid morphogenesis, we identified a recessive mutation in the mab21l2 gene, and in a subset of mab21l2 mutants the lens does not form. Utilizing these “lens-less” mutants, we determined whether the lens was required for hyaloid morphogenesis. Our data demonstrate that the lens is not required for Stage I of hyaloid formation; however, Stages II and III of hyaloid formation are disrupted in the absence of a lens, supporting a role for the lens in hyaloid maturation and maintenance. Taken together, this study provides a foundation on which the cellular, molecular and embryologic mechanisms underlying hyaloid morphogenesis can be elucidated.

© 2014 Elsevier Inc. All rights reserved.

In vivo analysis of hyaloid vasculature morphogenesis in zebrafish: A role for the lens in maturation and maintenance of the hyaloid

Introduction

The eye is a highly metabolic organ that requires substantial oxygen and nutrients for normal development. Two vascular networks nourish the embryonic eye as it develops – the hyaloid vasculature, located at the anterior of the eye between the retina and lens, and the choroidal vasculature, located at the posterior of the eye, surrounding the optic cup (reviewed in Saint-Geniez and D’Amore (2004)). The hyaloid artery enters the eye through the optic fissure in the ventral optic cup, passes into the vitreous, and contacts the posterior pole of the lens. Here, the hyaloid artery branches over the posterior hemisphere of the lens to form the hyaloid network (known as the tunica vasculosa lentis). More anteriorly, the pupillary membrane branches from the hyaloid and covers the anterior of the lens. The hyaloid network connects to the choroidal network at the annular vessel, located along the anterior aspect of the optic cup, thereby providing drainage from the hyaloid, which contains no veins. The hyaloid vasculature is thought to form via angiogenesis, although recent data from human eyes indicate that the hyaloid system in humans may develop through hemo-vasculogenesis from a pool of hemangio-blast precursors (McLeod et al., 2012). In mammals, the hyaloid system regresses during late stages of eye development, concomitant with the formation of the retinal vasculature (reviewed in Saint-Geniez and D’Amore (2004)). Defects in hyaloid regression result in persistent fetal vasculature, a congenital disorder that affects vision and may result in glaucoma, retinal detachment, cataract and strabismus if left untreated (reviewed in Shastri (2009)).

Little is known about hyaloid morphogenesis in the human eye, or in any animal model systems used to study eye development. Avian fate mapping studies have demonstrated that neural crest and mesoderm-derived cells contribute to the hyaloid vasculature, located at the anterior of the eye between the retina and lens, and the choroidal vasculature, located at the posterior of the eye, surrounding the optic cup. Little is known about hyaloid development and morphogenesis, however. To begin to identify the morphogenetic underpinnings of hyaloid formation, we utilized in vivo time-lapse confocal imaging to characterize morphogenesis of the zebrafish hyaloid through 5 days post fertilization (dpf). Our data segregate hyaloid formation into three distinct morphogenetic stages: Stage I: arrival of hyaloid cells at the lens and formation of the hyaloid loop; Stage II: formation of a branched hyaloid network; Stage III: refinement of the hyaloid network. Utilizing fixed and dissected tissues, distinct Stage II and Stage III aspects of hyaloid formation were quantified over time. Combining in vivo imaging with microangiography, we demonstrate that the hyaloid system becomes fully enclosed by 5 dpf. To begin to identify the molecular and cellular mechanisms underlying hyaloid morphogenesis, we identified a recessive mutation in the mab21l2 gene, and in a subset of mab21l2 mutants the lens does not form. Utilizing these “lens-less” mutants, we determined whether the lens was required for hyaloid morphogenesis. Our data demonstrate that the lens is not required for Stage I of hyaloid formation; however, Stages II and III of hyaloid formation are disrupted in the absence of a lens, supporting a role for the lens in hyaloid maturation and maintenance. Taken together, this study provides a foundation on which the cellular, molecular and embryologic mechanisms underlying hyaloid morphogenesis can be elucidated.

© 2014 Elsevier Inc. All rights reserved.

In vivo analysis of hyaloid vasculature morphogenesis in zebrafish: A role for the lens in maturation and maintenance of the hyaloid

Introduction

The eye is a highly metabolic organ that requires substantial oxygen and nutrients for normal development. Two vascular networks nourish the embryonic eye as it develops – the hyaloid vasculature, located at the anterior of the eye between the retina and lens, and the choroidal vasculature, located at the posterior of the eye, surrounding the optic cup (reviewed in Saint-Geniez and D’Amore (2004)). The hyaloid artery enters the eye through the optic fissure in the ventral optic cup, passes into the vitreous, and contacts the posterior pole of the lens. Here, the hyaloid artery branches over the posterior hemisphere of the lens to form the hyaloid network (known as the tunica vasculosa lentis). More anteriorly, the pupillary membrane branches from the hyaloid and covers the anterior of the lens. The hyaloid network connects to the choroidal network at the annular vessel, located along the anterior aspect of the optic cup, thereby providing drainage from the hyaloid, which contains no veins. The hyaloid vasculature is thought to form via angiogenesis, although recent data from human eyes indicate that the hyaloid system in humans may develop through hemo-vasculogenesis from a pool of hemangio-blast precursors (McLeod et al., 2012). In mammals, the hyaloid system regresses during late stages of eye development, concomitant with the formation of the retinal vasculature (reviewed in Saint-Geniez and D’Amore (2004)). Defects in hyaloid regression result in persistent fetal vasculature, a congenital disorder that affects vision and may result in glaucoma, retinal detachment, cataract and strabismus if left untreated (reviewed in Shastri (2009)).

Little is known about hyaloid morphogenesis in the human eye, or in any animal model systems used to study eye development. Avian fate mapping studies have demonstrated that neural crest and mesoderm-derived cells contribute to the hyaloid vasculature, located at the anterior of the eye between the retina and lens, and the choroidal vasculature, located at the posterior of the eye, surrounding the optic cup. Little is known about hyaloid development and morphogenesis, however. To begin to identify the morphogenetic underpinnings of hyaloid formation, we utilized in vivo time-lapse confocal imaging to characterize morphogenesis of the zebrafish hyaloid through 5 days post fertilization (dpf). Our data segregate hyaloid formation into three distinct morphogenetic stages: Stage I: arrival of hyaloid cells at the lens and formation of the hyaloid loop; Stage II: formation of a branched hyaloid network; Stage III: refinement of the hyaloid network. Utilizing fixed and dissected tissues, distinct Stage II and Stage III aspects of hyaloid formation were quantified over time. Combining in vivo imaging with microangiography, we demonstrate that the hyaloid system becomes fully enclosed by 5 dpf. To begin to identify the molecular and cellular mechanisms underlying hyaloid morphogenesis, we identified a recessive mutation in the mab21l2 gene, and in a subset of mab21l2 mutants the lens does not form. Utilizing these “lens-less” mutants, we determined whether the lens was required for hyaloid morphogenesis. Our data demonstrate that the lens is not required for Stage I of hyaloid formation; however, Stages II and III of hyaloid formation are disrupted in the absence of a lens, supporting a role for the lens in hyaloid maturation and maintenance. Taken together, this study provides a foundation on which the cellular, molecular and embryologic mechanisms underlying hyaloid morphogenesis can be elucidated.

© 2014 Elsevier Inc. All rights reserved.

Please cite this article as: Hartsock, A., et al., In vivo analysis of hyaloid vasculature morphogenesis in zebrafish: A role for the lens in maturation and maintenance of the hyaloid. Dev. Biol. (2014), http://dx.doi.org/10.1016/j.ydbio.2014.07.024
McLeod et al., 2012; Zhu et al., 2000), primates (Hamming et al., 1977), mouse (Balazs et al., 1980; Gage et al., 2005; Poche et al., 2009), hamster, rabbit, cow, cat (Balazs et al., 1980) and zebrafish (Alvarez et al., 2007; Kitambi et al., 2009), most of these studies utilized fixed samples and were therefore unable to identify any of the dynamic cellular behaviors that underlie hyaloid formation.

With an interest in the cellular dynamics underlying hyaloid morphogenesis we re-examined hyaloid formation in zebrafish. Utilizing transgenic embryos that express GFP or RFP in the vasculature under control of the fltlα (Lawson and Weinstein, 2002) and kdr1 (Wang et al., 2010) promoters, respectively, we performed in vivo time-lapse imaging of hyaloid formation. In combination with fixed sample analyses and microangiography, we describe and quantitify dynamic aspects of hyaloid morphogenesis that were heretofore unknown. Specifically, hyaloid formation can be segregated into three distinct morphogenetic stages: Stage I – arrival of hyaloid precursor cells and formation of the “hyaloid loop”, Stage II – formation of a branched hyaloid network, and Stage III – refinement and maturation of the network.

Studies in mice have shown that hyaloid development is influenced by both lens- and retina-derived ligands, and the prevailing model is that tight control of VEGF availability is critical for normal hyaloid development (Ash and Overbeek, 2000; Garcia et al., 2009; Gerhardt et al., 2003; Mitchell et al., 2006; Rutland et al., 2007; Saint-Geniez et al., 2009). Zebrafish lens ablation studies suggest that the lens is not required for recruitment of hyaloid precursor cells to the eye (Semina et al., 2006); however, these lens ablations were performed at 24 hpf, a time point after hyaloid precursor cells have already arrived at the lens (this study). Moreover, it is unknown whether the lens is required for later stages of hyaloid development. To address these questions, we cloned the affected locus in a recessive zebra fish mutant in which a subset of homozygous embryos lack a lens (mab212zm100) (Lee et al., 2012), and used these lens-less mutants to assay hyaloid formation. Our results support a model in which hyaloid precursor recruitment and Stage I of hyaloid development do not require a lens, while Stage II and Stage III hyaloid growth and maturation are lens-dependent.

Materials and methods

Fish maintenance

Zebrafish (Danio rerio) were maintained at 28.5°C on a 14-h-light/10-h-dark cycle. Embryos were obtained from the natural spawning of wild-type or heterozygous carriers set up in pairwise crosses. Alleles utilized in this study were Tg(fli1α:GFP118); (Lawson and Weinstein, 2002), Tg(kdr1:mCherry125) (Wang et al., 2010) and mab212zm100 (Lee et al., 2012). Animals were treated in accordance with University of Texas at Austin Institutional Animal Care and Use Committee provisions.

Cloning of mab212zm100 and mRNA rescue

au10 Heterozygous carriers (AB background) were outcrossed with wild-type TU fish. Heterozygous carriers of the au10 mutation were identified and then incrossed to generate homozygous mutant embryos. 50 mutant zebrafish embryos from 4 parental pairs were collected, genomic DNA was isolated (DNeasy Blood & Tissue Kit, Qiagen), and 1ug was used for Illumina sequencing at the UT Genomic Sequencing and Analysis Facility. 150 million paired-end 100 bp sequences were generated on an Illumina HiSeq 2000 machine, for an average genome coverage of 19 x. To identify putative mutations, sequencing reads were analyzed using the BSSseq mapping pipeline on MegaMapper (Obholzer et al., 2012). Candidate SNPs were confirmed by cDNA sequencing. Full-length wild-type and mutant mab212 were cloned from reverse transcribed zebrafish mRNA and then subcloned into CS10R-GFP for mRNA synthesis. RNA was synthesized using mMESSAGE mMACHINE SP6 Transcription kit (Life Technologies). One-cell embryos from au10 were injected with 100 pg of mRNA of mab212-GFP and scored for lens defects; a subset of these were genotyped to verify rescue.

Cryosectioning and confocal imaging

Embryos were prepared for cryosectioning as previously described (Uribe and Gross, 2007). 20 μm sections were generated and imaged using a Leica TCS SP5 II fixed stage microscope with a 40 × (NA 1.3) objective taking 1 μm optical slices. Images were processed using Fiji.

In vivo imaging

One hour prior to imaging, embryos were anesthetized in 0.0015 M tricaine in fish water. 16 hpf-3 dpf embryos were submerged in 0.0015 M tricaine in 1% Low-Melt Agarose (LMA; UltraClean Agarose LM; #15005) while 3 dpf and older embryos were submerged in 0.0015 M tricaine in 1.2% LMA. Embryos were mounted in a glass-bottom imaging dish just below the LMA surface for imaging on an upright microscope equipped with immersion objectives, and at the glass surface for imaging on an inverted microscope. After 5 min at room temperature, mounted embryos were completely submerged in fish water with tricaine (0.0015 M). Embryos were either imaged using a Leica TCS SP5 II (upright) microscope using a 25 × immersion objective (NA 1.8) or a Zeiss Pascal (inverted) microscope under a 20 × objective (NA 0.8). 50–100, 1 μm optical slices were acquired every 10–15 min (see movie legends for details). Each stack was compressed to a maximum projection, and projections compressed to AVI formatted time series using Leica LAS or Zeiss LSM software. AVI files were adjusted for optimal viewing (i.e. brightness and contrast) using Fiji. Still images from movies were extracted post-optimization using Fiji. All movies play at 6 frames/s.

Lens dissections and imaging

Embryos were anesthetized in tricaine, fixed in 4% PFA and stored at 4°C for 1–10 days. For dissection, embryos were submerged in PBS. Using insect pins the majority of retina was removed leaving a minimal amount of distal retinal tissue for orientation purposes, and lenses were then transferred to a glass-bottom imaging dish. Lenses were imaged on a Zeiss Pascal microscope under a 40 × objective (NA 1.3). Depending on lens size, 30–100 1 μm optical slices were obtained and maximum projections created using Zeiss LSM software. Lenses were imaged from the outer most visible edge to mid-hemisphere as indicated by the posterior coalescence of vessels.

Quantification of hyaloid formation in fixed lenses

Zeiss LSM software was used to measure the chord length (c) defined as the longest distance from the posterior hyaloid coalescence to the anterior most edge of the hyaloid (Fig. S1). The number of optical slices to image the outer most edge of the hyaloid to the most posterior coalescence of vessels was determined to be the radius (r). Arc length (A) was calculated as \( A=r\theta \), where \( \theta=2\sin^{-1}(c/2r) \) (Fig. S1). Branch number was also counted from maximum projections images. A student t-test was used to evaluate statistical significance (Microsoft Excel). Data are representative of one half of the hyaloid, imaged from a transverse view (Fig. S1).
Microangiography and post-injection imaging

GFP
+ embryos were pre-screened in fl1a:GFP or mab21l2 au10; fl1a:GFP backgrounds. One hour prior to desired injection time, embryos were anesthetized using 0.0015 M tricaine in fish water. Embryos were then submerged in 0.0015 M tricaine in 2% LMA in fish water at room temperature against stacked and glued coverslips. Using a quartz 1.0 mm diameter microinjection needle, embryos were injected with 4.5 nL of 2.5% concentration of Rhodamine–dextran (50 kD) in 0.5 M KCl. Embryos 2 and 3 dpf were injected in the heart, while embryos 4 and 5 dpf were injected in the posterior caudal vein (Schmitt et al., 2012). Embryos were then re-mounted in 0.0015 M tricaine in 1% LMA on a glass coverslip for an inverted confocal scope. After 5 min at room temperature to solidify LMA, embryos were submerged in 0.0015 M tricaine in fish water. Utilizing a Zeiss Pascal confocal microscope under a 20 /C2 objective (NA 0.8), 30–100 μm optical slices were obtained and maximum projections generated using Zeiss LSM software. Maximum projections were utilized to create iso-surface renderings in Imaris.

Results

Overview of in vivo imaging experiments

To image the developing hyaloid we utilized fl1a:GFP (Lawson and Weinstein, 2002) and kdrl:mCherry (Wang et al., 2010) transgenic lines, both of which label the developing hyaloid vasculature (Fig. S2). However, when utilizing kdrl:mCherry transgenics, mCherry was not detected within the hyaloid until 22 hpf and therefore all images prior to 22 hpf necessarily utilized fl1a: GFP. 22–72 hpf Embryos were mounted on their sides and optical slices were taken through the lens along its anterior–posterior axis (Fig. 1A-sagittal imaging). 3–5 dpf Embryos were mounted either dorsal-down or ventral-down and optical slices were collected along the dorsal–ventral axis of the lens (Fig. 4A-transverse imaging).

Stage I: arrival of hyaloid cells at the eye and formation of the hyaloid loop

Stage I of hyaloid formation results in the recruitment of hyaloid precursor cells to the vitreous/lens and the formation of the primitive hyaloid at the posterior of the lens. At 18–20 hpf, fl1a:GFP
+ cells arrive at the ventral eye and migrate through the choroid fissure toward the ventral lens (Fig. 1B; Supplementary Movie 1); at 21 hpf, they appear as a tube with multiple filopodial extensions emanating from the tip of the tube. At 24 hpf, the primitive hyaloid bifurcates, with one sprout angled nasally (Fig. 2A – arrowhead; Supplementary Movie 2), and the second sprout angled temporally (Fig. 2A – arrows, Supplementary Movie 2). The nasal branch continues to grow dorsally around the lens, until it meets cells from the temporal branch, which also extend
Stage I: hyaloid loop formation.

Stage II: formation of a branched hyaloid network

Stage III: refinement of the hyaloid network
Quantification of hyaloid growth

*In vivo* time-lapse imaging enables observations of dynamic cellular movements during hyaloid formation, but it is not practical to collect large sample sizes for quantification and statistical analyses of hyaloid formation. Therefore, we utilized lens dissections to quantify distinct aspects of hyaloid morphogenesis, specifically Stage II anterior progression and Stage III vessel refinement. 48 hpf was the earliest time point at which the lens could be removed from the optic cup with the hyaloid vasculature remaining attached. Stage II anterior progression was defined as the distance covered by the hyaloid basket along the posterior to anterior axis of the lens over time, and this was calculated by measuring the arc length of hyaloid vessels from maximum projection images of one half of the lens (Figs. 5A and S1; see “Materials and methods”). From 2 to 3 dpf, the hyaloid increases almost 400% in arc length, and this anterior growth continues from 3 to 5 dpf with statistically significant increases every 24 h (Fig. 5B). Stage III vessel refinement was defined as the reduction in branch points within the network over time, and was quantified by counting the number of identifiable hyaloid branches in maximum projection images. A statistically significant decrease in the number of branches occurs from 3 to 5 dpf (Fig. 5C).

The hyaloid system is fully enclosed at 5 dpf

Previous studies have attempted to determine when the zebrafish blood–retinal barrier (BRB) is formed and becomes functional by correlating tight junction localization and microangiography data (Hyoun Kim et al., 2011; Jeong et al., 2008; Xie et al., 2010). Results from these studies are confounding; the first reported a functional BRB that blocked a 4 kDa fluorescein dextran from...
leaking into the retina at 3 dpf (Xie et al., 2010); the second reported that a 10 kDa rhodamine dextran was not contained by the BRB at 3 dpf (Jeong et al., 2008); and the third reported that a 2000 kDa FITC–dextran was not contained at 7 dpf (Hyoun Kim et al., 2011). To distinguish between these studies we combined microangiography, using rhodamine dextran (50 kDa) injections and in vivo imaging in flt1a:GFP transgenic embryos through which we could immediately and unambiguously assess whether leakage into the retina or vitreous occurred. At 2 dpf, rhodamine was present throughout the vitreous, outside of leakage into the retina or vitreous occurred. At 2 dpf, rhodamine was detected within hyaloid vessels (Fig. 6A), demonstrating that the hyaloid was not enclosed at this early time point (Fig. 6A). At 3 dpf, rhodamine was detected within hyaloid vessels (Fig. 6B – arrowheads), specifically those at the anterior limit of the hyaloid, but it was also detected outside of the hyaloid vessels more posteriorly (Fig. 6B – arrows). At 4 dpf, rhodamine was contained within dorsal hyaloid vessels (Fig. 6B – arrowheads), while ventral vessels remained leaky, with rhodamine detected within some vessels, but outside of others (Fig. 6B – arrows). By 5 dpf, rhodamine was completely contained within the hyaloid vessels (Fig. 6B). Thus, microangiography analyses demonstrate that hyaloid vessels steadily become lumenized, with the dorsal hyaloid vessels closing prior to ventral vessels, and both becoming fully enclosed by 5 dpf.

**mab21l2au10 mutants do not form a lens**

Hyaloid development can be influenced by lens-derived signals (Ash and Overbeek, 2000; Garcia et al., 2009; Rutland et al., 2007). However, a previous study utilizing a lens ablation paradigm did not identify a requirement for the lens in the recruitment of hyaloid precursor cells to the eye (Semina et al., 2006). In this study, lens ablations were performed at 24 hpf, and our in vivo imaging data demonstrate that recruitment of hyaloid precursor cells occurs at ~18–20 hpf, and the nascent hyaloid enters the eye at 21 hpf (Fig. 1B, Supplementary Movie 1). Thus, lens ablations were performed several hours after the nascent hyaloid had already entered the eye, making it unclear if, in fact, the lens is involved in the early stages of hyaloid development. To address this question, we utilized a recessive zebrafish mutant that lacked a lens, which had been identified from a forward genetic screen for mutations that affected eye development (Lee et al., 2012). The mutant, au10, is variably penetrant with ~6.66 ± 1.2% of total embryos (~30% of mutants) possessing no obvious lens at 24 hpf (Fig. 7A-severe), and 16.41 ± 1.4% of total embryos (~70% of mutants) possessing a lens that is substantially reduced in size (Fig. 7A-mild). Histological analysis of the severe class of au10 mutants at 1 and 4 dpf highlights the complete absence of the lens (Fig. 7B). In addition to lens defects, the shape of the eye in au10 appears to be more oblong than round, and some embryos present with mild colobomas (Fig. 7A and data not shown).

To identify the gene mutated in au10, we utilized next-generation sequencing and SNP mapping (Obholzer et al., 2012), generating ~19 × average genome-wide sequencing coverage. Using the “MegaMapper” analysis pipeline, a 3 Mb window was identified on chromosome 1 that was predicted to harbor the mutation. The only “high-priority” candidate mutation within this region was an A to T transversion at nucleotide 301 of the mab21l2 coding sequence (Fig. 7C). This mutation generates a premature stop codon at amino acid 101 (K101STOP; Fig. 7D), truncating the protein by ~66%. Sequencing of genomic DNA and cDNA from au10 mutants verified that the mutation segregated with all phenotypically mutant embryos (Fig. 7C).
Originally identified in *C. elegans* as required for formation of sensory rays in the male tail (Chow et al., 1995), mab-21-family genes have been studied in a variety of organisms. In vertebrates, two mab-21 genes exist: mab21l1 and mab21l2. Mab21l1 is expressed in the mouse lens, and Mab21l1 knockouts do not form a lens (Yamada et al., 2003). Mab21l2 expression has been observed in the optic vesicle of mice (Wong et al., 1999), *Xenopus* (Baldessari et al., 2004) and zebrafish (Wong and Chow, 2002), and it is detected in human retinal extracts (Margolis et al., 1996). In zebrafish, mab21l1 and mab21l2 are both expressed in the developing optic cup at 11 hpf, with mab21l1 becoming enriched in the retina at 24 hpf, and mab21l2 expressed in the lens and dorsal and nasal aspects of the retina (Cederlund et al., 2011). These published data are consistent with the hypothesis that au10 possesses a mutation in mab21l2, and to validate this, mRNA rescue was performed. At 4 dpf, lens formation was normal in all embryos derived from au10 incrosses injected with mab21l2-GFP (*n* = 51; Fig. 7F). A subset of these were genotyped, and lens development was rescued in mab21l2 mutants (*n* = 6; Fig. 7E), supporting that au10 is a mutation in mab21l2 (hereafter referred to as mab21l2au10).

Little is known about the cellular functions of mab21l2; a myc-tagged version of mouse Mab21l2 is localized to the nucleus when expressed in NIH3T3 cells (Mariani et al., 1999), and our GFP-tagged mab21l2 was also detected in the nucleus (data not shown). In a heterologous cell culture expression system, *Xenopus* Gal4-tagged Mab21l2 acts as a transcriptional repressor (Baldessari et al., 2004). The position of the nonsense mutation in mab21l2au10 makes its analysis challenging.

---

**Fig. 5.** Quantification of hyaloid growth. (A) DIC and GFP maximum projections images of dissected lenses from *fl1a*:GFP embryos at 12 h intervals from 2 to 5 dpf. Dashed line indicates location of the lens. Scale bars = 50 μm. D: Dorsal, V: Ventral, La: Lens anterior, Lp: Lens posterior. (B) Quantification of anterior progression of the hyaloid over time. (C) Reduction in hyaloid vessel branching over time. *p* ≤ 0.01, †*p* ≤ 0.001, ‡*p* ≤ 1 × 10⁻¹⁰; *n*—number of samples analyzed.

**Fig. 6.** Microangiography demonstrates that the hyaloid vessel is fully enclosed by 5 dpf. (A) Maximum projection images of the eye of a *fl1a*:GFP (green) embryo injected with 50 kDa rhodamine dextran (red). Merged image highlights that rhodamine is detected throughout the vitreous. Iso-surface renderings of maximum projections collected from the eyes of 3–5 dpf *fl1a*:GFP (green) embryos injected with 50 kDa rhodamine dextran (red) and imaged either dorsally or ventrally. Arrowhead highlights rhodamine containment in a *fl1a*:GFP vessel and arrows highlight rhodamine outside of vessels. hh:mm. Scale bars = 50 μm. D: Dorsal, V: Ventral, N: Nasal, T: Temporal, La: Lens anterior, Lp: Lens posterior.

Please cite this article as: Hartsock, A., et al., *In vivo* analysis of hyaloid vasculature morphogenesis in zebrafish: A role for the lens in maturation and maintenance of the hyaloid. Dev. Biol. (2014), http://dx.doi.org/10.1016/j.ydbio.2014.07.024
The lens is required for Stages II and III of hyaloid formation through which we could address these outstanding questions.

The lens is required for Stage I to progress in mild and severe mab2l2au10; embryos. Stage I hyaloid loop formation was normal, indicating that while there is a lumenized and enclosed vessel leading into the hyaloid, the hyaloid vasculature in the distal retina is not possessed by all the mab2l2au10 mutants possess defects in lens formation. (A) Images of phenotypically wild-type sibling and mab2l2au10 mutants at 4 dpf. High-magnification views of the eyes of mild and severe mab2l2au10 mutants. (B) Transverse cryosections of wild-type and severe mab2l2au10 mutants at 1 and 4 dpf highlighting the lack of a lens in severe mab2l2au10 mutants. Scale bars = 50 μm. (C) Genomic sequences from wild-type, heterozygous and mab2l2au10 mutants. mab2l2au10 mutants possess an A→T transversion at position 301, resulting in a premature stop codon at amino acid 101. (D) Schematic of protein length of wild-type and mab2l2au10 mutant embryo injected with mab2l2-GFP (rescue). (F) Quantification of lens phenotype after mab2l2-GFP injection.

As discussed above, hyaloid development can be influenced by lens-derived signals, but it is unclear whether lens is required for the recruitment of hyaloid precursor cells to the eye, or how the lens facilitates hyaloid maturation. With this in mind, we wanted to utilize mab2l2au10 mutants that lacked a lens as a tool through which we could address these outstanding questions. mab2l2au10; fli1a:GFP embryos were generated, and we performed in vivo imaging of hyaloid formation, as above. Hyaloid arrival is delayed by ∼2 h in mab2l2au10 mutants (Fig. 8A; Supplementary Movie 7), although by ∼26 hpf, the hyaloid of mab2l2au10 mutants was indistinguishable from that of wild-type embryos. Stage 1 hyaloid loop formation was normal, indicating that while lack of a lens may slightly delay the arrival of hyaloid precursor cells, the lens is not required for Stage 1 to progress (Supplementary Movie 8 and Fig. 8B up to 29:00, compare to Fig. 2A). In vivo imaging of Stages I–II of hyaloid formation in mab2l2au10 mutants revealed hyaloid cells that were highly active, possessing multiple filopodial extensions in all directions, but these cells did not assemble into obvious vessels (Fig. 8B; Supplementary Movies 7–9). In addition, the network appears collapsed and is skewed in the temporal direction (Fig. 8B; Supplementary Movie 8, compare to Fig. 2A). Interestingly, this phenotype was observed in both mab2l2au10 mutants that lacked a lens, as well as mab2l2au10 mutants with smaller lenses (Fig. S3A), suggesting that collapse of the hyaloid was not solely due to the lack of a lens (i.e. not the lack of a physical substrate). At 3 dpf, the mab2l2au10 mutant hyaloid extended to the distal retina and formed connections to the annular ring (Fig. 8D – arrowhead; Supplementary Movies 10 and 11), similar to wild-type embryos, but the hyaloid network retained its collapsed appearance (Fig. 8D – arrowheads; Supplementary Movies 10 and 11, compare to Figs. 1B and 2A). Importantly, examination of intersegmental vessel formation revealed no defects in the severe class of mab2l2au10 mutants (Fig. 5B), suggesting that the mab2l2au10 mutation itself does not result in general vascular defects. Finally, microangiography analyses of mab2l2au10 mutants demonstrated that while there is a lumenized and enclosed vessel leading into the hyaloid, the hyaloid vasculature in the distal retina is not enclosed (Fig. 8E).

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2014.07.024.

Discussion

The goal of this study was to characterize the highly dynamic morphogenesis of the hyaloid vasculature of the vertebrate eye, and to use these data to begin to dissect the molecular and cellular underpinnings of hyaloid development. Capitalizing on the strengths of the zebrafish system for in vivo imaging, we were able to observe hyaloid formation in vivo from ∼1 to 5 dpf and identify key phases of this process, something that has not been performed previously in humans or any vertebrate model system. Indeed, data from previous studies in zebrafish, utilizing fixed and dissected lens samples, suggested that hyaloid formation initiates

Please cite this article as: Hartsock, A., et al., In vivo analysis of hyaloid vasculature morphogenesis in zebrafish: A role for the lens in maturation and maintenance of the hyaloid. Dev. Biol. (2014), http://dx.doi.org/10.1016/j.ydbio.2014.07.024
at ~48–60 hpf (Alvarez et al., 2007). Our in vivo data, however, demonstrate that hyaloid development starts far earlier than this, with the first vascular cells detected in the eye between 18 and 20 hpf, and a morphologically obvious hyaloid present by 30 hpf. Moreover, from our in vivo data, in comparison to the Alvarez et al. (2007) study, the earliest phases of hyaloid development are far more complex than what can be identified from fixed samples (Alvarez et al., 2007). These data are consistent with studies by Kitambi et al. (2009) who detected fil1a::GFP cells around the lens at 24 hpf and a complex hyaloid at 48 hpf (Kitambi et al., 2009).

Our data segregate hyaloid formation into three distinct stages (summarized in Fig. 9). During Stage I, hyaloid precursor cells arrive at the lens and form the hyaloid loop at the posterior of the lens (Fig. 9A). In Stage II, the hyaloid system substantially...

Please cite this article as: Hartsock, A., et al., In vivo analysis of hyaloid vasculature morphogenesis in zebrafish: A role for the lens in maturation and maintenance of the hyaloid. Dev. Biol. (2014), http://dx.doi.org/10.1016/j.ydbio.2014.07.024
elaborates into a branched hyaloid network (Fig. 9B). Throughout Stage III, the hyaloid network is redefined, with an overall decrease in number of branches and the system becomes fully enclosed (Fig. 9C). In addition, utilizing fixed samples, we were able to demonstrate quantifiable landmarks that coincide with Stage II (anterior progression) and Stage III (branch point reduction). Finally, utilizing a recessive mab21l2 mutant that does not form a lens, we were able to determine that the lens is not required for Stage I of hyaloid formation, but is required for Stages II and III of hyaloid maturation.

The mechanisms facilitating hyaloid formation have not been well studied. Highlighting this point, a recent study in zebrafish demonstrated that choroid fissure closure can be affected by defects in the developing hyaloid (Weiss et al., 2012). In this study, the hyaloid was severely dilated in lmo2 mutants and this physically impeded closure of the choroid fissure, resulting in colobomas. Thus, not only is a functional hyaloid system required for growth of the eye, defects in hyaloid formation can contribute to other ocular malformations.

Angiogenic sprouting has been studied extensively in a variety of contexts including zebrafish intersegmental vessels and the mouse retina (reviewed in Eilken and Adams (2010)). While prevailing models suggest that hyaloid growth occurs through angiogenesis (e.g. Saint-Geniez and D’Amore, 2004), recent data in the human eye suggest that hemo-vasculogenesis may build the hyaloid (McLeod et al., 2012). Our in vivo imaging data support an angiogenic mechanism for hyaloid development in zebrafish, and to our knowledge, these data are the first to demonstrate hyaloid angiogenesis in vivo in any vertebrate system. During angiogenesis, multi-cellular extensions emanate from existing vessels, and these extensions are polarized along their proximal–distal axes, with the proximal region comprised of stalk cells and the distal region containing a tip cell (Adams and Alitalo, 2007; Geudens and Gerhardt, 2011). The tip cells possesses numerous filopodial extensions that guide growth and branching of the vessels as they presumably encounter pro-and anti-angiogenic factors (reviewed in (Lamalice et al., 2007). From in vivo imaging of hyaloid formation, Stages I and II showed a tip cell sprouting mechanism to form the hyaloid loop and to increase the complexity of the vessel network, and this is similar to that observed in zebrafish intersegmental vessels (Lawson and Weinstein, 2002), and angiogenesis in the mouse retina (Auszprunk and Folkman, 1977; Fruttiger, 2007). Recently it was shown that during angiogenesis of the aorta and retina, tip cells are sometimes overtaken by stalk cells and this results in cell mixing during vessel formation (Arima et al., 2011). During Stage I of hyaloid morphogenesis, we did not detect any stalk cells overtaking tip cells, however we cannot rule out the possibility that stalk cells overtake tip cells during Stage II and contribute to the anterior progression of the hyaloid. Lineage tracing studies will be able to resolve this issue and determine the fates of individual hyaloid cells within the vessel network, as well as the morphogenetic mechanisms underlying hyaloid expansion.

Stage III of hyaloid formation involves a remodeling of the hyaloid vasculature network by vessel pruning, leading to a
reduction in overall vasculature complexity. This quantifiable reduction in vascular complexity could result from retraction of vessel contacts within branches or from apoptosis of hyaloid cells within the vessels. Results from in vivo imaging indicate that branches retract, and this is similar to what has been observed during vascular remodeling in the zebrfish (Chen et al., 2012) and mouse brain (Wang et al., 1992). Interestingly, vasculature remodeling in the zebrfish brain results from a lack of blood flow into the vessels that are being pruned (Chen et al., 2012). Additionally, observations of Nasal Ciliary Artery pruning in zebrfish demonstrate that a pruned vessel becomes unicellular and elongated as it regresses from the maintained vessel network (Kochhan et al., 2013), similar to our observations of vessel remodeling in the hyaloid (Fig. 4). Our in vivo imaging of filha:GFP embryos indicates the final stage of hyaloid development is comprised of a reduction in vasculature complexity due to reduction in cell–cell connections. Although microangiography analysis provides insight into lumenization of the hyaloid, additional studies utilizing a transgenic line in which blood cells are labeled would be required to determine if lack of blood flow precedes hyaloid remodeling.

To begin to dissect the tissues required for hyaloid formation, we cloned a recessive mutation in mab21l2, and ~30% of mab21l2au10 mutants lacked lenses. Morpholino-mediated knockdown of mab21l2 in zebrfish resulted in a range of ocular defects that included microphthalmia and delays in retinal differentiation (Kennedy et al., 2004). Mab21l2 morphants also displayed substantially elevated levels of apoptosis in their retinas at 24 hpf, and regions of persistent death at the retinal periphery at 3 dpf. While a lens did form in mab21l2 morphants, the lens epithelium was abnormal and possibly undergoing apoptosis. Hyaloid vessels in mab21l2 morphants were also reported to be thicker and poorly patterned at 5 dpf (Alvarez et al., 2007). These phenotypes are different than those observed in mab21l2au10 mutants, and could reflect maternal compensation in the mutants, or compensation from mab21l1, whose expression overlaps that of mab21l2 in many regions of the embryo, including the retina (Cederlund et al., 2011). Despite truncating the protein by roughly two-thirds, the mab21l2au10 mutation could also be hypomorphic, and the truncated protein retains some of its endogenous function. Mab21l2 is not predicted to possess any conserved protein domains, and beyond localization to the nucleus when ectopically expressed (Mariani et al., 1999); CL, unpublished observations), little is known about the molecular functions of the protein. From a gene regulatory network perspective, mab21l2 expression is regulated by Pax6 (Wolf et al., 2009) and it is thought to act upstream of lens formation (Cvekl and Duncan, 2007; Yamada et al., 2004). Future studies will be needed to determine whether mab21l2 acts as a transcription factor, whether it binds DNA directly or as part of a complex, and what targets it regulates during lens formation.

With respect to hyaloid formation and the influence of the lens, our data demonstrate that hyaloid precursor cells were recruited to the optic cup/vitreous in mab21l2 mutants although they were slightly delayed in their arrival, supporting a model in which the lens is not required for hyaloid precursor cell recruitment, though it may contribute to the process. Late Stage I (hyaloid loop formation) and Stages II and III of hyaloid morphogenesis were disrupted in mab21l2 mutants, indicating that the lens is required for sustained hyaloid growth and maturation. VEGF expression within the lens is known to influence hyaloid development (Ash and Overbeeck, 2000; Garcia et al., 2009; Rutland et al., 2007), so it is possible that in the absence of a VEGF source, growth and maintenance of the hyaloid are perturbed in mab21l2 mutants. Alternatively, the lens is enclosed by the lens capsule, an extracellular matrix (ECM)-rich basement membrane on which the developing hyaloid resides. Previous studies in mice and zebrfish have demonstrated that ECM components of the lens capsule such as laminin alpha 1 and collagen are required for hyaloid development (Fukai et al., 2002; Rutland et al., 2007; Saint-Geniez et al., 2009). While mouse knockouts for many ECM components are embryonic lethal prior to lens formation (Alpy et al., 2005; Edwards et al., 2010; Francis et al., 2002; George et al., 1997, 1993; Miner et al., 2004), many zebrfish ECM mutants survive to 5–7 dpf providing models through which the later roles of these ECM components can be studied (Amsterdam et al., 2004; Karlstrom et al., 1996; Koshida et al., 2005; Parsons et al., 2002; Trinh and Stainier, 2004). The zebrfish lens capsule contains laminin-111 (Hayes et al., 2012; Lee and Gross, 2007), fibronectin (Hayes et al., 2012) and multiple collagens (Fang et al., 2010; Xiao and Baier, 2007). Thus, it is likely that these components are required for the growth and maturation of the hyaloid by providing a substrate on which the hyaloid cells migrate, or a signal that shapes their morphogenesis. Indeed, preliminary analyses of zebrfish fibronectin1b mutants suggest that the hyaloid is malformed (AH, unpublished observations). It will be interesting to utilize other zebrfish ECM mutants to determine how hyaloid formation is affected in the absence of distinct ECM components.

In summary, this study establishes a framework for in vivo imaging of hyaloid vasculature development in the zebrfish eye, providing a vertebrate model through which the cellular, molecular and embryologic mechanisms underlying distinct phases of hyaloid formation can be elucidated. Furthermore, it provides quantifiable landmarks that can be used for identifying defects in hyaloid morphogenesis. Finally, the results support a role for the lens in the maturation and maintenance of the hyaloid, but not in the initial stages of hyaloid formation.

Acknowledgements

We are grateful to Tom Waits for technical assistance, members of the Gross lab for helpful comments and criticisms on this work, Nikolaus Obholzer for advice on using Megamapper and to Jeff Essner for providing kdrt::mCherry15 transgenics and helpful advice. AH thanks Dave Raible, Andres Collazo and Jim Fadool for advice on live imaging experiments provided during the Zebrafish Genetics and Development course at the Marine Biological Laboratories in Woods Hole, MA. This work was funded by the National Institutes of Health/National Eye Institute Grants R01-EY18005, EY18005-04S1 and R21-EY22770 to JMG, and the University of Texas at Austin Undergraduate Research Fellowship to VA. In vivo imaging was performed on a confocal microscope funded by the NIH S10-RR028951. Zebrfish were obtained from ZIRC, which is supported by the NIH-NCRR Grant P40 RR012546.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.07.024.

References