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# Origin and turnover of ECM proteins from the inner limiting membrane and vitreous body

W Halfter[1], S Dong[1], A Dong[1], AW Eller[2] and R Nischt[3]

**Abstract**

**The inner limiting membrane (ILM) and the vitreous body (VB) are two major extracellular matrix (ECM) structures that are essential for early eye development. The ILM is considered to be the basement membrane of the retinal neuroepithelium, yet *in situ* hybridization and chick/quail transplant experiments in organ-cultured eyes showed that all components critical for ILM assembly, such as laminin or collagen IV, are not synthesized by the retina. Rather, ILM proteins, with the exception of agrin, originate from the lens or (and) ciliary body and are shed into the vitreous. The VB serves as a reservoir providing high concentrations of ILM proteins for the instant assembly of new ILM during rapid embryonic eye growth. The function of the retina in ILM assembly is to provide the cellular receptor proteins for the binding of the ILM proteins from the vitreous. The VB is a gelatinous ECM structure that fills the vitreous cavity of the eye. Its major structural proteins, collagen II and fibrillin, originate primarily from the ciliary body. Reverse transcription–PCR and western blotting show that the rate of synthesis of structural, monomeric ILM and VB proteins, such as laminin, collagen IV and II is very high during embryogenesis and very low in the adult. The downregulation of ILM and VB protein synthesis occurs during early postnatal life, and both ILM and VB are from then on maintained throughout life with minimum turnover. Our data explain why ILM and VB do not regenerate after vitrectomy and ILM peeling.**
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## ILM

The inner limiting membrane (ILM) is a basement membrane (BM) that defines histologically the border between the retina and the vitreous cavity. Functionally, the ILM is more appropriately considered as an adhesive sheet that facilitates the connection of the vitreous body (VB) with the retina. Identical to other BMs,[1–3] the ILM consists of about ten high-molecular weight extracellular matrix (ECM) proteins that include members of the laminin family, nidogen1 and 2, collagen IV and three heparan sulphate proteoglycans, agrin, perlecan and collagen XVIII.[4,5] The ILM is invisible by conventional light microscopy but can be readily visualized by immunocytochemistry using antibodies to any of the BM components (Figure 1a).

In high-resolution transmission electron micrographs (TEM), the ILM appears as a thin sheet of ECM with two lamina lucidas sandwiching an electron-dense lamina densa (Figure 1c). Based on TEM micrographs, the ILM of the embryonic chick eye has a thickness of $<100$ nm (Figure 1c). Studies on native ILMs using atomic force microscopy (AFM), however, showed that the real thickness of the hydrated ILM is with 400 nm much thicker than previously thought.[5] The size difference is due to the shrinkage that tissues undergo when they are dehydrated for plastic embedding and thin sectioning. The shrinkage of the ILM during preparation for TEM is best appreciated by AFM measurements of the ILM before and after dehydration revealing a 93% reduction in thickness from 404 to 52 nm[5]. It is reasonable to expect that BMs undergo more severe shrinkage than other tissue components during dehydration due to the presence of three highly hydrated proteoglycans in BMs (agrin, perlecan and collagen XVIII). AFM measurement also showed that the mechanical strength of the ILM

[1]Department of Neurobiology, University of Pittsburgh, Pittsburgh, PA, USA

[2]Department of Ophthalmology, University of Pittsburgh, Pittsburgh, PA, USA

[3]Department of Dermatology, University of Cologne, Cologne, Germany

Correspondence: Dr W Halfter, Department of Neurobiology, University of Pittsburgh, 1402E Biological Science Tower, Pittsburgh, PA 14262, USA
Tel: +1 412 648 9424; Fax: +1 412 648 1441.
E-mail: whalfter@pitt.edu

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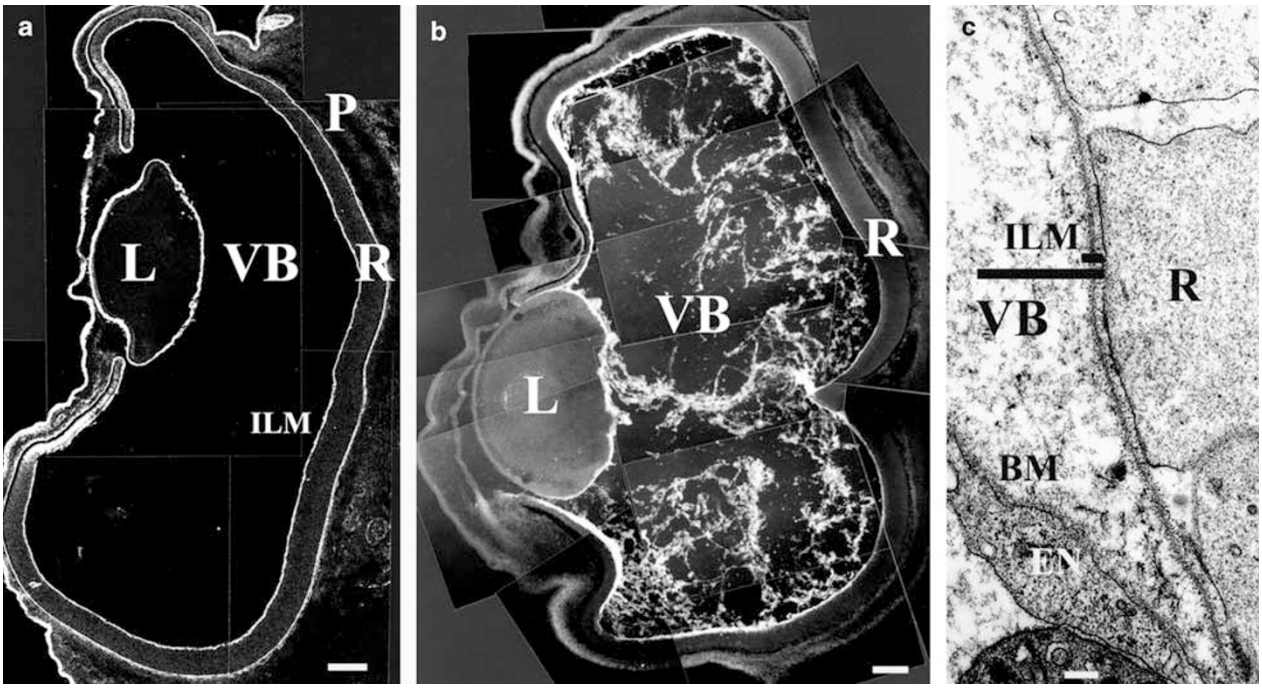


**Figure 1** Basement membranes (BMs) of the embryonic chick eye as shown by immunostaining for laminin-1 (a). The lens capsule (L), inner limiting membrane (ILM) and the BM of the pigment epithelium (P) are strongly labelled in this cross section of an E7 eye. R, retina. The fibrillar structure of the vitreous body (VB) extracellular matrix (ECM) in an E7 eye was visualized by staining for fibrillin (b). A transmission electron (TEM) micrograph of the retino-vitreal border of an E18 mouse embryo is shown in (c). The micrograph shows the ILM in close apposition to the retinal neuroepithelial cells (R) and the vitreous (VB). Part of a hyaloid blood vessel with its endothelial lining (EN) and its endothelial BM (BM) is shown in the lower left corner. The thickness of the ILM is indicated by the short black bar. The thickness of the ILM as measured by atomic force microscopy (AFM) is indicated by the long black bar, illustrating the enormous shrinkage of the ILM during dehydration of the sample for TEM. Scale bar (a, b) 150 $\mu$m; (c) 100 nm.

is in the MPa range,[5] similar to articular cartilage and about a 1000-fold stronger than cell layers.

The presence and stability of the ILM and other BMs of the eye is essential for normal eye development, evident by the ocular phenotypes of mice and fish with mutations of BM proteins.[6–9] In mutant mice with defective ILMs, for example, retinal cells herniated through gaps into the vitreous cavity.[5–7,9] Further, mutant mice with defective BMs have ocular haemorrhages due to ruptures of hyaloids blood vessels demonstrating that the mechanical stability of BMs is essential for tissues to withstand internal pressure or for tissues under stress.[5,6] The fact that in mice with a defective ILM 50% of ganglion cells undergo apoptosis[6] indicates that the BMs also have a signalling function that promotes the differentiation and survival of nearby cells. This signalling function could be mediated by BM protein/receptor binding[10] or by the sequestering of heparin-binding growth factors to the three heparan sulphate proteoglycans that are abundant in the ILM. It is of note that mutations of different BM proteins lead to very similar ocular phenotypes, showing that loss of stability or breaks of the ILM structure are responsible for the phenotypes rather than the loss of a specific protein. A very similar retinal phenotype as seen in the mouse and zebrafish mutants was also detected in chick embryos after enzymatic deletion of the ILM, further emphasizing the importance of the ILM as a structure in retinal development.[11,12] The enzymatic disruption of the ILM in chick embryos also led to an increase in eye size by 30%.[13] The fact that mutations of collagen XVIII, one of the three proteoglycans in the ILM, causes congenital high myopia[14] indicates that the ILM may also have a function in regulating eye size during embryogenesis. In the adult, the ILM is dispensable and its surgical removal is even considered beneficial for patients undergoing macular hole surgery.

## Origin of the ILM proteins in the developing eye

Based on its tight connection to the retina the ILM is considered to be the BM of the retinal neuroepithelium, and in textbooks the retinal glial cells are stated to be responsible for the synthesis of ILM proteins.[15,16] *In situ* hybridization in mouse,[17–19] chick[20] (Figure 2a and b) and human (Figure 3), however, has shown that the ILM

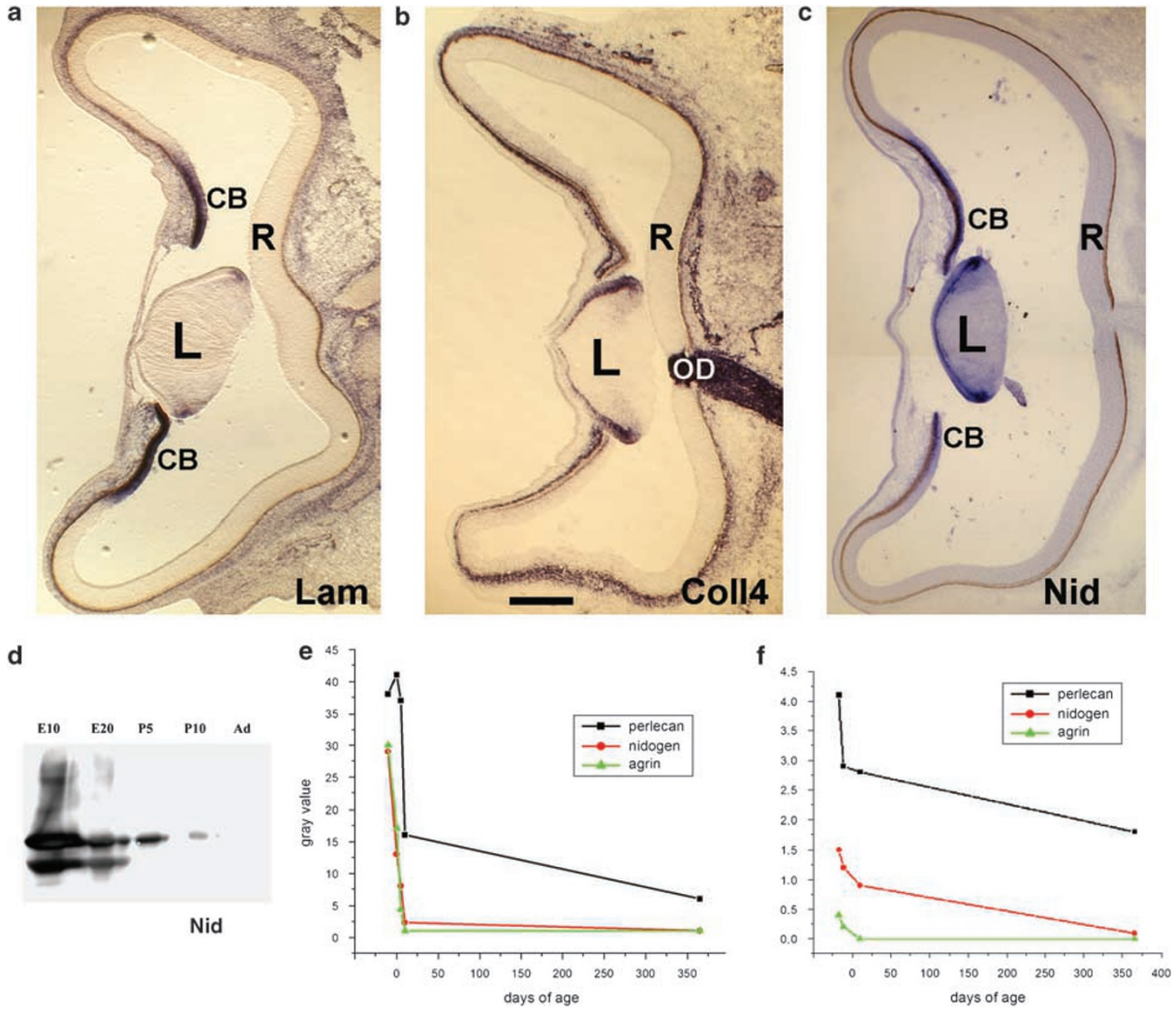


**Figure 2** *In situ* hybridization of E6 chick eye with cRNA probes for the laminin $\gamma$1 chain (a), collagen IV $\alpha$1 (b) and nidogen-1 (c). The collagen IV $\alpha$1 mRNA is localized in the lens (L) and optic disc (OD; b), the laminin $\gamma$1 mRNA is most prominent in the ciliary body (CB; a) with little contribution from the lens and the nidogen is expressed in lens and CB (c). The neural retina does not express collagen IV, laminin or nidogen mRNA. All inner limiting membrane (ILM) proteins were detectable in vitreous body (VB). Nidogen-1, for example (d), was most abundant in E10 VB, and its concentration declined from E20 to P10. No nidogen-1 was detectable in adult VB. Densitometry quantification of the protein bands from the western blots (e) confirmed the high concentration of nidogen-1 in the embryonic VB, its sharp decline at late embryogenesis to a very low concentration in the adult. Similar time courses were seen for agrin and perlecan (e). Quantification of nidogen-1, collagen IV $\alpha$1 and perlecan mRNA in lens by densitometry of reverse transcription (RT)–PCR bands (f) showed a similar time course, with high concentrations at early stages of eye development and very low levels in the adult lens. Scale bar 200 $\mu$m.

proteins do not originate from the retina but from the lens and (or) the ciliary body (Figures 2a–c and 3c). A comprehensive mapping study for the mRNA expression of ILM proteins in chick embryos showed that laminin $\beta$1 and $\gamma$1 (Figure 2a), collagen IV $\alpha$1 (Figure 2b), nidogen-1 (Figure 2c), perlecan and collagen XVIII are synthesized by the lens and (or) the ciliary body.[20] Some of the proteins are also synthesized by the optic disc (collagen IV; Figure 2b), and only agrin originates from the retina. The most dominant site of ILM protein synthesis is the ciliary body followed by the lens. Interestingly, each of the ILM proteins has its unique mRNA expression pattern in the eye (Figure 2, compare a, b and c). It is of note that several non-BM laminin trimers are synthesized by the retina. These laminin family members are not present in the ILM, and they have a function in the survival of photo receptors.[21]

The origin from extra-retinal tissues infers that the ILM proteins are shed from ciliary body and lens into the vitreous to have access to the retinal neuroepithelium. Indeed, western blot analysis in chick and human confirmed that all ILM proteins are present in relative

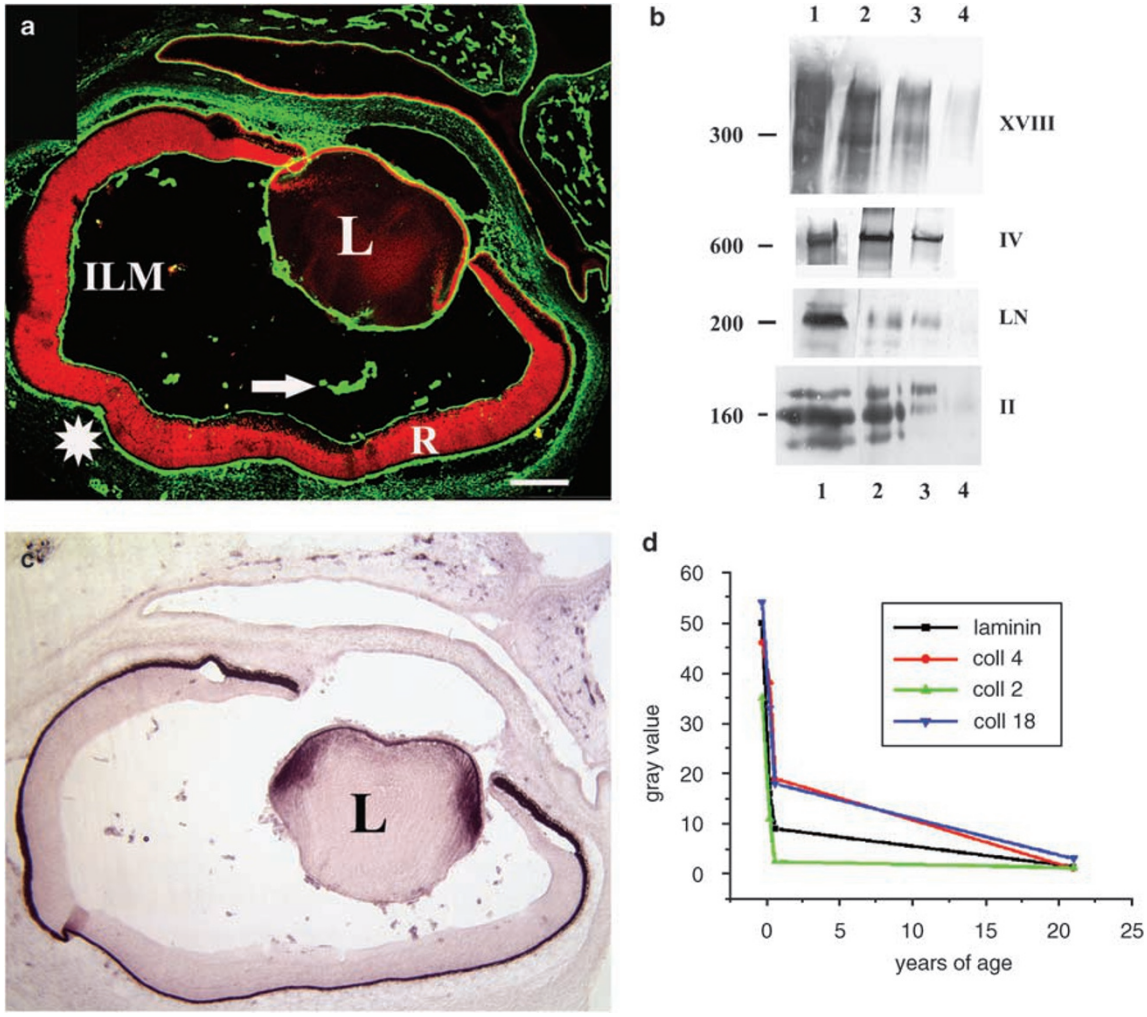


**Figure 3** Distribution of collagen IV protein (a) and collagen IV $\alpha$1 mRNA (c) in the 10-week foetal human eye. Immunostaining for collagen IV protein (green) labels all basement membranes (BMs) of the eye, including the inner limiting membrane (ILM), lens (L) capsule, BMs of the pigment epithelium (white star) and of the blood vessels in the periocular connective tissue and the vitreous (a, white arrow). The section was counter-stained for Pax6. *In situ* hybridization shows that the most dominant source for collagen IV within the eye is the lens followed by the endothelial cells of the hyaloids vessels (white star). The retina does not express collagen IV mRNA (c). Western blots (b) confirm a high concentration of all known ILM proteins in vitreous body (VB), including collagen XVIII, collagen IV and laminin. The abundance of the ILM proteins drops shortly after birth to a very low level as shown in the graph (b, d). A similar time course was also found for the major VB protein, exemplified by a series of western blots for collagen II (b) and the time course of collagen II protein expression (d). Scale bar 200 $\mu$m.

high concentrations in the embryonic VB[22] (Figures 2d and 3b). It appears that the VB serves as a reservoir for ILM proteins during embryogenesis to allow a very rapid and continuous ILM assembly as the retina increases in size by up to factor of 20 in only a few days.

Independent evidence that the retina does not contribute in the synthesis of proteins that are essential for new ILM assembly comes from chick/quail transplantation experiments; by using organ-cultured E3 quail host eyes and chick retinal transplants, it was found that laminin in newly assembled segments of the ILM originated exclusively from the quail host eye and not the chick retinal graft (Figure 4). Species-specific antibodies were used in these experiments to discriminate between the chick and the quail laminin.

While the lens and ciliary body provide the ILM proteins via the VB, the retina expresses the cellular receptors that are essential for ILM assembly. Best evidence for this notion comes from the finding that the deletion or mutation of cellular potential receptors for BM proteins leads to similar phenotypes as deletions or mutations of BMs proteins. Receptor candidates are members of the integrin family and dystroglycan. Deletion of the $\alpha$6 integrin subunit, for example, causes breaks in the ILM and ectopia of retinal cells into the vitreous.[23] Surprisingly, mouse mutants with a

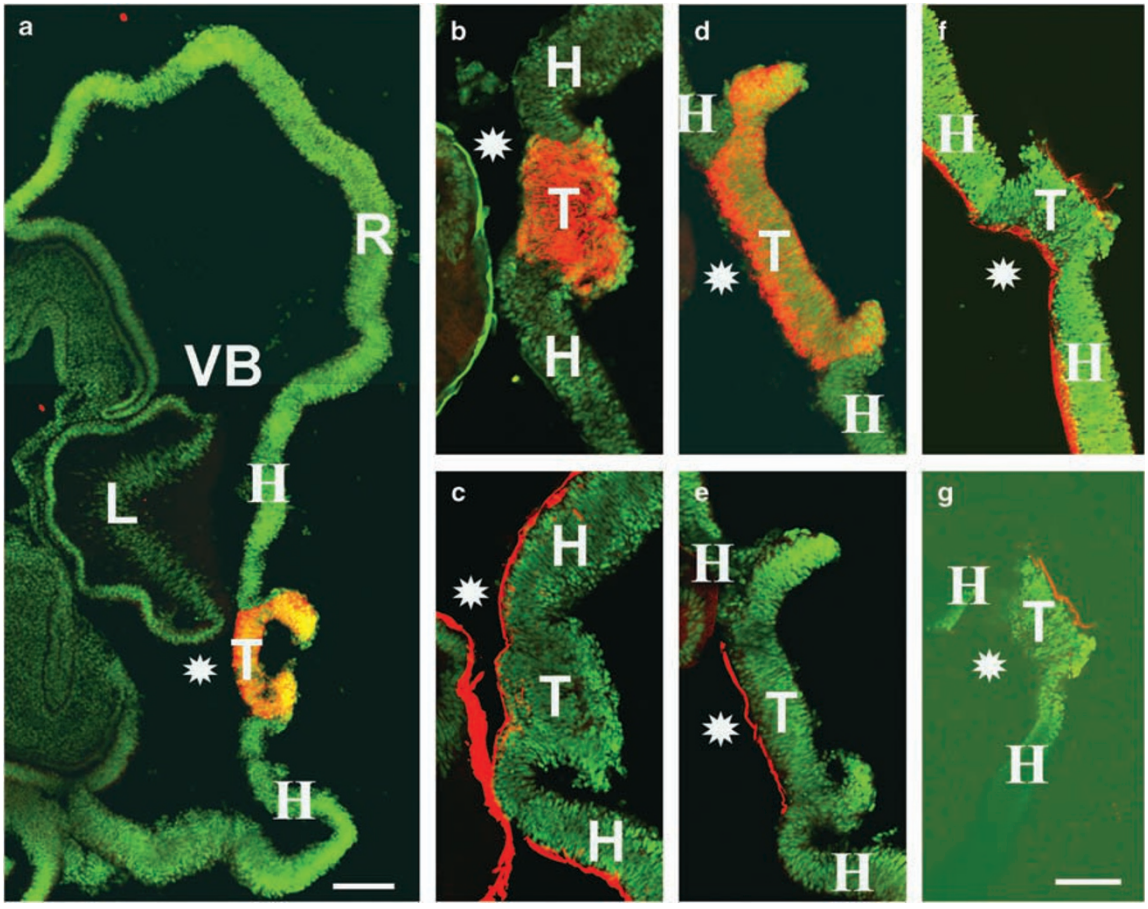


**Figure 4** The retinal neuroepithelium does not contribute in the *de novo* assembly of inner limiting membrane (ILM). Transplantation of chick retinal tissue in organ-cultured quail host eyes shows a tight integration of the graft into the host after 24 h of culture (a). The chick transplants (T) were labelled with an antibody that labels chick but not quail tissue (a: low magnification; b, d: high magnification). Staining of transplant and host retina with a pan-anti-laminin antibody showed a continuous ILM over the graft (c, f). Staining with an antibody specific to chick laminin showed that the ILM-labelling remained localized to the chick transplant and did not extend into the adjacent quail host retina (e). When the transplant was flipped and inserted with its ventricular surface facing the vitreous of the host, a new ILM formed over the normally basement membrane (BM)-free ventricular surface of the graft as shown with the pan-anti-laminin antibody (f). This newly assembled ILM was continuous with the ILM of the host retina (f). Staining an adjacent section with the chick-specific anti-laminin antibody showed that the chick graft retina had not contributed in the assembly of the new ILM (g). The original ILM of the inverted graft is still visible and confirms the inverted orientation of the grafted retina (f, g). The white stars indicate the vitreal surface of the host retina. L, lens; VB, vitreous body; R, retina. (b–g) show adjacent sections. Scale bar (a) 200 $\mu$m; (b–g) 100 $\mu$m.

conditional deletion of $\beta$1 integrin in the central nervous system[24] showed no breaks in the ILM and a normally layered retina, making it unlikely that an integrin with the $\beta$1 chain serves as a receptor for ILM assembly. Thus, the chain composition of the integrin required for ILM assembly needs to be worked out. It is of note that the deletion of $\beta$1 integrin has a very strong phenotype in lens development with a severe disruption of the lens capsule, and mutant mice are aphakic shortly after birth.[24] The fact that the deletion of an enzyme that is essential for the glycosylation of dystroglycan leads to ILM ruptures indicates that dystroglycan contributes in ILM assembly.[25]

## Vitreous and the origin of VB proteins in the eye

The VB is a transparent, gel-like ECM structure that fills the vitreal cavity of the eye[26] (Figure 1b). While tiny in mice, the VB is a very large structure in chick and human eyes. In E10 chick, for example, the VB accounts for over 40% of the weight of the entire eye. The vitreal proteins include collagen II, fibrillin (Figure 1b), collagen IX, tenascin, hyaluronic acid and a series of serum proteins, such as albumin, fibronectin and transferrin.[27–31] Collagen II is essential for the gel character of the vitreous.[31,32] The dominant origin of the vitreous proteins is the ciliary body,[30,31,33–36] with little contribution of the

inner retina for the synthesis of collagen II at early stages of eye development.[30,37,38]

### Turnover of the ILM and VB proteins

Western blotting showed that the ECM proteins from ILM and VB are very abundant in the embryonic vitreous but almost undetectable in adult VB. A subsequent time course analysis of ILM and VB proteins in chick and human[22] showed the highest concentrations of these proteins during embryonic development and a dramatic decline during late embryonic and early postnatal stages. In human, the decline occurs in the fist few months after birth, and plotting the data shows that the synthesis comes almost to a halt by the first or second year of life. Quantification of mRNA for VB proteins in mouse eyes by northern blotting[30] and reverse transcription (RT)–PCR of ILM protein mRNA in chick lens[22] showed a decline of the mRNA with a similar time course as the proteins (Figure 2d) confirming that ILM and VB protein synthesis is dramatically tuned down at late embryogenesis. Not all proteins in VB decrease in concentration over time. Transferrin, a protein typical for serum, is present in large quantities in VB. It is synthesized by the ciliary body,[34,39] and its concentration in VB remains steady from embryogenesis to old age.[22] A similar time course was detected for fibronectin.[22] $\alpha$2 macroglobulin, a serum protease inhibitor, even increases in concentration from embryonic to adult stages. It appears that the downregulation of VB protein expression is prominently targeted to ECM proteins with potentially long half-lives (W Halfter, unpublished).

We propose that the presence of VB and ILM protein monomers in the VB is a sign of active ILM and VB *de novo* assembly, and their absence reflects the cessation in the assembly of both structures. The chick eye, for example, grows between E2 and E20 by a factor of 20. During this time, the rapidly expanding retina is continuously covered by new ILM that has the full complement of all ILM proteins and the vitreous cavity is entirely filled with the fibrillar network assembled from monomeric VB proteins. The VB serves at these stages as a storage site for monomeric ILM and VB proteins, and the abundance of ILM and VB protein monomers guarantees an instant assembly of new ILM and VB during rapid eye expansion. We also propose that the low abundance of monomeric ILM and VB proteins in the adult reflects lack of turnover of both structures and that ILM and VB are maintained at minimum *de novo* synthesis of ILM and VB protein monomers.

We speculate that the limited synthesis of ILM and VB proteins in the adult eye is due to the long half-life of many of the ECM proteins in the body. Several studies have shown that collagens in articular cartilage have half-lives of up to a century,[40–42] and a continued synthesis of very slowly metabolized ECM proteins in the eye would probably lead to cloudiness of the vitreous and thickening of the ILM that might interfere with vision. Indeed, the half-life of collagen IX in human VB has been estimated to 11 years.[43] It is of note that BM thickening during diabetes is considered deleterious and might be one of the reasons for the uncontrolled vasculogenesis in the eyes.[44,45] We also assume that with the shorter lifespan of humans in ancient times the evolutionary pressure was for a one-time assembly of connective tissue structures that lasted for 3–4 decades. Continued production of slowly metabolized ECM proteins may carry a greater risk of storage disorders that outweighs the benefits of continuously regenerating matrix structures.

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