CHAPTER 8

Basement Membrane Diseases in Zebrafish

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Abstract

Basement membranes (BMs) are a complex, sheet-like network of specialized extracellular matrix that underlies epithelial cells and surrounds muscle cells. They provide adherence between neighboring tissues, permit some flexibility of these adherent structures, and can act as a store for growth factors and as a guide for cell

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migration. The BM is not just a static structure; its deposition and remodeling are important for many processes including embryonic development, immune response, and wound healing. To date, dysfunction in BM deposition or remodeling has been linked to many human congenital disorders and diseases, affecting many different tissues in the body, including malformations, dystrophies, and cancer. However, many questions remain to be answered on the role BM proteins, and their mutations, play in the pathogenesis of human disease. In recent years, the zebrafish (*Danio rerio*) has emerged as a powerful animal model for human development and disease. In the first part of this chapter, we provide an overview of described defects caused by BM dysfunction in zebrafish, including development and function of notochord, muscle, central nervous system, skin, cardiovascular system, and kidney. In the second part, we will describe details of methods used to visualize and assess the structure of the BM in zebrafish, and to functionally analyze its different components.

I. Introduction

Basement membranes (BMs) are dense, acellular, 60- to 300-nm thick specialized, sheet-like extracellular matrix structures that underlie all epithelia, and in higher organisms BMs also underlie endothelial cells and surround muscle cells, nerve-supporting cells, and adipocytes (Kalluri, 2003; Paulsson, 1992; Yurchenco *et al.*, 2004). Many vertebrate extracellular matrix (ECM) genes evolved rather recently within the deuterostome lineage, involving an expansion and elaboration of pre-existing families (e.g. laminins and collagens), as well as the invention of novel proteins (e.g. fibronectin and tenascins). This most likely is a reflection of the versatile roles of the ECM in different species and biological processes (Hynes, 2009). In contrast the core BM components are much more ancient and found in most metazoa, suggesting that BMs were crucial for metazoan evolution (Hynes, 2009; Whittaker *et al.*, 2006). This ancient BM "toolkit" includes collagen IV, laminin, nidogen, the heparin sulfate proteoglycan (HSPG) perlecan, and type XV/XVIII collagen (Figure 1A). Laminins are heterotrimers composed of one α, one β, and one γ chain. In mammals, at least 18 isoforms isoforms have been identified, which are composed of 11 different subunits (α1–5, β1–3, γ1–3) (Colognato and Yurchenco, 2000; Durbeej, 2010). Collagen subunits are also made up of three polypeptide strands that form a triple helix and self-associate to form a cooperative quaternary structure stabilized by numerous hydrogen bonds. So far, 28 types of collagen have been identified and described (I–XXVIII), which are present at many sites throughout the body (Ricard-Blum, 2011). Each collagen is encoded either by a single or by multiple genes. Mammalian type IV collagen, the major component of BMs, is encoded by six different genes, *COL4A1–COL4A6* (Hudson *et al.*, 1993, 2003). Of these six different chains, α1 and α2 are present in all mammalian BMs, whereas the remaining chains show distinct tissue-specific expression patterns (Hudson *et al.*, 2003; Kleppel *et al.*, 1989; Nakano *et al.*, 1999, 2001; Ninomiya *et al.*, 1995). Both laminins and collagen IV contribute to proper mechanical stability within the BM. In

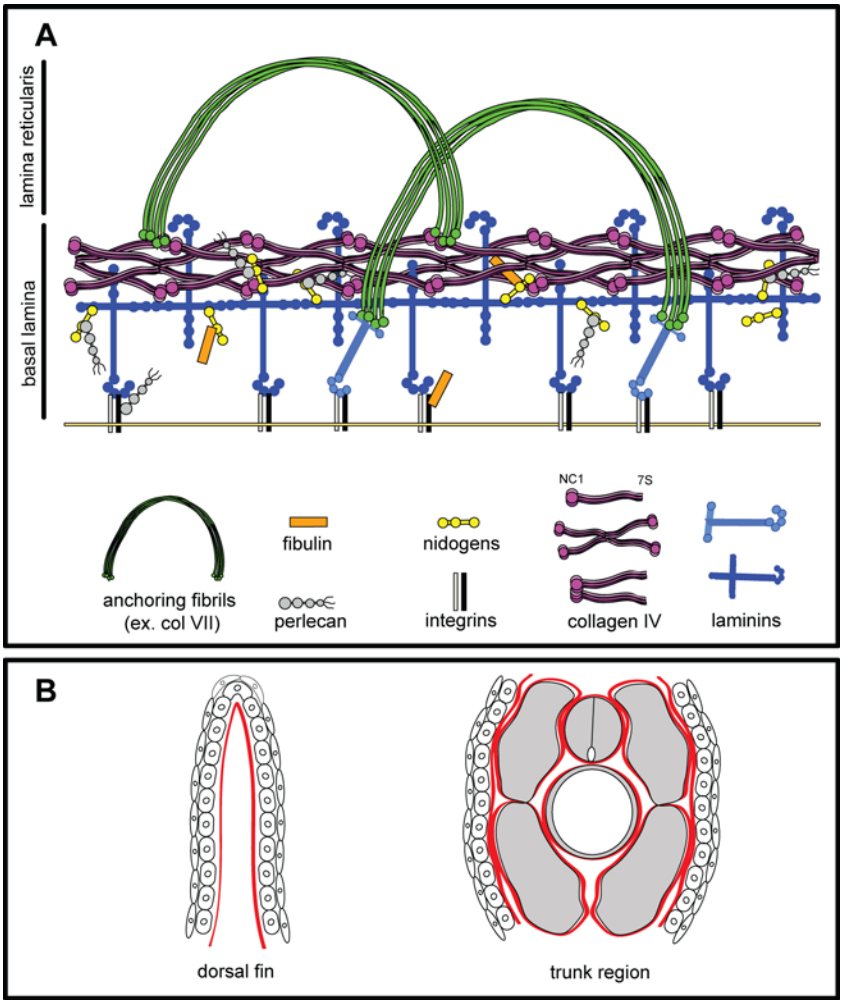

Fig. 1 (A) Simplified basement membrane (BM) diagram composed of basal lamina and lamina reticularis. This diagram contains the major components of the BM: type IV collagen, laminin, HSPGs (perlecan), and nidogen; two examples of minor components: type VII collagen and fibulins; and integrins as examples of cell adhesion molecules that bind directly to BM proteins. The localization of the molecules in the BM represents their binding partners; however, not all interactions are shown, such as type IV collagen binding to dimers of integrins. Two types of laminin are shown: the "classical" laminin structure (dark blue) and laminin5 (light blue) that has truncations of all three short arms. Type IV collagen is shown as a protomer (a trimer of α-chains) that can associate via its carboxy-terminal NC1 domain to form dimers, or via its glycosylated amino-terminal 7S region to form tetramers (diagram based on Cheng *et al.*, 1997; Kalluri, 2003; Masunaga, 2006; Yurchenco *et al.*, 2004). (B) Diagrams showing the BM of the fin and trunk of a zebrafish embryo. At this stage, the epidermis consists of two layers: the inner basal layer and the outer periderm. The BM is shown as a red line on the basal surface of the epidermis. The dermis at this time point is acellular. Adjacent BMs are connected via cross-fibers (not shown here). In the trunk of the embryo, the BM surrounds the main structures: epidermis, somites, notochord, and spinal cord. The skin is also composed of one layer of epidermis and one layer of periderm. At this stage, the BM of the epidermis is in very close proximity to the BM of the somites. (See color plate.)

addition, they can mediate attachment to adjacent epithelial cells by binding to transmembrane receptors such as integrins or dystroglycan (Fig. 1A; see also below for more details). Minor BM components include SPARC, fibulins, agrin, and type VII, XV, and XVIII collagens (Fig. 1A) (Kalluri, 2003; Yurchenco *et al.*, 2004; Yurchenco and Schittny, 1990). The BM has many vital functions including to both connect and separate epithelia and their neighboring connective tissue, as a substrate for cell migration, storage, and presentation of growth factors and as a nonimpenetrable barrier to cell invasion (Aviezer *et al.*, 1994; Gohring *et al.*, 1998; Iozzo, 2005; Kramer, 2005; Marinkovich, 2007; Rowe and Weiss, 2008; Sherwood, 2006).

Although the major BM molecules are always present, the composition of the BM can vary depending on where it is assembled, as demonstrated by tissue-specific expression patterns for several different BM component isoforms (Aumailley and Krieg, 1996; Hallmann *et al.*, 2005; Maatta *et al.*, 2004). It has also, more recently, been suggested that the expression patterns of BM components and, therefore, BM composition can not only be spatially but also temporally regulated during development (Hudson *et al.*, 2003; Saito *et al.*, 2010).

To date, much of the work performed on the composition and distribution of BM components has been performed on human tissue samples or mammalian animal models; nevertheless, zebrafish BM structure and the components of this structure are highly similar to those found in humans and mammalian models that, along with the plethora of other advantages of a lower vertebrate model, makes the zebrafish a wonderful system to study BM homeostasis, formation, and disease. Indeed, in the last few years, aided by powerful techniques of genetic manipulation and transplantation, zebrafish researchers have made crucial contributions to BM research, most of which are also relevant for human diseases, ranging from the developmental multisystem disorder Fraser syndrome (Carney *et al.*, 2010) to muscular dystrophies (Jacoby *et al.*, 2009; Thornhill *et al.*, 2008) and diabetic retinopathies (Alvarez *et al.*, 2010). Here, we will first review current zebrafish literature dealing with different developmental processes and pathologies involving BM function and dysfunction, followed by a detailed description of methods used to study BM composition and function in zebrafish.

II. Basement Membrane-Related Zebrafish Pathologies

The zebrafish BM represents a powerful biological tool for the study of the adherent properties and dynamic regulation of this specialized extracellular matrix structure as well as providing valuable insight into the pathogenesis of BM-related human disease. Forward genetic screening (see below; Driever *et al.*, 1996; Haffter *et al.*, 1996), for which zebrafish has become the predominant vertebrate model system, has identified multiple BM mutants that are related to human disease (see below). Like in other vertebrates, the homeostasis of the BM is important to maintain the flexibility and stability of a tissue. Any disruption to the connection between epithelial cells and the BM or between the BM and the underlying connective tissue

can lead to blistering, dysfunction, and even degeneration of the tissue (Carney *et al.*, 2010; Masunaga, 2006; Olasz and Yancey, 2008). In addition, basic morphogenetic processes of development can be affected, such as cell migratory processes that require the BM as a substrate or guiding structure. An overview of known zebrafish mutants with defects of BM formation, stability, or function is presented in Table I. These mutants and their phenotypes will be discussed in more detail below, together with the phenotypes of morphants, in which genes encoding BM components, binding partners, or modifiers have been knocked down via antisense morpholino oligonucleotides.

As in other vertebrates, including mammals, BMs or BM-like ECM structures are present at multiple sites of the developing zebrafish embryo, around the notochord, the somites, and the neural tube, on the basal side of endothelial cells in the walls of blood vessels, and on the basal side of the basal epidermal layer of the skin, separating it from the underlying dermis (Fig. 1B). For all of these sites, zebrafish mutants or morphants have been described.

A. Notochord

The notochord is a transient rod-like structure located at the developing midline, ventral to the neural tube and dorsal to the dorsal aorta. It is composed of a single column of large vacuolated cells surrounded by a thick BM. Studies of zebrafish mutants showed that this BM plays a fundamental role in notochord development

Table I
Zebrafish mutants in basement membrane components

Mutant	Gene	Affected structures	References
bashful	*laminin α1*	Notochord, intersegmental blood vessels, somatic muscle	Pollard *et al.* (2006)
candyfloss	*laminin α2*	Notochord, intersegmental blood vessels, somatic muscle	Hall *et al.* (2007)
fransen	*laminin α5*	Fin skin (epidermis–BM junction)	Carney *et al.* (2010), Webb *et al.* (2007)
grumpy	*laminin β1a*	Notochord, intersegmental blood vessels, somitic muscle	Parsons *et al.* (2002b), Snow *et al.* (2008)
softy	*laminin β2*	Somitic muscle	Jacoby *et al.* (2009)
sleepy	*laminin γ1*	Notochord, intersegmental blood vessels, somitic muscle	Parsons *et al.* (2002b), Snow *et al.* (2008)
gulliver	*collagen 8α1a*	Notochord	Gansner and Gitlin (2008a)
dragnet	*collagen 4α5*	Retinotectal axonal targeting	Xiao and Baier (2007)
pinfin	*fras1*	Fin skin (BM–dermis junction)	Carney *et al.* (2010)
blasen	*frem2a*	Fin skin (BM–dermis junction)	Carney *et al.* (2010)
rafels	*frem1a*	Fin skin (BM–dermis junction)	Carney *et al.* (2010)
nagel	*hmcn1*	Fin skin (BM–dermis junction)	Carney *et al.* (2010)
puff daddy	*fibrillin2b*	Notochord, blood vessels, fin skin (BM–dermis junction)	Xiao and Baier (2007)

and function (see for review Scott and Stemple, 2005). Transmission electron microscopy (TEM) reveals that the notochordal BM is composed of at least three layers of organized fibers: an inner layer that most likely contains laminins, a thicker intermediate layer with dense collagen fibers, and an outer layer with perpendicular collagen fibers that run in parallel to the notochord (Parsons *et al.*, 2002b; Scott and Stemple, 2005; Stemple, 2005). In *grumpy* and *sleepy* mutants, which carry loss-of-function mutations in the laminin β1 and γ1 chain genes *lamb1a* and *lamc1*, respectively, all three layers are disorganized, accompanied by failed notochordal differentiation and subsequent death of chordamesodermal cells (Parsons *et al.*, 2002b). This indicates that signaling by BM components to the chordamesoderm is required for notochordal differentiation and survival, although the receptors on chordamesodermal cells mediating these effects are unknown so far (Scott and Stemple, 2005). During early vertebrate development, the notochord serves both structural and signaling functions (Stemple, 2005). It is important for the elongation of the embryonic axis. As the notochord cells become vacuolated, the notochord stiffens and elongates. Accordingly, it has been proposed that the perinotochordal BM has to withstand high hydrostatic pressures, and is therefore comparable to the glomerular basement membrane (GBM) of the mammalian kidney (see below) (Parsons *et al.*, 2002b). In *lamb1a* and *lamc1* mutants, notochordal cells fail to differentiate and vacuoles do not inflate, resulting in a severe shortening of the embryonic axis. In addition, the notochord plays a critical function in muscle development during the first stages of somitogenesis (approximately 10 hours postfertilization (hpf)), when notochord-derived Hedgehog (Hh) proteins induce medial paraxial mesoderm, also known as adaxial cells, to become muscle pioneer and slow-twitch muscle fibers (Wolff *et al.*, 2003). *lamb1a* and *lamc1* mutants lack these specific muscle cell types, although expression of the *hh* genes in chordamesodermal cells is unaltered or even upregulated (Parsons *et al.*, 2002b; Stemple *et al.*, 1996), suggesting that laminins are crucial for proper lateral transport of the signaling proteins.

A similar, although weaker, phenotype is present in *lama1* (*bashful*) mutants (Pollard *et al.*, 2006). The α1, β1, and γ1 chains are essential components of Laminin111 (formerly called Laminin1; Aumailley *et al.*, 2005). The weaker defects of the *lama1* mutants are most likely due to functional redundancy between the α1 and other laminin α chains. Thus, phenotypes as strong as in *lamb1a* and *lamc1* mutants were obtained when *lama1* mutants were injected with antisense morpholinos against *lama4* or *lama5*, which, in association with laminin β1 and laminin γ1, form laminin 411 (formerly called laminin 8) and laminin 511 (formerly called laminin 10) (Pollard *et al.*, 2006).

In addition to laminins, essential roles for zebrafish notochord development have been demonstrated for collagen VIII (Gansner and Gitlin, 2008a), collagen XV (Pagnon-Minot *et al.*, 2008), collagen-modifying lysyl oxidases (Gansner *et al.*, 2007), and Fibrillin 2 (Gansner *et al.*, 2008b), a member of a family of extracellular matrix proteins that form 10-nm-diameter microfibrils and can bind to integrins and growth factors such as bone morphogenetic proteins (BMPs) (Hubmacher *et al.*, 2006). Like the laminins, all of these ECM proteins are made by notochordal cells.

fibrillin 2b (*puff daddy*) mutants display rather late and moderate notochord deficiencies, with a specific disorganization of the outer layer of the perinotochordal BM, whereas the medial and inner layers appear normal (Gansner *et al.*, 2008b). Morpholino-mediated knockdown of *col15a1a* causes a disorganization of all perinotochordal BM layers, defective notochordal differentiation, and alterations in somitic muscle patterning. Although the notochord and BM defects are similar to those in the laminin mutants, the somite defects appear different. In contrast to the laminin mutants, *col15a1a* morphants had unaltered numbers of muscle pioneer cells, whereas the number of fast-twitch muscle fibers, which also depend on Hh signals (Wolff *et al.*, 2003), was substantially increased, suggesting that collagen XV might normally hinder, rather than facilitate, the transport of Hh proteins (Pagnon-Minot *et al.*, 2008). Comparable later and weaker notochordal and BM defects were observed in mutants (*gulliver*) and morphants in *col8a1a*. Here, only the medial layer of the BM seems affected, with disorganized collagen fibrils, and notochordal cells form inflated vacuoles and survive, with an undulated shape of the notochord (Gansner and Gitlin, 2008a). A similar phenotype was obtained on morpholino-based knockdown of the lysyl oxidase *lox* (Reynaud *et al.*, 2008), on dual knockdown of *loxl1* and *loxl5a*, two lysyl oxidase-like genes expressed in the developing zebrafish notochord (Gansner *et al.*, 2007), and on genetically or chemically induced copper deficiency (Mendelsohn *et al.*, 2006). Lysyl oxidases are copper-dependent enzymes involved in the cross-linking of collagen subunits that strengthens collagen fibers. Together, these findings provide an interesting link between copper metabolism, BM organization, and developmental defects caused by nutritional deficiencies and the inherited human copper metabolism disorders, Menke's disease, and Wilson's disease (Mendelsohn *et al.*, 2006). In addition, the integrity of the different layers of the zebrafish perinotochordal BM seems to be dependent on different proteins, which confer different functional properties on the structure.

B. Somites and Muscle

In skeletal muscle, the BM surrounds each myofiber and provides the elasticity that enables the sarcolemma to withstand the mechanical stress of repeated contraction, as well as allows proper attachment between adjacent muscle fibers (myomuscular junctions) and between muscle fibers and tendons (myotendinous junctions (MTJ)). In young zebrafish embryos, MTJ-like junctions are formed between somatic fast muscle fibers and the myosepta, BM-like structures of the ECM surrounding the somites. Zebrafish mutations in *lamb1a* (*grumpy*) and *lamc1* (*sleepy*) display compromised anchoring of the ends of somatic muscle fibers to the myosepta, a process called boundary capture, which in turn stops muscle fiber elongation (Snow *et al.*, 2008). Genetic mosaic experiments in laminin-deficient embryos further revealed that boundary capture is a cell-autonomous phenomenon, indicating that individual muscle cells are sufficient to condition properly their respective attachment sites (Snow *et al.*, 2008). Similar, but comparably later, essential roles

for proper MTJ formation, skeletal muscle integrity, and the prevention of muscle dystrophies have been revealed for zebrafish laminin α2, laminin α4, and laminin β2 in *lama2* (*candyfloss*) mutants (Hall *et al.*, 2007), *lama4* morphants (Postel *et al.*, 2008), and *lamb2* (*softy*) mutants (Jacoby *et al.*, 2009), respectively, for lysyl oxidases, which are involved in collagen stabilization (see above) (Reynaud *et al.*, 2008), and for the HSPG perlecan (Zoeller *et al.*, 2008), a major structural constituent of BMs (see Fig. 1A) and key regulator of several growth factor signaling pathways. All of these laminins, as well as collagens and perlecan, are components of the myosepta. Muscle cells bind to these BM components via plasma membrane receptors such as integrin $\alpha7\beta1$ (laminins and collagens) and dystroglycan (laminins). Integrins and dystroglycan, in turn, are associated with muscular actin cytoskeleton binding partners and intracellular signaling pathway components such as integrin-linked kinase (Ilk) and dystrophin. Accordingly, zebrafish morphants in *itga7* (Postel *et al.*, 2008), *dystroglycan* (Parsons *et al.*, 2002a), or *fukutin-related protein* (*fkrp*), an O-linked glycosyltransferase involved in the glycosylation of dystroglycan (Thornhill *et al.*, 2008), as well as mutants in *ilk* (*lost-contact*) (Postel *et al.*, 2008) or *dystrophin* (*sapje*) (Bassett *et al.*, 2003), display compromised MTJ formation, reduced mechanical stability of skeletal muscles, and muscle dystrophies of various strengths. The zebrafish *dystrophin* gene mutated in *sapje* mutants is the orthologue of the X-linked human Duchenne muscular dystrophy (DMD) gene, mutations in which cause Duchenne or Becker muscular dystrophies (Bassett *et al.*, 2003). In addition, human mutations in *FKRP* or genes encoding other known or putative O-linked glycosyltransferases have been shown to cause severe forms of congenital muscular dystrophy associated with structural brain defects and variable eye involvement such as Fukuyama congenital muscular dystrophy, muscle–eye–brain disease, and Walker–Warburg syndrome (Thornhill *et al.*, 2008). Furthermore, laminins have been implicated in muscle dystrophy in mammals, mediating the attachment between muscle cells and tendons (Snow *et al.*, 2008). Together, this makes the zebrafish a powerful model to elucidate further the molecular and cellular basis of human muscle dystrophies, possibly even providing the basis for new therapeutic strategies to inhibit myofiber loss (Jacoby *et al.*, 2009).

C. Blood Vessels

In addition to the notochord defects and muscle dystrophy described above, zebrafish mutants in *lama1* (*bashful*), *lamb1a* (*grumpy*), and *lamc1* (*sleepy*) also display failed migration of sprouts of intersegmental blood vessels along intersegmental, normally laminin-rich myoseptal regions (Parsons *et al.*, 2002b; Pollard *et al.*, 2006). As described above for the notochord phenotype, the intersegmental vessel defects of *lama1* mutants are weaker than those of *lamb1a* and *lamc1* mutants, because laminin 111 (formerly called laminin 1) acts in partial redundancy with laminin 411 (formerly called laminin 8) and laminin 511 (formerly called laminin 10), all of which share the same β and γ chains, but have different α chains

(Pollard *et al.*, 2006). These defects are most likely due to compromised formation of myoseptal BMs, which serve as a substrate for endothelial cells at the tip of the vessel sprouts. Essential functions during zebrafish angiogenesis have also been described for the integrin α2 subunit (San Antonio *et al.*, 2009), and for perlecan (Zoeller *et al.*, 2008, 2009), the major HSPG of BMs. *itga2* as well as *perlecan* morphants display compromised sprouting of intersegmental vessels and other vessel systems (San Antonio *et al.*, 2009; Zoeller *et al.*, 2008). In *perlecan* morphants, these defects are caused by an inappropriate localization of the vascular endothelial growth factor VEGF-A, providing an attractive mechanism by which BM components could direct endothelial migrations underlying angiogenesis (Zoeller *et al.*, 2009). Itga2 might in turn be involved in the same processes by assisting VEGFR signaling in migratory endothelial cells, consistent with similar results obtained for integrin $\alpha 2\beta 1$ in mammalian systems (Senger *et al.*, 1997).

In addition to these roles during blood vessel morphogenesis, other reports have demonstrated roles of BM components during blood vessel function. Laminin 8 and laminin 10 are the two major endothelial BM laminins in mammals. In mouse, *Lama4* mutants display weakened capillary BMs, leading to rupturing of microvascular walls and widespread hemorrhaging during embryonic and neonatal periods, whereas later, these vascular defects disappear, most likely due to the onset of laminin 10 expression (Thyboll *et al.*, 2002). Similar cardiovascular defects, characterized by blood vessel dilation and ruptures due to thinning of the endothelial wall, together with severe hemorrhaging, were observed in zebrafish *lama4* morphants (Knöll *et al.*, 2007), pointing to an essential role of laminin 411 in endothelial BMs of the zebrafish. A similar role was also revealed for integrin-linked kinase, a likely intracellular mediator of laminin-integrin signaling (see above) (Knöll *et al.*, 2007). Interestingly, the same study reports mutations in the human LAMA4 and ILK orthologues to be associated with, and most likely causative of, endothelial-based dilated cardiomyopathies (Knöll *et al.*, 2007). Furthermore, potentially conserved functions during blood vessel development in zebrafish and human could be revealed for Fibrillin 1 and Fibrillin 2, major components of microfibrils that are present in the extracellular matrix of the vessel walls, possibly attached to the endothelial BM, lending elasticity to the tissue. In humans, mutations in *FBN1* cause Marfan syndrome, characterized by dilation or dissection of the aorta and mutations in *FBN2* cause congenital contractural arachnodactyly, a rare disease with autosomal inheritance characterized by joint contractions, arachnodactyly, kyphoscoliosis, and vascular abnormalities. Consistently, zebrafish *fbn2b* (*puff daddy*) mutants (Gansner *et al.*, 2008b) and morphants in *fbn1* or the microfibril-associated glycoprotein *MAGP1* (Chen *et al.*, 2006) display reduced branching and dilation of various blood vessels. The exact molecular mechanisms underlying these defects are unclear; however, they may involve reduced integrin–matrix interaction (Chen *et al.*, 2006) and altered growth factor signaling to endothelial cells (Gansner *et al.*, 2008b). Finally, recent work has shown that the transcription factor Foxc1 is required for proper BM integrity in diverse blood vessels (De Val *et al.*, 2008), including vessels of the developing eye, which could underlie the glaucoma

(optic nerve degeneration) and anterior chamber eye defects caused by *FOXC1* alterations in humans (Skarie and Link, 2009). Concomitant morpholino injection experiments further revealed a genetic interaction between zebrafish *foxc1a* and *lama1*, shedding first light onto possible mechanisms of Foxc1 function during BM biology, and suggesting that Foxc1 might be involved in the transcriptional regulation of *laminin* genes (Skarie and Link, 2009). Together, these results underline the value of the zebrafish model to elucidate human diseases, in this case related to endothelial BMs and associated ECM components.

D. Kidney

The physiological functions of the zebrafish pronephros are blood plasma filtration and osmoregulation. Vascularization of the glomerulus involves the intimate association between podocytes and endothelial cells and the formation of an intervening GBM. In the zebrafish mutant *cloche*, where endothelial cell development is blocked at an early stage, glomerular epithelial cells initially differentiate into podocytes and form a GBM; however, the integrity of this GBM is not maintained, suggesting that the mature glomerular filtration barrier requires the presence of endothelial cells (Majumdar and Drummond, 1999). As in mammals, proper filtration in the zebrafish pronephros requires the formation of so-called slit diaphragms, consisting of specialized cell junctions between podocyte foot processes on one side and fenestrated endothelial cells on the other side of the GBM. These slit diaphragms normally form between 3 and 4 days postfertilization (dpf). Their formation and selective glomerular filtration are compromised on morpholino-mediated inactivation of the podocyte-specific *nphs1l* and *nphs2* transcripts, disease genes implicated in congenital nephropathies and proteinuria in humans (Kramer-Zucker *et al.*, 2005). Elegant *in vivo* imaging studies further revealed an instrumental role of the GBM during nephron morphogenesis that could underlie tubule segment dysfunctions found in several human kidney pathologies including Fanconi syndrome, renal tubular acidosis, and renal tubular dysgenesis (Vasilyev *et al.*, 2009). Similar to intersegmental vasculogenesis described above, the pronephric tubule BM serves as a substrate for the collective migration of differentiated pronephric epithelial cells accounting for the proximal shift in nephron segment boundaries and proximal tubule convolution (Vasilyev *et al.*, 2009). Finally, morpholino-mediated knockdown of the zebrafish orthologues of the human polycystin genes *PKD1 and PKD2*, responsible for autosomal dominant polycystic kidney disease (ADPKD), the most common heritable human disease, resulted in a range of developmental defects, including the formation of pronephric cysts (Mangos *et al.*, 2010). Although the ultrastructural basis of cyst formation was not investigated, the authors showed that the axis curvature phenotype of polycystin zebrafish morphants is linked to increased *col2a1a* expression, and concomitant *col2a1a* knockdown rescued the defects, pointing to a role of polycystins as components of a negative feedback loop to attenuate the expression of collagens and/or ECM/BM maturation (Mangos *et al.*,

2010). Future studies will reveal whether similar alterations in BM integrity may be a primary defect underlying ADPKD tissue pathologies.

E. Central Nervous System

The neuroepithelial BM plays essential roles during various processes of neural development, serving as an anchor for neuroepithelial cells and as a substrate for migrating neurons or axons. In the neural tube, neuroepithelial cells are organized along the apical–basal (medial–lateral) axis of the tube, with cell contacts to the BM on the basal (lateral) sides (Ciruna *et al.*, 2006; Geldmacher-Voss *et al.*, 2003). Time-lapse *in vivo* recordings in zebrafish revealed that during cell division, neuroepithelial cells round up and divide apically along the medial–lateral axis of the neural keel and their daughter cells become incorporated into opposite sides of the neural tube. The more basal daughter cell maintains contact with the BM through a thin cellular process and returns to its original position within the neuroepithelium. In contrast, the more apical daughter cell loses contact with the BM, becomes polarized along the medial–lateral axis, and intercalates across the midline into the contralateral side of the neural keel. Mutant analyses further revealed that proper neuroepithelial cell reorientation and integration after cell division requires components of both the epithelial (apical–basal) (Geldmacher-Voss *et al.*, 2003) and planar (Ciruna *et al.*, 2006) cell polarity systems. In addition, it is very likely, although not directly investigated, that the BM itself is absolutely essential for proper spatial organization of dividing neuroepithelial cells and the neural keel. In addition, *lama1*, *lamb1a*, and *lamc1* are required for later processes of brain morphogenesis, such as the shaping of the midbrain–hindbrain boundary, allowing proper basal constrictions and apical extensions of neuroepithelial cells that drive the inward bending of the neuroepithelium (Gutzman *et al.*, 2008; Lowery *et al.*, 2009). Furthermore, the BM serves as a crucial substrate for migrating neurons and outgrowing axons within the central nervous system, as revealed by studies in *lama1* mutants (Grant and Moens, 2010; Paulus and Halloran, 2006; Sittaramane *et al.*, 2009). Interestingly, in the case of facial branchiomotor neuron (FBMN) migration in the hindbrain, *lama1* displays a genetic interaction with components of the planar cell polarity system (*vangl2*) (Sittaramane *et al.*, 2009) and the epithelial cell polarity system (aPKCλ, aPKCξ, and Pard6gb) (Grant and Moens, 2010), possibly pointing to an instructive role of laminin to allow neural cell migration by regulating cell polarity. In addition, the BM without doubt serves crucial permissive roles as both a substrate and a boundary that constrains FBMNs to the appropriate migratory path. In line with the latter notion, disruption of the PAR–aPKC complex members aPKCλ, aPKCξ, and Pard6gb results in an ectopic ventral migration in which facial FBMNs escape from the hindbrain through holes in the Laminin-containing BM (Grant and Moens, 2010).

Several other reports deal with essential roles of BMs during eye development and retinotectal projections. Several BMs are present in the developing eye, including the lens capsule, the inner limiting membrane, and Bruch's membrane (Lee and Gross,

2007). *lamb1a* and *lamc1* mutants exhibit defects in the lens capsule and the inner limiting membrane, resulting in lens dysplasia and small retinal ectopias that extend from the retina into the interstitial space between the retina and the lens. In contrast, Bruch's membrane is largely unaffected (Lee and Gross, 2007). In addition, *lamb1*, *lamc1*, and, to a lower extent, *lama1* mutants show alterations in ganglion cell layer organization and optic nerve fasciculation, severe shortening of outer photoreceptor segments, irregular synapse formation, and increased cell death (Biehlmaier *et al.*, 2007). Furthermore, retinotectal axon pathfinding is affected, with failed midline crossing of the anterior and postoptic commissures (Karlstrom *et al.*, 1996; Paulus and Halloran, 2006). An essential later role for the final targeting of ingrowing retinal axons to specific tectal layers has been revealed for the a5 chain of type IV collagen, based on elegant work with the *col4a5* mutant *dragnet* that was isolated during a GFP-based forward genetic screen (Xiao and Baier, 2007; Xiao *et al.*, 2005). Col IV is a BM component normally lining the surface of the tectum, in close association with HSPGs. In wild-type zebrafish, axons choose one of four retinorecipient layers on entering the tectum and remain restricted to this layer. In *col4a5* mutants, by contrast, HSPGs are dispersed throughout the tectum, and a large fraction of retinal axons aberrantly trespass between layers (Xiao and Baier, 2007). Similar defects were observed in *extl3* (*boxer*) mutants, which are deficient in HSPG synthesis. Together, this suggests an essential role of the BM and collagen IV to anchor secreted factors at the surface of the tectum, which serve as guidance cues for retinal axons. Mutations in human COL4A5 cause Alport syndrome, which combines BM defects in kidney, ear, and eyes (Barker *et al.*, 1990; Hudson *et al.*, 2003). Lens degeneration is also observed in zebrafish *col4a5* mutants, whereas no pronephric defects have been reported (Xiao and Baier, 2007). The latter might be due to functional redundancy with other *col4a* genes. In contrast, little is known about the cellular basis of the CNS abnormalities described for some individuals with Alport syndrome. Here, the identified role of zebrafish *col4a5* in ordering synapses might be helpful to elucidate these neurological symptoms of human patients (Xiao and Baier, 2007).

F. Skin and Fins

In our own laboratory, we focus predominantly on the BM of the skin, which separates the epidermis from the dermis. The skin of the trunk and the fin are large and easily accessible areas of zebrafish embryos and adults, making them perfect tissues in which to explore BM function (Fig. 1B). During the first 3 weeks of development, the zebrafish epidermis consists of two epithelial cell layers, an outer periderm, and a layer of basal keratinocytes, which are attached to the underlying BM via hemidesmosome-like junctions (Le Guellec *et al.*, 2004; Sonawane *et al.*, 2005). During metamorphosis, the epidermis becomes multilayered (four to six layers on the dorsal side, three to four layers on the ventral side) (Le Guellec *et al.*, 2004). The dermis is initially relatively thin, but becomes progressively thicker. In the trunk, it contains only rather few flat and elongated fibroblast-like

cells, called dermal endothelial cells, which line the BM at the surface of somatic muscle cells, whereas most of the dermal collagen is generated by epithelial cells (Le Guellec *et al.*, 2004). During metamorphosis, when the thickness of the dermis has already increased several-fold, high numbers of fibroblasts invade the dermis and are mainly involved in scale production (Le Guellec *et al.*, 2004; Sire and Akimenko, 2004).

In the developing body fins, development is slightly different, as best investigated in the caudal fin (Dane and Tucker, 1985). At approximately 24 hpf, the six to nine epidermal basal cells at the apical surface of the tail develop a wedge shape and form an apical ectodermal ridge-like structure that detaches from the underlying mesoderm. Later the basal cells at the apical tip of the ridge maintain this wedge shape and are referred to as cleft cells (Dane and Tucker, 1985). The other epidermal cells in the ridge change their shape, becoming rectangular, and form two apposed epidermal walls (Dane and Tucker, 1985). Already at 27 hpf, much earlier than in the trunk, discontinuous BM stretches become morphologically visible via TEM underneath the epidermal sheets, leading to two epidermal sheets almost directly opposed to each other via their BMs that are separated by only a very thin, completely acellular ECM (Dane and Tucker, 1985). In this space, a set of uncharacterized fibers recognized via TEM crosses the basal lamina and directly contacts the cell membrane, possibly accounting for proper attachment and elasticity between the two opposing BMs (Dane and Tucker, 1985). These cross-fiber structures, together with the earlier establishment of the BM, might be special features of the fins to ensure the integrity and rigidity of this tissue, which despite its thinness must remain erect while subject to high mechanical forces. This might also be the reason why already at approximately 48 hpf, much earlier than in the trunk, fin mesenchymal cells, as marked by expression of *hmcn2* (see below), migrate into the space between the two BMs of the fin epithelia (Carney *et al.*, 2010), where they form the actinotrichia and the fin skeleton (Dane and Tucker, 1985).

Not too surprisingly, multiple mutants have been isolated during forward genetic screens that display specific defects in the skin of the fins, whereas the skin in the rest of the larval body appears unaffected. By morphological criteria, these fin mutants can be further subdivided into two phenotypic classes: fin dysmorphogenesis and fin blistering (Carney *et al.*, 2010). The fin dysmorphogenesis in *fransen* and *badfin* mutants is caused by loss-of-function mutations in the genes encoding the lamininα5 and integrinα3 chains, respectively (Carney *et al.*, 2010; Webb *et al.*, 2007). TEM analyses of *lama5* mutants revealed failed epidermal BM formation, pointing to a crucial role of Lama5 and Itga3b in the epidermis–BM junction (Webb *et al.*, 2007), most likely by binding of Laminin 511 from the BM to integrin $\alpha3\beta1$ receptors in basal epidermal cells (Carney *et al.*, 2010). Studies in epithelial cell culture systems have revealed the involvement of integrin $\alpha3$ in intracellular signaling to promote the assembly and maintenance of adherence junctions, thereby enhancing cell–cell adhesion within the epithelium (Chartier *et al.*, 2006). Accordingly, zebrafish *lama5* and *itga3b* mutants display massively compromised epithelial integrity and a disaggregation of the fin epidermis (Carney *et al.*, 2010; Webb *et al.*, 2007).

This is in striking contrast to the fin blistering mutants, in which the fin epidermis and the underlying BM remain largely intact, whereas blistering occurs between the sublamina densa and the underlying dermis (Carney *et al.*, 2010). Positional cloning revealed that three of these blistering mutants, *pinfin*, *blasen*, and *rafels*, are null mutations in the Fraser syndrome genes *fras1*, *frem2a*, and *frem1a*, respectively (Carney *et al.*, 2010; Gautier *et al.*, 2008; Kiyozumi *et al.*, 2006; Petrou *et al.*, 2005, 2008). Fraser syndrome is a recessive, polygenic, congenital human disorder that is now known to be caused by skin fragility during embryonic development, resulting in blister formation on the limbs and developing eyelids. These blisters later heal but result in the characteristic phenotypes observed at birth, namely, syndactyly of the digits and cryptophthalmos (Petrou *et al.*, 2008; Slavotinek and Tifft, 2002). Studies in corresponding mouse mutants revealed that Fras1, Frem1, and Frem2 form a protein complex that is localized in the sublamina densa of some, but not all regions of the developing skin, pointing to a role of the proteins during the establishment of BM–dermis junctions in areas of the embryo that are subject to particular morphogenetic processes.

In addition to *fras1*, *frem1a*, and *frem2a*, other zebrafish mutants with similar fin blistering defects were identified, caused by mutations in Hemicentin1 (Hmcn1; *nagel*), a member of the fibulin family of ECM proteins that has thus far been functionally analyzed only in the nematode *C. elegans*, or fibrillin 2b (Fbn2b; *puff daddy*), a component of microfibrils (see above) (Carney *et al.*, 2010; Gansner *et al.*, 2008b). However, in contrast to Frem2, Hmcn1 is dispensable for Fras1 stability (Carney *et al.*, 2010). This, together with the known role of fibrillins in elastic fibers, and known physical interactions between other fibulin and fibrillin family members, suggests that Hmcn1 and Fibrillin 2 might be involved in the anchorage of the BM to the aforementioned cross-fibers to allow proper attachment between the two opposing epidermal BMs of the fin fold (Carney *et al.*, 2010). Interestingly, fin mesenchymal cells migrating into the space between these two epidermal sheets express *hmcn2*, a paralog of *hmcn1* (Carney *et al.*, 2010). Therefore, we speculate that to break such cross-fibers, invading mesenchymal cells might secrete similar proteins (Hmcn2) that could replace cross-fiber components (Hmcn1). Further experiments will be necessary to test this notion. In addition, it has to be investigated whether mutations in human *HMCN1* or *FBN2* might contribute to thus far unresolved cases of Fraser syndrome.

III. Methodology for Zebrafish Studies of Basement Membrane Composition and Function

In this section, we introduce the main methods used in the study of the zebrafish BM during embryonic development. For the most part, these are well-established techniques in other model systems, which have not been widely described for use in the zebrafish model system. These techniques can also be used to study the BM of

adult zebrafish, a relatively untouched field, to assist in the elucidation of the role of BMs in adult tissue homeostasis and disease.

A. Embedding Samples and Sectioning

The visualization of BM protein distribution following whole-mount staining of zebrafish embryos or larvae can be difficult. For this reason, sectioning of the sample is fundamental. There are several protocols for embedding zebrafish tissue that include the use of various embedding media, the most common being paraffin wax and JB4 (Nüsslein-Volhard and Dahm, 2002). In the case of paraffin wax, the immunostaining or *in situ* hybridization is usually performed after embedding and sectioning the sample. However, if the sectioning can be done following whole-mount immunostaining, then epoxy resin embedding can be considered because it provides very good preservation of the morphology of the tissue.

Epoxy resins were introduced for sectioning and use in electron microscopy in the 1950s specifically because of the fine preservation of cellular and intercellular connections (Glauert *et al.*, 1956). These early protocols were improved some years later (Finck, 1960) and are still popular now for electron microscopy and in cases where the morphological structure of the sample is particularly important. One example is the developmental atlas from the Driever laboratory, available in the Zebrafish Model Organism Database, ZFIN (http://zfin.org). The epoxy resin most commonly used is known as Araldite. It is a medium based on the mixture of Araldite casting resin M, containing dibutyl phthalate as plasticizer, hardener grade 964, and accelerator grade 964 (Glauert *et al.*, 1956). Araldite with these components is also known as Durcupan.

In our laboratory, we routinely use Durcupan embedding and subsequent sectioning of embryos following whole-mount immunostaining. Besides preserving the tissue, Durcupan does not destroy the fluorescent signal, and beautiful pictures of immunofluorescent specimens can be obtained using a fluorescence microscope or laser confocal microscope (Figs. 2C and 4C and D). In the case of *in situ* hybridization labeling followed by immunostaining, the structure of more fragile tissues, such as the fin, is compromised during the *in situ* hybridization procedure. Therefore, sections in Durcupan are not so advantageous in this case (Carney *et al.*, 2010) (Fig. 4A). Below we provide a detailed protocol for Durcupan resin embedding and sectioning.

1. Durcupan Embedding Protocol

Materials

- Acetone
- Ethanol absolute

- Durcupan ACM Fluka cat. no. 44610, one kit, Fluka Chemika
- Flat embedding mold (silicone) (G369) Agar Scientific (made from silicone rubber with 24 numbered cavities 12.5 mm long × 4.5 mm wide × 3 mm deep)
- Slides SuperFrost Plus (Menzel-Gläser)

Protocol

All steps are performed at room temperature unless otherwise stated. Following immunostaining or *in situ* hybridization, the embryos first pass through dehydration steps. If samples have been stored in a viscous solution of glycerol, then they should be washed with 1× phosphate buffered saline (PBS) containing 0.1% Tween 20 overnight before starting the protocol.

- The samples need to be slowly dehydrated. They pass gradually to a more concentrated ethanol solution: 50, 70, and 90% ethanol for 10–15 min at room temperature in each solution. These incubation times are for small samples, such as 1–4 dpf embryos; older or larger specimens will require longer dehydration steps.
- Wash the samples twice in 100% ethanol for 10 min each and then transfer the tissue into acetone. Wash twice in acetone for 15 min each.
- The samples are incubated open overnight in acetone:Durcupan 1:1 under the hood to allow the slow evaporation of the acetone.
- The samples should then be placed in an embedding mold and new Durcupan solution added, orientated, and incubated overnight or longer at 65–70 °C to allow for complete hardening of the Durcupan.
- Sections of 5–8 μm can be done using a rotary microtome. Glass or disposable high-profile blades can be used.

B. Localization of BM Components

To understand the role of BM components, methods to visualize the site of mRNA and/or protein expression can be used. Techniques such as *in situ* hybridization and antibody labeling (Nüsslein-Volhard and Dahm, 2002) are broadly used by the zebrafish community for the visualization of mRNA and protein expression, respectively, and can successfully be used for the localization of BM components (Carney *et al.*, 2010). In the case of *in situ* hybridization, a standard protocol can be used for the localization of BM components. There are well-established protocols available for antibody labeling; however, variations guarantee the success of the localization of a particular protein in a specific tissue and cell region. In this section, some important points of antibody labeling methods used for the localization of BM components are described.

1. Fixation

For the study of BM component localization, special attention should be paid to the choice of fixative agent for your particular tissue. A fixative should preserve cells and tissue structures as close as possible to the biological state. The best fixative choice for a specific sample depends on the purpose of the analysis, for example: mRNA or protein localization, or simple histological analysis. There are several types of commonly used fixative agents and these are discussed below.

Paraformaldehyde (PFA) is normally used in the laboratory as 4% formaldehyde in PBS solution, pH 7. It is a noncoagulant fixative that fixes proteins and does not separate them from the surrounding water (Baker, 1966; Foster *et al.*, 2006). Formaldehyde fixation of the tissue can produce cross-links within and between proteins, which, particularly in the case of extracellular matrix proteins, can mask the antibody epitope preventing antibody binding (Foster *et al.*, 2006; Helander, 1994; Hopwood *et al.*, 1989). If PFA fixation is to be used, it is recommended to fix for the shortest period possible.

Other fixative agents are available that may produce better results for the localization of BM components. In cases where formaldehyde fixation masks the epitope, coagulant fixatives, such as methanol, which do not form cross-links between proteins, can be used (Foster *et al.*, 2006; Hall *et al.*, 2007). Another alternative is Carnoy's fixative, which is an ethanol, chloroform, and acetic acid (6:3:1) treatment that fixes tissue extremely rapidly and without cross-linking (Puchtler *et al.*, 1968; Stickland, 1975). Carnoy's fixative should always be prepared fresh when needed, and the tissue should then be washed directly through absolute ethanol, cleared, and embedded in paraffin.

If immunohistochemical analysis on fixed, paraffin-embedded tissue does not work, it is also possible to cryopreserve zebrafish tissue for cryosections. The cryoembedded tissue only undergoes a brief methanol or ethanol treatment of the cryosection before immunohistochemical analysis; therefore, antibody epitopes should still be available for antibody binding. However, in the majority of cases, tissue preservation of cryoembedded samples is not as satisfactory as Epon, Araldite, or paraffin-embedded tissue. The various embedding techniques used for zebrafish tissue have been described in the section "Embedding Samples and Sectioning".

2. Histological Staining

Histological analysis is the most classical staining method used to assess the structures and properties of cells and the surrounding environment. Histological stains can be used very effectively to study the pathogenesis of human disease and also to help identify the underlying causes of these diseases in animal models. The BM and the zebrafish are no exceptions to this rule. Periodic acid-Schiff (PAS) staining has long been used to visualize glycogen in tissues, staining structures with a high proportion of carbohydrates such as connective tissue, mucous, and the basal lamina (Bancroft and Stevens, 1977; Rambourg and Leblond, 1967; Swift and

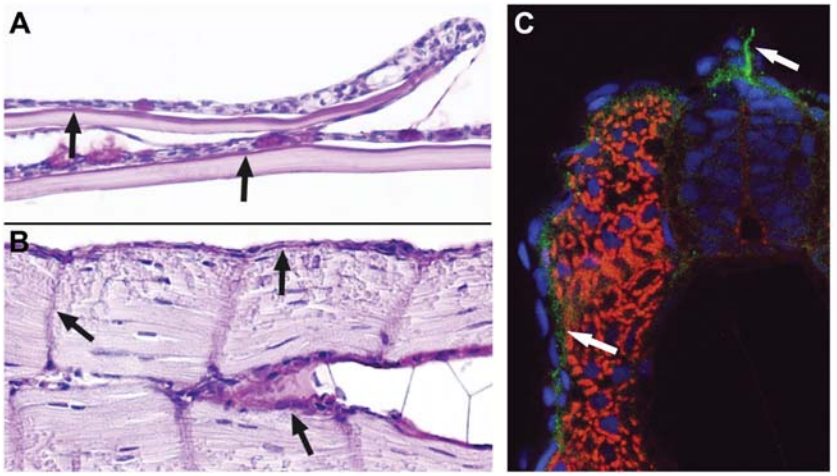

Fig. 2 Analyzing the BM through histological techniques and immunofluorescence. (A and B) PAS staining of larval and adult zebrafish tissues. (A) Adult zebrafish skin consists of a multilayered epidermis covering overlapping scales. The basement membrane appears as a thin magenta line between each scale and the overlying epidermis (arrows). (B) In the 6-day zebrafish larva, longitudinal sections along the trunk reveal PAS staining of the BM surrounding the notochord, underlying the epidermis and surrounding the muscles. (C) Immunofluorescent analysis of BM components in the zebrafish embryo. A transverse section through the trunk of an embryo at 30 hpf embedded in Durcupan. The embryo was stained with laminin to observe the BM (green) (white arrows), phalloidin, staining mainly the somites (red) and DAPI to counterstain the nuclei (blue). (See color plate.)

Saxton, 1967). Using this stain, the BM can be visualized as a magenta line separating two tissues (Fig. 2A and B). We used a PAS staining kit purchased from Merck Chemicals (1.01646 PAS staining kit), and the staining procedure was performed according to the manufacturer's directions. This commonly used histological stain can be used effectively on larval and adult zebrafish, and PFA-fixed and paraffin-embedded tissue (Fig. 2A and B).

3. Antibody Staining

Immunohistochemistry, cytochemistry, or fluorescence analysis is fundamental to the visualization of any protein that is produced by a cell and later exported to the matrix. It indicates where the BM component is assembled and, therefore, active. Extracellular matrix proteins can be transported or diffuse away from the original expressing cell, and therefore localization by antibody binding represents a more accurate method to discover where the protein exerts its function. For example, antibodies against laminin or different collagen chains have been widely used (see Table II and Fig. 2C). Another recent interesting example is the localization of Fras1, a component of the extracellular BM-associated Fraser complex, which, when

Table II
Antibodies used for detection of different zebrafish basement membrane components

Antibody	Tissue	Host species	Company	Reference
Collagen II	Notochord	Mouse	DSHB, II-II6B3	Yamamoto *et al.* (2010)
Collagen IV	Gills Kidney	rabbit	–	MacDonald *et al.* (2006)
	Kidney	Goat	Southern Biotechnology Inc., 1340-01	Majumdar and Drummond (1999)
Collagen XII	Various	Rabbit	–	Bader *et al.* (2009)
		Guinea pig	–	Bader *et al.* (2009)
Collagen XV	Notochord Skeletal muscle	Rabbit	–	Pagnon-Minot *et al.* (2008)
Laminin	Notochord	Rabbit	Sigma, L9393	Hawkins *et al.* (2008), Parsons *et al.* (2002b), Yamamoto *et al.* (2010)
	Skeletal muscle	Rabbit	Sigma, L9393	Hall *et al.* (2007), Jacoby *et al.* (2009), Parsons *et al.* (2002a)
	Ocular vasculature	Rabbit	Sigma, L9393	Skarie and Link (2009)
	Various	Rabbit	Sigma, L9393	Bader *et al.* (2009)
	Midbrain–hindbrain boundary	Rabbit	Sigma, L9393	Gutzman *et al.* (2008)
	Epidermis Fins	Rabbit	Sigma, L9393	Webb *et al.* (2007)
	Eye	Rabbit	Sigma, L9393	Lee and Gross (2007)
	Retinal ganglion cells	Rabbit	Sigma, L9393	Zolessi *et al.* (2006)
	Hindbrain neuroepithelium	Rabbit	Thermo Scientific, RB-082-A	Grant and Moens (2010)
Fras1	Epidermis, intersomitic space	Rabbit	–	Carney *et al.* (2010)
AMACO	Various	Rabbit	–	Gebauer *et al.* (2010)

disrupted, results in blistering of vertebrate embryonic skin and Fraser syndrome in humans (Carney *et al.*, 2010; Petrou *et al.*, 2008; Vrontou *et al.*, 2003). Fras1 protein is produced by epidermal cells, but the Fraser complex, consisting of Fras1 and its associated partners, the Frems, is assembled in the lamina densa of the BM and also in the subepidermal region of the dermal mesenchyme (Kiyozumi *et al.*, 2006; Petrou *et al.*, 2005). Interestingly in zebrafish, Fras1 was detected via antibody labeling close to the expressing cell but also, via unknown mechanisms, far away from this original producing cell (Carney *et al.*, 2010) (Fig. 4A and C).

There are also some adaptations of known protocols for labeling whole-mount embryos to identify proteins located in the BM of the skin. The main adaptation to a standard immunological labeling protocol is the length of time of the permeabilization treatment. Although for the localization of extracellular and BM components, permeabilization of cells should not strictly be required, it can aid antibody binding in whole-mount specimens and is also required for transmembrane proteins. The treatment used and the length of treatment can depend on the tissue, the specific

antibody, and the expected localization of the protein. For example, during embryonic stages, the zebrafish epidermis is composed of just two layers of cells: one layer of basal epidermal cells and an outer layer of peridermal cells. Therefore, permeabilization treatments of the embryos do not need to be harsh to visualize protein localization within this thin epidermis. In our laboratory, we usually employ two different permeabilization techniques: one mild and therefore suitable for analysis of the epidermis, and one harsher, which is better suited for BM labeling of deeper tissues such as around the notochord. For the mild protocol, embryos are washed twice for 10 min in MilliQ water (distilled and deionized water) at room temperature prior to washing in 1× PBS and the start of conventional immunostaining protocols. A harsher permeabilization protocol first requires the dehydration of PFA-fixed embryos into methanol in which they can be stored at −20 °C until required. The embryos are then transferred to ice-cold acetone and incubated at −20 °C for 7 min. The embryos can then be washed in 1× PBS and the immunolabeling protocol continued as normal. Increased permeabilization and improved nonspecific background antibody labeling can also be achieved by addition of 0.5% Triton X-100 or Tween 20, nonionic, nondenaturing detergents, to the 1× PBS used for washing the samples. Immunohistochemical or immunofluorescence analysis of adult zebrafish tissue is usually performed on PFA-fixed paraffin-embedded sections. In our laboratory, we perform antigen retrieval on sections to ensure removal of any PFA cross-links, which might prevent antibody binding. We perform antigen retrieval by incubating tissue sections in prewarmed 10 mM citric acid (pH 6.0) at 70 °C for 1 h. The sections are then allowed to cool to room temperature, washed in 1× PBS, and the immunostaining protocol continued as normal.

C. Transmission Electron Microscopy

To understand BM composition, structure, and, therefore, function fully, it is fundamental to observe its ultrastructure, something best achieved with TEM. The ultrastructure of the BM reveals in more detail how the misassembly of the extracellular matrix could cause a specific phenotype, such as blisters. Although this type of phenotype can be observed macroscopically, it is impossible to distinguish by light microscopy where the rupture between the two tissues occurs, either at the epidermal to BM junction or between the BM and the underlying connective tissue. TEM analysis allows discrimination of the different layers within a BM, as described in more detail for the particularly thick perinotochordal BM (see above). But different layers can be distinguished even in "regular" BMs, which usually consist of the lamina densa and the lamina rara of the basal lamina, and of the underlying lamina reticularis (Vracko, 1974; Yurchenco and Schittny, 1990). The lamina densa consists of a self-assembled rich matrix of collagen IV, separated from epithelial cells by a lighter zone (lamina rara or lucida), which is rich in laminin and integrins. On the opposite side of the lamina densa is a zone named the lamina reticularis, sublamina densa, or subbasal lamina, characterized by large anchoring fibrils and

BM-associated proteins such as collagen VII, fibulins, fibronectin, and fibrillins (Kalluri, 2003; Yurchenco *et al.*, 2004; Yurchenco and Schittny, 1990). In the literature, the basal lamina and the BM are sometimes considered the same structure (Vracko, 1974). However, here we refer to the BM, also called the basement membrane zone (BMZ), as the structure formed by both the basal lamina and the anchoring fibrils or lamina reticularis (Fig. 1A) (Yurchenco and Schittny, 1990).

Electron microscopy has been successfully used to study zebrafish tissues (see Fig. 3 for our own examples). The fixation solution used for samples to be analyzed by electron microscopy usually contains glutaraldehyde (GA) (Bader *et al.*, 2009; Dane and Tucker, 1985; Le Guellec *et al.*, 2004; Parsons *et al.*, 2002b; Slanchev *et al.*, 2009; Sonawane *et al.*, 2005; Webb *et al.*, 2007). This aldehyde, like PFA, forms cross-links but it also fixes tissue very rapidly and so preserves the fine

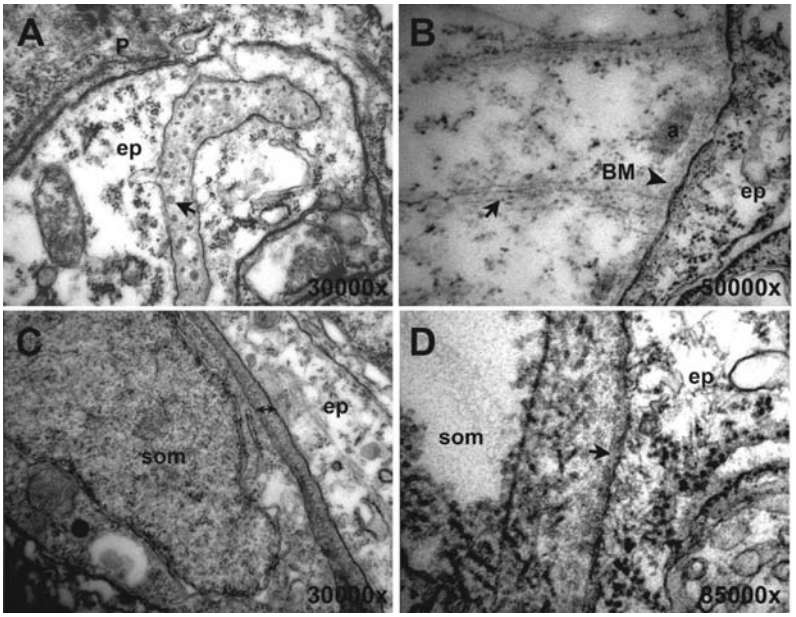

Fig. 3 BM ultrastructure of the fin and trunk at 30 hpf. (A) TEM picture of the most apical part of the fin showing the cleft cell (epidermis) and the extracellular space with assembling actinotrichia (arrow). (B) At higher magnification, the cross-fibers (arrow) are visible. The BM (arrowhead) has a dense appearance and is in close proximity to the actinotrichia. (C and D) Trunk epidermal–somite adhesion. (C) In the trunk at 30 hpf, between the epidermis and the somites, there is a dense matrix (double-headed arrow) but it is difficult to observe the BM. (D) At higher magnification, the BM is visible within this dense matrix (arrow). Collagenous fibers assemble in the extracellular space and assemble separately from the actinotrichia. P, periderm; som, somite; ep, epidermis; BM, basement membrane; a, actinotrichia. Overview at (A and C) 30,000×, (B) 50,000×, and (D) 85,000× magnification.

ultrastructural details of a tissue, making it a valuable addition to any EM fixation protocol. In the case of samples rich in water-soluble ECM components, such as proteoglycans, the samples can be fixed in a solution of aldehydes (2% PFA and 1.5% GA) with different cationic dyes and tannic acid (Dubový and Bednárová, 1999). This treatment allows for more detailed structural analysis, also of soluble molecules. However, some antigens are sensitive to GA fixation, and its concentration should, thus, be decreased or it should be completely removed if immunogold electron microscopy techniques are to be used. The immunogold EM method is used to visualize protein localization on an ultrastructural level via a normal immunolabeling protocol but with specific labeling of the secondary antibody with gold particles (Bader *et al.*, 2009; Nixon *et al.*, 2009). Immunogold EM is the best method available to analyze the ultrastructural localization of a protein. Recently, immunogold EM has also been successfully used on cryosections of zebrafish tissue, reducing the problems caused by cross-linking fixation solutions (Nixon *et al.*, 2009). In addition, immunogold EM has been used for detection of mRNA after *in situ* hybridization, which can be helpful during analyses of the skin, where cells are extremely flat (Le Guellec *et al.*, 2004).

D. Transplantation Experiments

Cell transplantation has been widely used in zebrafish to investigate various aspects of embryo development. It is a well-known technique in the zebrafish community first described in 1990 (Ho and Kane, 1990), and is described in detail in the zebrafish manual edited by Nüsslein-Volhard and Dahm (2002). Labeled cells from transgenic or fluorochrome-injected donor embryos are usually transplanted into unlabeled wild-type host embryos at the late blastula stage (Kimmel *et al.*, 1995). Using this type of experiment, it is possible to test the commitment of cells to their fate in early zebrafish embryos, and to investigate whether a gene product acts in a cell-autonomous fashion, affecting only the cells in which it is generated, or in a non-cell-autonomous fashion, also affecting other cells. It can also nicely be used to label specific cells and to analyze their role in BM formation, function, and BM component production. Recently, using transplantation of wild-type cells into *fras1*$^{-/-}$ mutants, combined with anti-Fras1 immunolabeling, we could show that Fras1 protein, which is produced by epidermal cells but localizes to the BM where it is critical for epidermal adhesion during embryonic stages in human, mouse, and fish, can be deposited in the BM far from the original producing cell (Carney *et al.*, 2010). This might appear surprising, because Fras1 is a transmembrane protein. However, doing the same transplantation experiments with cells from *sturgeon* mutant donors, lacking the proprotein convertase FurinA, we could further show that this localization of Fras1 remote from its site of production requires cell-autonomous furin shedding activity in Fras1-producing cells to enable the release of Fras1 protein from the plasma membrane (Carney *et al.*, 2010) (Fig. 4B–D).

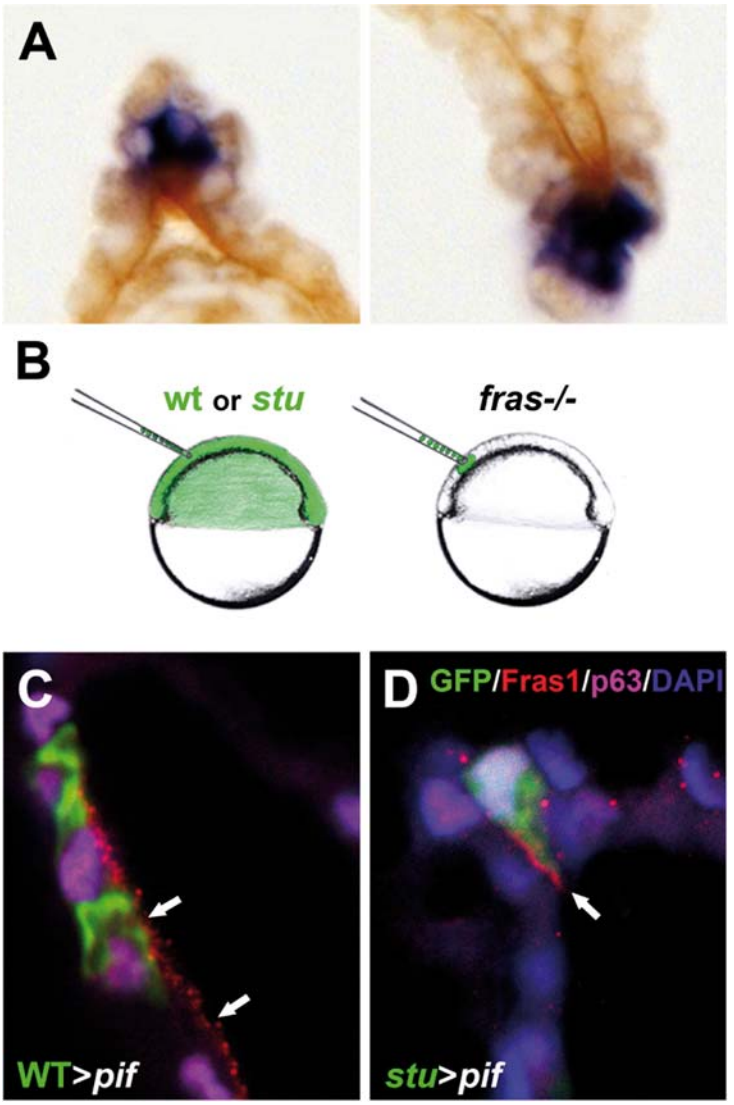

Fig. 4 Transplant techniques can elucidate specific aspects of BM protein deposition. (A) Transverse section through the fin of a wild-type 30 hpf embryo where *fras1* mRNA is localized by *in situ* hybridization (purple) and Fras1 protein distribution is demonstrated using a specific anti-Fras1 antibody (brown). Fras1 protein is localized to a broader region than the mRNA. (B) Diagram depicting an example of a transplant experiment, where GFP-labeled cells (green) are collected from a donor embryo and injected into a host embryo. (C and D) Transverse section through the posterior medial fin of a 30 hpf *fras1*$^{-/-}$ mutant embryo following transplantation of GFP-positive cells from a WT donor (C) or from a *stu* mutant cell (D) at 6 hpf. (C) Fras1 protein from WT cells can be found in the BM several cell diameters proximal of its wild-type source (GFP-positive cells) (white arrows). (D) Interestingly, Fras1 from *stu* mutant cells lacking FurinA remains restricted to the basal surface of the donor cell (white arrow). The embryos were immunolabeled for Fras1 (red), p63 (pink), and GFP (green), and nuclear DNA was counterstained with DAPI (blue). (See color plate.)

E. Forward and Reverse Genetics and Transgenesis

Any disruption to BM structure and stability, for example, the loss of a critical BM component, generally leads to specific and dramatic phenotypes, some of which can be easily analyzed by light microscopy in zebrafish embryos. This, along with the high numbers of zebrafish embryos produced from a single mating, makes the zebrafish an excellent and important tool for forward genetic screens. Although other techniques such as insertional or irradiation-induced mutagenesis are available, forward genetic screening is usually performed by mutagenizing male zebrafish with ethylnitrosourea (ENU), to introduce stable DNA changes throughout the genome, and subsequently screening their F3 progeny for specific phenotypes (Driever *et al.*, 1996; Haffter *et al.*, 1996; Mullins *et al.*, 1994; Solnica-Krezel *et al.*, 1994). BM defects lead to a range of phenotypes, which can be detected using different screening assays. Some phenotypes resulting from mutations in BM component genes can be easily assessed by binocular microscope observation, for example, the fin blistering or fin dysmorphogenesis caused by mutations in the BM components *fras1* and *laminin α5*, respectively (Carney *et al.*, 2010; van Eeden *et al.*, 1996), and the notochord degeneration or muscle detachment and muscular atrophy caused by *laminin α1, α2, β1, β2, or γ1* mutations (Granato *et al.*, 1996; Odenthal *et al.*, 1996; Stemple *et al.*, 1996). In contrast, more subtle phenotypes, for instance, during blood vessel development (Covassin *et al.*, 2009), guidance of neural migrations, retinotectal axonal pathfinding (Grant and Moens, 2010; Karlstrom *et al.*, 1996; Xiao *et al.*, 2005), or BM function in Schwann cell biology and axonal myelination (Pogoda *et al.*, 2006), might be detectable only via more specific and sensitive assays employing transgenic lines or histological techniques to visualize specific cell types. In addition, to search for genes contributing to polygenic or multifactor traits, screens in a mutant background or in combination with environmental insults (such as altered copper concentrations; see above; Gansner *et al.*, 2007) might be feasible.

In addition to forward genetics, zinc finger nuclease (ZNF) technology (Doyon *et al.*, 2008; Meng *et al.*, 2008) and Targeted Induced Local Lesions in Genomes (TILLING) reverse genetic approaches have been established in zebrafish. For TILLING, libraries of ENU-mutagenized F1 genomes are screened for mutations in particular genes via PCR amplification and sequencing (Draper *et al.*, 2004; Wienholds *et al.*, 2003). This technology is offered as a free-of-charge service by various centers in the zebrafish community, in particular the Sanger Center in Cambridge, UK, and similar services are currently being set up for the ZNF technology. In contrast, recombinant gene targeting technology as in the mouse is not available as yet, due to the lack of suitable embryonic stem cell lines (but see Fan *et al.*, 2004).

However, transgenic approaches can be used for spatially and/or temporally controlled overexpression studies, fluorochrome labeling of specific cell types, or specific cell ablations. Depending on the choice of the expression-driving elements, both temporally and spatially controlled overexpression studies are possible. Spatial control is obtained by using *cis*-regulatory elements driving expression in the cell type of interest, very much as is also done in mice. For temporal control, the

inducible and ubiquitously active heat shock protein 70 (hsp70) promoter is widely used, taking advantage of the "cold-blooded" (ectothermic) nature of the zebrafish. Thus, transgenes under the control of this promoter are inactive as long as the fish are kept at normal temperature (28 °C), whereas the transgene can be rapidly activated on transferring of the fish to warmer medium (usually 39 °C). Under some circumstances, it is even possible to activate the hsp70 transgene in individual cells by focusing a sublethal laser microbeam onto them (Halloran *et al.*, 2000). Combined spatial and temporal control of transgene activation can also be obtained via a double transgenic approach, combining, for example, a transgene in which the recombinase Cre is under the control of the hsp70 promoter, with a floxed (Cre-activatable) transgene under the control of a spatially restricted promoter. Alternatively to the *hsp70:Cre* transgene, a ligand-inducible CreER(T2) under the control of any promoter can be used, so that Cre is activated and recombination in the other transgene induced only on administration of the ER-ligand tamoxifen (Hans *et al.*, 2009), comparable to current techniques used in mouse. Such transgene-driven overexpression studies could, for example, be used to verify developmental defects associated with increased production of BM components, as described for the *polycystin* morphants mentioned above (Mangos *et al.*, 2010). They could also be used to address some open questions of human skin pathology. For example, in mouse and human, mutations in the genes encoding laminin $\alpha 3$, $\beta 3$, or $\gamma 3$ chains or the integrin $\alpha 6$ or $\beta 4$ cause epidermolysis bullosa junctionalis, a very severe skin blistering disorder, indicating that laminin 332 and integrin $\alpha 6\beta 4$ are required for anchoring epidermal cells to the underlying BM (Aumailley *et al.*, 2006; Burgeson and Christiano, 1997). However, mutants and patients also show an upregulation of other laminin chains and integrin subunits, and it is currently unclear why these do not compensate for the loss of the missing subunits. According to one hypothesis, such subunits made in excess remain in the endoplasmic reticulum, triggering the unfolded protein response and activating diverse ER stress pathways that could lead to cellular dysfunction and apoptosis. Zebrafish transgenesis could help to elucidate the contribution of such unfolded stress responses to the etiology of epidermolysis. By applying low amounts of tamoxifen, this approach can also be used to generate mosaic animals, in which only a subset of cells expresses the transgene, addressing cell autonomy issues and offering an alternative to transplantation approaches (see above).

A variant of this transgenic approach can also be used for temporally controlled ablation of specific cells, again comparable to a well-established strategy in mouse, where the floxed cDNA encodes a cytotoxin such as the diphtheria toxin A chain. An alternative approach for conditional cell ablation recently established in the zebrafish uses the nitroreductase (NTR) metronidazole (Mtz) system (Curado *et al.*, 2007; Pisharath *et al.*, 2007). Mtz is a predrug, which can be administered to fish at any stage of development and which normally is harmless, unless it is converted to a cytotoxic DNA cross-linking agent in cells expressing the transgene-encoded Mtz enzyme. To increase the effectiveness of cell ablation, a bimodular variant of this system has been invented, in which a spatially restricted promoter drives expression of the yeast Gal4 transcription factor, while within the same cassette, NTR is under

the control of the Gal4-inducible upstream activating sequence (UAS) (Zhao *et al.*, 2009). Such approaches could, for example, be used to study the extent of epithelial versus mesenchymal cell contribution to BM formation and function, a question that in many systems is still a matter of debate.

Transgenic labeling of specific cell types with cytoplasmic, nuclear, or membrane-tagged fluorescent proteins is also widely used for live *in vivo* imaging, another major strength of the zebrafish compared to other vertebrate systems, taking advantage of the transparency of its embryos and larvae, and the availability of mutant lines that are largely transparent even in adulthood (White *et al.*, 2008). In a variant of this approach, histone gene promoters have been used to drive expression of histone–mCherry or histone–GFP fusions, labeling all nuclei or mitotic figures in living fish (McMahon *et al.*, 2009; Pauls *et al.*, 2001). Using a similar approach for a laminin would result in fluorescent labeling of all BMs in live fish, thereby allowing *in vivo* recordings of BM formation and remodeling.

IV. Summary

The BM is a complex, specialized extracellular matrix that is critically important for the development and maintenance of many tissues in the body. BM ultrastructure has been extensively studied in mammalian tissues; however, there remain many questions to be answered particularly related to BM dysfunction in human disease. Several elegant recent studies in the zebrafish, *Danio rerio*, shed important new light on the pathogenesis of human disease in a variety of tissues. These studies, utilizing the techniques for analyzing the BM outlined in this chapter, demonstrate the importance and validity of the zebrafish system for BM research. The conservation of BM components, assembly sites, and function between humans and zebrafish, coupled with the multitude of advantages of this lower vertebrate system, including the ease of transgenesis and transplantation studies as well as the suitability for forward genetic screening, makes the zebrafish a valued addition to the future study of BM function.

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