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Beta-1 integrin is important for the structural maintenance and homeostasis of differentiating fiber cells

David A. Scheiblin^a, Junyuan Gao^b, Jeffrey L. Caplan^c, Vladimir N. Simirskii^{a,d}, Kirk J. Czymmek^{a,e}, Richard T. Mathias^b, and Melinda K. Duncan^{a,f}

^aDepartment of Biological Sciences, University of Delaware, Newark, DE 19716

^bDepartment of Physiology and Biophysics, State University of New York at Stony Brook, Stony Brook, NY, New York 11794-8661

^cDelaware Biotechnology Institute, University of Delaware, Newark, DE 19716

Abstract

β 1-integrin is a heterodimeric transmembrane protein that has roles in both cell-extracellular matrix and cell-cell interactions. Conditional deletion of β 1-integrin from all lens cells during embryonic development results in profound lens defects, however, it is less clear whether this reflects functions in the lens epithelium alone or whether this protein plays a role in lens fibers. Thus, a conditional approach was used to delete β 1-integrin solely from the lens fiber cells. This deletion resulted in two distinct phenotypes with some lenses exhibiting cataracts while others were clear, albeit with refractive defects. Analysis of “clear” conditional knockout lenses revealed that they had profound defects in fiber cell morphology associated with the loss of the F-actin network. Physiological measurements found that the lens fiber cells had a two-fold increase in gap junctional coupling, perhaps due to differential localization of connexins 46 and 50, as well as increased water permeability. This would presumably facilitate transport of ions and nutrients through the lens, and may partially explain how lenses with profound structural abnormalities can maintain transparency. In summary, β 1-integrin plays a role in maintaining the cellular morphology and homeostasis of the lens fiber cells.

Keywords

Lens fiber cells; β 1-integrin; lens; differentiation; cytoskeleton; connexins

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^fTo whom correspondence should be addressed Melinda K. Duncan, Ph.D. Department of Biological Sciences University of Delaware 327 Wolf Hall Newark, DE 19716 Tel: 1-302-831-0533 Fax:1-302-831-2281. duncanm@udel.edu.

^dPresent address: Koltzov's Institute of Developmental Biology, Russian Academy of Sciences, 26 Vavilov Street, 119334 Moscow, Russia

^ePresent address: Carl Zeiss Microscopy, LLC, One Zeiss Drive, Thornwood, NY 10594

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1. Introduction

The crystalline lens is a transparent tissue consisting of a basement membrane, the lens capsule, which surrounds two cell types, lens epithelial cells (LECs) on the anterior surface, which proliferate and differentiate into lens fiber cells (LFCs) that make up the majority of the lens (Kuszak et al., 1988, Piatigorsky, 1981). Similar to other tissues (Ingber, 2003a, b), the cells of the lens must interact with both the extracellular matrix and each other to maintain the structural integrity of the tissue, and to regulate cell function (Yamada et al., 2000). While lens cells express a wide array of cell adhesion molecules capable of mediating such interactions, relatively little is known about the function of many of these molecules in the lens and how these functions change as LECs differentiate into lens fibers.

Integrins are heterodimeric transmembrane proteins comprised of an α - and β - subunit, which play major roles in cell-ECM adhesion and cell signaling in all vertebrate tissues (Hynes, 1992). Currently, 18 alpha and 8 beta subunits are found in mammals which assemble into 24 functional heterodimers (Hynes, 2004). Each heterodimer has discrete functions resulting from both differences in their interactions with ECM components and other ligands, as well as the cell signaling pathways that they regulate (Hynes, 1987). Thus, integrins are important players in both the maintenance of tissue integrity and the regulation of cellular identity in animals.

The lens expresses a diverse complement of integrins which have been ascribed various functions in lens development and disease (Walker and Menko, 2009). Mice lacking both the $\alpha 3$ and $\alpha 6$ integrin genes exhibit profound defects in the lens epithelium (De Arcangelis et al., 1999), while conditional deletion of the $\beta 1$ -integrin gene from all lens cells after primary fiber cell elongation leads to disorganization of the lens epithelium with ectopic expression of α -smooth muscle actin at E16.5, and finally inappropriate LEC apoptosis by birth (Simirskii et al., 2007). $\beta 1$ -integrins are also expressed by LFCs where they have been proposed to be crucial for fiber cell-lens capsule interactions (Bassnett et al., 1999). Notably, $\beta 1$ -integrins are also present on the lateral membranes of cortical fiber cells (Duncan et al., 2000b, Menko and Philip, 1995, Simirskii, Wang, 2007) while $\alpha 6$ -integrin, a major partner of $\beta 1$ -integrin in embryonic chicken lens fibers (Menko and Philip, 1995, Walker and Menko, 1999), can co-signal with the IGF receptor and regulate fiber cell differentiation in primary cell culture (Walker et al., 2002). However, while deletion of $\beta 1$ -integrin from all embryonic lens cells did result in severe lens fiber cell defects and lens degeneration (Samuelsson et al., 2007, Simirskii, Wang, 2007), it was not possible to separate direct functions of $\beta 1$ -integrin in the lens fibers from indirect ones caused by loss of $\beta 1$ -integrin from the lens epithelium. Here, $\beta 1$ -integrin was solely deleted from the LFCs, while leaving lens epithelial expression intact, revealing a role for $\beta 1$ -integrin in lens fiber cell structure and physiology.

2. Methods

2.1 Animals

All animal experiments described here conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. *Itgb1^{tm1Efu}/J* mice, which harbor an allele of

the $\beta 1$ integrin gene in which exon 3 is flanked by LoxP sites (Raghavan et al., 2000), were obtained from The Jackson Laboratory (Bar Harbor, Maine) and genotyped as described (Simirskii, Wang, 2007). MLR39-cre mice on a FVB/N background expressing Cre recombinase in LFCs from E12.5 onward (Zhao et al., 2004) were obtained from Michael L. Robinson (Miami University, Oxford, Ohio) and genotyped as described (Zhao, Yang, 2004). *Itgb1^{tm1Efu}/J* mice were bred to MLR39 mice to create mice lacking $\beta 1$ integrin in lens fibers (cKO) (Simirskii et al., 2013). All conditional knockout mice are mutant at the CP49 locus consistent with the genetic background of the founder strains of these mice (Alizadeh et al., 2004, Sandilands et al., 2003, Simirskii et al., 2006). The wild type mice used in these studies were of either the CP49 + C57Bl/6<Har> strain or the CP49 – FVBN strain (designated in the figure legends) (Simirskii, Lee, 2006). All mice were maintained under specific pathogen free conditions at the University of Delaware animal facility under a 14/10-hour light/dark cycle. Embryos were staged by designating the appearance of a vaginal plug in the dam as 0.5 days embryonic (E). All experiments were done using closely age matched experimental and control animals; the ages of the animals used in each experiment are noted in the figure legends.

2.2 Gross Morphology

Eyes were enucleated, lenses dissected, and placed into pre-warmed tissue culture Medium 199, 1X (with Earle's salts & L-glutamine) (Cellgro, Mediatech, Inc. Manassas, VA). Transparency was assessed by photographing lenses using a Zeiss Stemi SV dissecting microscope fitted with a darkfield base. The refractive properties of lenses were assessed by placing them on a 200 mesh electron microscopy grid then photographing them using brightfield with a dissecting microscope, optimizing focus upon the grid, as previously described (Shiels et al, 2007).

2.3 Histology

Eyes (postnatal mice) were removed immediately after sacrifice and fixed in Pen-Fix (Richard Allan Scientific, Kalamazoo, Michigan) for 2 hours. Samples were then transferred into 70% ethanol and paraffin embedded. Six-micron thick sections were prepared, stained by hematoxylin and eosin (H&E) and imaged on a Zeiss Axiophot fitted with a digital Nikon camera.

2.4 Immunofluorescence

Fluorescent immunolocalization on longitudinal eye sections was done as previously described (Reed et al., 2001). Briefly, eyes were removed, embedded directly in Optimum Cutting Temperature media (OCT, Tissue Tek, Torrance, California), 16 μ m thick sections created on a Leica cryostat (Leica) and mounted on slides (ColorFrost Plus, Fisher Scientific Hampton, New Hampshire). Slides were immersion fixed in 1:1 acetone-methanol at -20°C for 15 minutes or 4% paraformaldehyde for 30 minutes depending on the antibody, and blocked in 1% BSA (Bovine Serum Albumin, Sigma Aldrich) for one hour at room temperature. Sections were then incubated with primary antibody diluted with blocking buffer under conditions described in table one. Sections were washed in either 1X PBS or 1X TBS depending on antibody, and incubated with the appropriate Alexa Fluor 568 or

Alexa Fluor 488 labeled secondary antibody (Invitrogen, Grand Island, NY) at a dilution of 1:200 and the DRAQ-5 nucleic acid stain (Biostatus Limited, Shepshed, Leicestershire, UK) at a dilution 1:2000 in either 1X PBS or 1X TBS as appropriate. Sections were washed again in 1X PBS or 1X TBS and then mounted in mounting media (10 ml of PBS with 100 mg of p-phenylenediamine to 90 ml of glycerol; final pH 8.0).

Equatorial sections were stained similarly, except that the sections were 20 μ m thick and a 1:200 dilution of Alexa Fluor 488 labeled wheat germ agglutinin (Invitrogen, Grand Island, NY), which binds N-acetyl-D-glucosamine and sialic acids, was added to the secondary antibody solution to visualize cell membranes (Ohno et al., 1986).

2.5 F-actin visualization

For whole mount analyses, lenses were dissected from the eye, fixed for 2 hours in 4% paraformaldehyde then washed in 1X PBS with 0.1% Triton X-100. Samples were stained in 1% BSA in 1X PBS with 0.25% Triton X-100, a 1:2000 dilution of DRAQ5 (Biostatus Limited, Shepshed, Leicestershire, UK), and a 1:200 dilution of Alexa Fluor 568 labeled phalloidin overnight at 4°C. Lenses were then washed in 1X PBS with 0.1% Triton X-100 and stored in the dark at 4°C until imaged.

Twenty micrometer thick equatorial sections were fixed in 4% paraformaldehyde for 30 minutes and blocked in 1% BSA for one hour at room temperature. Sections were then incubated with Alexa Fluor 568 labeled phalloidin (Invitrogen, Grand Island, NY) at a 1:200 dilution for 1 hour at room temperature then washed in 1X PBS. Sections were counterstained in a solution containing a 1:200 dilution of Alexa Fluor 488 labeled wheat germ agglutinin (Invitrogen, Grand Island, NY) and a 1:2000 dilution of the nucleic acid stain DRAQ5 for 1 hour at room temperature. Sections were washed again in 1X PBS, then mounted.

For β 1 integrin and F-actin co-staining sixteen micrometer thick longitudinal sections were fixed in 4% paraformaldehyde for 30 minutes and blocked in 1% BSA for one hour at room temperature. Sections were incubated with primary β 1 integrin antibody (described in table one). Sections were washed in 1X PBS then incubated with appropriate Alexa Fluor 488 antibody (1:200), Alexa Fluor 568 labeled phalloidin (1:200), and DRAQ 5 (1:2000). Sections were washed again in 1X PBS, then mounted.

2.6 Confocal image collection and analysis

Zeiss LSM780 upright, Zeiss LSM510 inverted and a Zeiss LSM5 DUO inverted confocal microscopes (Carl Zeiss Inc., Jena, Germany) were used in this study. All samples (wild type and conditional knockout) for a particular experiment were imaged using identical conditions on the same instrument. In some cases, brightness and/or contrast of images were adjusted in Adobe Photoshop for optimum viewing on diverse computer screens, however, in all cases, such adjustments were applied equally to both experimental and control images to retain the validity of comparison.

F-actin area was determined using a customized measurement protocol to find objects in Volocity 5.0.3. Volocity uses Otsu's method to perform image thresholding to exclude low

intensity noise. Additional extraneous noise was removed by excluding objects at or below the size of a single pixel ($0.05 \mu\text{m}^2$). The mean area of signal above the threshold (μm^2) was obtained from the remaining objects found in the image and used as a measure of the amount of F-actin present. Results are presented as the mean of the sum of the area (μm^2) $\pm$ the standard error of the mean and were statistically analyzed by a Student's t-test.

2.7 Super Resolution Structured Illumination Microscopy

Slides were prepared using the immunofluorescence protocols described above for equatorial sections. A Zeiss Elyra PS1 (Carl Zeiss Inc., Jena, Germany) equipped with a Plan-Apochromat 63x/1.4 NA Oil objective lens was used in this study. Image stacks were typically taken at $0.101 \mu\text{m}$ z-interval with 25 images per plane (5 phases, 5 angles) and a total z distance of 5 – $15 \mu\text{m}$. Raw images were processed and reconstructed to obtain high-resolution structures and channels were aligned using ZEN software (Carl Zeiss Microimaging) based on structured illumination algorithms. Reconstructed super resolution images of single z-sections are represented in the figures.

2.8 Scanning Electron Microscopy

Eyes were immersion fixed in 0.08M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Hatfield, PA), 1.25% glutaraldehyde (Electron Microscopy Sciences), and 1% paraformaldehyde (Electron Microscopy Sciences) for five hours, the lens excised, then transferred to fresh fixative for an additional 48 hours. After fixation, the lenses were washed in 1X PBS, the lens capsule was removed from the anterior pole to the posterior pole, and around half the circumference of the lens. The superficial fiber cell layers were removed with fine forceps to visualize the cortical fiber cells at reproducible depths by measuring the distance from the lens capsule to the layer being imaged. Peeled lenses were transferred to an ethanol dehydration series (25%, 50%, 75%, 100%) followed by one overnight, and two 2.5-hour, incubations in fresh 100% ethanol. Peeled lenses were critical point dried using hexamethyldisilazane (HMDS) (Electron Microscopy Sciences) as previously described (Duncan et al., 2000a). Samples were mounted on aluminum stubs, coated with gold/palladium for 2.5 minutes and viewed with a Hitachi S-4700 Field Emission Scanning Electron Microscope (FESEM) (Tokyo, Japan).

2.9 Impedance Measurements

Impedance measurements were taken in both WT and cKO lenses as described previously (Mathias et al., 1981). Briefly, mice were killed by intraperitoneal injection of sodium pentobarbital solution (57.1mg/kg of weight), lenses excised and mounted on a silicone base (Sylgard; Dow Corning, Midland, MI). A current microelectrode was placed in the central fiber cells and used to inject a stochastic current of selected bandwidth while a second electrode recorded the induced voltage at varying depths. Impedance was calculated in real time by a fast Fourier analyzer (model 5420A; Hewlett-Packard Co., Palo Alto, CA). Membrane conductances and the effective extracellular resistivity were determined by curve fitting the impedance data to a model for the lens. Results are presented as mean $\pm$ standard deviation and differences between groups were assessed by a Student's t-test. The series resistance at several depths was recorded in each of several lenses by advancing the voltage electrode towards the center of the lens. Data were pooled and fit with a structurally based

model to determine the gap junction coupling conductance as previously described (Shi et al., 2011). The mean value of the data at each location is based on the best fit of the structurally based model, whereas the standard deviations can be estimated from the scatter of the data from the mean at each location.

2.10 Pressure Analysis

Intracellular hydrostatic pressure measurements were taken in both WT and cKO lenses using a microelectrode/manometer based system as described previously (Gao et al., 2011). Results are presented as raw pressure data recorded at several depths in each of several lenses from WT and cKO mice. The data were pooled and mean pressure at each location was estimated by the best fit of a structurally based model, whereas the standard deviations can be seen by the scatter of the data from the curve fit.

3. Results

Prior work on the function of $\beta 1$ -integrins in the lens demonstrated an essential role for these proteins in lens epithelial phenotype and survival (Simirskii, Wang, 2007). However, while $\beta 1$ -integrin null lenses also had fiber cell defects (Samuelsson, Belvindrah, 2007, Simirskii, Wang, 2007), the role of $\beta 1$ -integrins in LFCs was still unclear since the lens epithelium is required for fiber cell homeostasis (Bassnett et al., 1994, Rae et al., 1996, Simirskii, Wang, 2007). Thus, to study the role of $\beta 1$ -integrins in LFCs, B6: 129-*Itgb1*^{tm1Efu}/J mice that carry an allele of the $\beta 1$ -integrin gene where exon 3 is flanked by LoxP sites (Raghavan, Bauer, 2000) were mated with MLR39-cre mice which express Cre recombinase in LFCs from 12.5 dpc onward (Zhao, Yang, 2004). The deletion scheme for the $\beta 1$ -integrin F/F MLR 39 CRE + mice is shown in (Figure 1A).

3.1 $\beta 1$ -integrin F/F MLR39 CRE+ mice have varying phenotypes

$\beta 1$ -integrin F/F MLR39 CRE+ mice ($\beta 1$ -integrin cKO mice) generated from our initial crosses resulted in two distinct phenotypes where lenses either exhibited an obvious cataract (Figure 1B) or were grossly normal by darkfield microscopy (Figure 1C). In contrast, the MLR39 Cre mice (unpublished observation and Robert deLongh personal communication) and the *Itgb1*^{tm1Efu}/J mice carrying the floxed allele (supplemental figure one) which were used to create these $\beta 1$ -integrin cKOs never developed obvious opacities at the ages studied, similar to what was observed for mice on the FVB/N and C57Bl/6 genetic backgrounds (supplemental figure one). Hematoxylin and eosin staining of paraffin sections from cataractous lenses demonstrated profound defects in fiber cell organization including the formation of large cortical vacuoles (Figure 1D). However, transparent lenses from $\beta 1$ -integrin cKO mice show no morphological defects at the light level (Figure 1E). However, the lenses of cKO mice that were grossly transparent by darkfield analysis show slightly more haze and refractive defects when placed on a 200 mesh EM grid (Figure 1G) compared to a wild type lens (Figure 1F).

Immunolocalization of $\beta 1$ -integrin in adult lenses revealed that $\beta 1$ -integrin expression was maintained in the lens epithelium of $\beta 1$ -integrin cKO mice (Figure 1H) at levels similar to that of wildtype lenses (Figure 1G), but $\beta 1$ -integrin protein was absent from cKO lens fibers

(Figure 1H) consistent with the lens fiber cell specific expression of the MLR39 cre transgene (Zhao, Yang, 2004).

3.2 Time course of $\beta 1$ -integrin expression in the embryonic lens and its loss from $\beta 1$ -integrin cKO lens fiber cells

At E12.5, wild type mice express $\beta 1$ -integrin protein in both lens epithelial and fiber cells with levels decreasing, but still detectable, in lens fibers as development proceeds (Figure 2A, C, E, E') consistent with prior reports (Menko and Philip, 1995, Simirskii, Wang, 2007). The cKO lenses still express $\beta 1$ -integrin protein in all cells at E12.5 (Figure 2B), although by E13.5, levels have decreased (Figure 2D) relative to wild type (Figure 2C) in the central lens fiber cells. At E16.5, $\beta 1$ -integrin is still detected in the newly forming cortical fibers of cKO lenses (Figure 2F, F'), although the protein is lost from these cells as they mature. While in some cases the protein expression of other integrins can upregulate when an integrin is lost (Walker and Menko, 2009), this is not seen here as two other β integrins are down regulated in the cKO lenses (Supplemental Figure 2). We do not believe this downregulation of $\beta 5$ and $\beta 8$ integrins, which function as obligate heterodimers with αV -integrin (Mamuya and Duncan, 2011), is functionally relevant to the phenotype since loss of αV -containing integrins from the lens does not affect lens morphology (Mamuya et al., 2014). However, it is possible that the phenotype observed in these cKOs arises from the simultaneous loss of $\beta 1$ -integrins and downregulation of αV integrins in lens fiber cells.

3.4 $\beta 1$ -integrin cKO fiber cells have abnormal morphology

Since $\beta 1$ -integrin is found on the lateral membranes of LFCs, we then assessed the morphology of these cells by scanning electron microscopy. The cortical fibers (150–350 μm from the lens capsule) of wild type mice are organized in discrete parallel layers, which interdigitate with their neighbors via elaborate membrane protrusions (Figure 3A, D, G). In contrast, clear lenses lacking $\beta 1$ -integrin from the lens fibers (cat-; Figures 3B, E, H) have disorganized fiber cell packing, and a loss of organized repeatable membrane protrusions. Cataractous lenses lacking $\beta 1$ -integrin from lens fibers have profoundly abnormal fiber cell structure with no clear demarcation between fiber cells (cat+; Figure 3C, F, I).

In the lens nucleus (analyzed at a distance of 550 μm –750 μm from the lens capsule), wild type lens fibers (Figure 4A) normally undergo structural rearrangements including the attenuation of membrane protrusions (Figure 4D) and the formation of membrane furrows (Figure 4G) on the previously smooth broad faces. In contrast, both transparent and cataractous lenses lacking $\beta 1$ -integrin from the lens fibers do not have clearly distinguishable LFCs (Figure 4B, C). Instead, in both phenotypes, the tissue has become amorphous with the only obvious feature being the development of a highly organized lattice on the fiber cell membrane (Figure 4E, F, H, I) that appears to be an extreme version of the membrane furrows observed in wild type nuclear lens fibers (Figure 4G). Overall, the morphology of cataractous and transparent lenses lacking $\beta 1$ -integrin from the lens fibers is quite similar, although cataractous lenses have more extreme changes. Unfortunately, the mice with the cataractous phenotype did not breed well and this phenotype was lost from the colony after a couple of generations. Thus, the remainder of this investigation describes findings obtained in cKO mice with grossly transparent lenses.

3.5 β 1-integrin cKO inner cortical fiber cells do not maintain an actin cytoskeleton network

Since many lens fiber cell membrane proteins are differentially localized between the long and short faces of the hexagonal fiber cell (Beebe et al., 2001, Maddala et al., 2011b, Nowak et al., 2009, Song et al., 2009, Straub et al., 2003), β 1-integrin expression in adult wild type lens fibers was determined by analyzing lens fiber cell cross sections (equatorial view). β 1-integrin is found on both the long and short sides of the hexagonal lens fiber cell with no apparent preference in localization (Figure 5B, E) although the staining is brighter in the outer lens cortex compared to the inner (Figure 5B). This staining is absent in all fibers from cKO lenses (data not shown) both confirming the antibody specificity and that the β 1-integrin protein is lost from these cells.

The cytoplasmic tails of integrins bind to a host of different proteins, many of which are involved in cytoskeletal organization, including F-actin polymerization (Lo et al., 1997a, Maddala et al., 2011a). Thus, intact whole lenses were stained with phalloidin and imaged on a confocal microscope to visualize F-actin. In wild type lenses, F-actin was most prominent in the lens epithelium and the apical tips of fiber cells, while lower levels are seen along the lateral membranes of fiber cells (Figure 6A, B, C). In cKO lenses, F-actin levels were reduced in cortical fiber cells, although further from the lens surface, some patchy regions of F-actin are maintained (Figure 6D, E, F). The timing of F-actin loss compared to the loss of β 1-integrin protein during cortical fiber cell differentiation was assessed by co-labeling sections for β 1-integrin (Figure 6G, J) and F-actin (Figure 6 H, K). This revealed that the F-actin staining is reduced concomitant with the loss of β 1-integrin protein from newly forming fibers (Figure 6L). The near complete loss of F-actin in ICFs (Figure 7E) is apparent by the time lens fiber cell organelle loss has occurred (Figure 6J, K, L).

This observation was confirmed by staining equatorial lens sections with phalloidin. LFCs are normally packed into organized columns and exhibit a hexagonal cross section characterized by two long and four short sides (Figure 7A–C) and as previously reported, F-actin is localized more strongly to the short sides and vertices of the hexagonal fiber cell cross section (Figure 7B) (Nowak, Fischer, 2009, Straub, Boda, 2003). In contrast, the cortical fibers of cKO mice exhibit disorganization of fiber cell packing (Figure 7D), and the inner cortical fibers (ICFs) do not stain appreciably with phalloidin, consistent with a loss of the F-actin network (Figure 7E). Quantification of the phalloidin intensity profiles revealed peaks of F-actin along the short sides and vertices of wild type fiber cell membranes (Figure 7G), while this is absent from cKO lens fibers (Figure 7H). Further, the overall ICF F-actin network was significantly reduced in cKO lenses lacking β 1-integrin in the fiber cells compared to wild type (Figure 7I). However, this is not due to loss of β -actin expression since similar levels normalized to total protein were detected in wildtype and cKO lenses (data not shown).

In other tissues, integrins regulate F-actin polymerization via interactions between their cytoplasmic tails and numerous adaptor proteins (Cammass et al., 2012, Lo, 2006). Integrin-linked kinase (ILK) is one of these integrin-binding proteins, which is expressed at relatively low levels in the lens epithelium, but upregulates upon fiber cell differentiation localizing along the fiber cell membrane with expression decreasing as these outer cortical fibers (OCFs) mature to ICFs (Figure 8A)(Cammass, Wolfe, 2012, Weaver et al., 2007). In cKO

lenses, ILK levels follow a similar pattern in fiber cells, although as fibers mature, the staining becomes more diffuse suggesting a change in localization away from the membrane (Figure 8B). Using a super resolution approach via structured illumination microscopy (SIM), ILK immunoreactivity was seen to decorate WT OCFs in a punctate pattern associated with both the long and short sides of the fiber cell membranes (Figure 8D, d), while ILK is found in both the cytoplasm and at some membranes in cKO OCFs (Figure 8G, g; H, h). ILK localization in cKO lens fibers with a moderate phenotype is shown here, similar results were seen in fibers exhibiting both milder and more severe morphological defects (Supplemental figure 3)

3.6 β 1-integrin cKO lens fiber cells have abnormal gap junction function and water permeability

The data above show that the lenses from cKO mice have abnormalities in fiber cell packing and loss of membrane protrusions in the cortical fibers while the nuclear fibers exhibit increased furrowed membranes. In order to assess the consequences of these morphological changes on lens physiology, the impedance and intracellular hydrostatic pressure of cKO lenses were compared to wild type. Unexpectedly, the gap junction-coupling conductance of the cKO lenses is almost two fold higher in both the differentiating (G_{DF}) and mature fibers (G_{MF}) compared to WT (Table 2). These conductances were derived from the series resistance data (Figure 9A), which are lower in cKO lenses than WT. In addition, the intracellular hydrostatic pressure is lower in cKO lenses compared to the WT (Figure 9B), as would be expected based on the higher gap junction coupling of cKO lenses. The effective extracellular resistivity (R_e), which depends on the volume fraction of the extracellular clefts, is higher in the WT than cKO lenses. These profound changes in cellular physiology may result in a defect in fluid homeostasis as cKO lenses were slightly, but significantly larger ($0.0076 \pm 0.0004g$) than wild type lenses ($0.0071 \pm 0.0004g$) (p-value = 0.02).

In other systems, deletion of β 1-integrin is associated with increased connexin (Cx) expression (Czyz et al., 2005) however, there was no convincing change in the levels of either Cx46 or Cx50 in cKO lenses (Figure 9C–N). Structured illumination microscopy revealed that Cx46 is found mainly on the broad sides of the fiber cell membranes with lower levels at the vertices in wild type lenses (Figure 9D, d). While a similar pattern of Cx46 distribution was seen in cKO lenses, the gap junctional plaques on the broad sides may be qualitatively larger than normal (Figure 9G, g). Cx46 localization in cKO lens fibers with a moderate phenotype is shown here, similar results were seen in fibers exhibiting both milder and more severe morphological defects (Supplemental figure 4). Cx50 is also found mainly on the broad sides of the fiber cell membranes of wild type lenses although some protein was randomly scattered along the short sides as well (Figure 9J, j). Notably, in cKO lenses, Cx50 was strongly detected on both short and long sides of the fiber cell membranes, and the overall size of each gap junctional plaque may be qualitatively larger as well (Figure 9M, m). Cx50 localization in cKO lens fibers with a moderate phenotype is shown here, similar results were seen in fibers exhibiting more severe morphological defects (Supplemental figure 5)

4. Discussion

This study investigates the phenotypic and molecular consequences of removing the $\beta 1$ -integrin gene from differentiating LFCs while leaving lens epithelial expression intact. Recombination of a floxed $\beta 1$ -integrin allele mediated by MLR39-cre ($\beta 1^{\text{flox/flox}}$ MLR39 Cre+) resulted in the downregulation of $\beta 1$ -integrin protein levels in LFCs by E13.5, consistent with the onset of detectable activity of MLR39-cre at E12.5 (Zhao et al, 2004) although a complete loss of $\beta 1$ -integrin protein in fibers was not observed until after birth. This slow diminution of $\beta 1$ -integrin staining probably reflects the relatively long half-life of $\beta 1$ -integrin in cells (Hotchin and Watt, 1992, Simirskii, Wang, 2007) compounded by the known slow turnover of proteins in lens fiber cells (Lynnerup et al., 2008, Stewart et al., 2013).

$\beta 1^{\text{flox/flox}}$ MLR39 Cre+ mice exhibited two distinct phenotypes; some animals developed cataracts while others retained grossly transparent lenses (Figure 1). Notably, these differences were heritable, cKO mice with transparent lenses did not produce offspring with cataracts, while cataractous cKO mice did produce cataractous offspring, although these animals did not breed well and we were unable to maintain them as a stock long term. Further, the severity of the observed defects seen in grossly transparent cKO lenses was also variable, ranging from near normal appearing fiber cell packing to severe morphological defects. Notably, other lens mutations are known to exhibit such variability. For instance, mice lacking the Cx46/Gja3 gene exhibit either nearly transparent lenses or develop cataracts due to heritable strain-dependent effects (Gong et al., 1999). We do not know the genetic basis of $\beta 1^{\text{flox/flox}}$ MLR39 Cre+ mice variability, however, we found no correlation with the presence of the phenotype and the known mutation in the lens specific intermediate filament protein CP49 which is found in both FVB/N (Simirskii, Lee, 2006) (the genetic background of MLR39cre mice) and 129 (Alizadeh, Clark, 2004, Fudge et al., 2011, Simirskii, Lee, 2006) (one of the founding backgrounds of the $\beta 1$ -integrin flox line). It is also possible that the $\beta 1$ -integrin cKO phenotype is influenced by the concomitant downregulation of $\beta 5$ - and $\beta 8$ -integrin expression in these lenses. However, removal of αV -integrin from the developing lens (which is the obligate α -integrin partner for both $\beta 5$ and $\beta 8$ -integrin) did not result in lens fiber cell structural defects (Mamuya, Wang, 2014) while $\beta 1$ -integrin was found to be the most abundant β -integrin message detected in the E15.5 embryonic mouse lens by RNA-seq (Manthey et al., 2014).

During the differentiation of LECs to fiber cells in the transition zone, lens cells rearrange their orientation to one another to form highly organized radial columns which optimize the packing of these hexagonal cells (Zampighi et al., 2000). As fiber cells begin their elongation, they maintain the initial hexagonal geometry along their cross section and also develop elaborate membrane protrusions from the vertices, which increases membrane interactions with neighboring cells, while presumably also facilitating packing and transparency (Kuszak et al., 2004, Nowak, Fischer, 2009). Late cortical fibers then develop higher order undulations that further increase interactions with adjacent cells (Blankenship et al., 2007). Finally, as fiber cells further mature to form the lens nucleus, they undergo further modifications, including the attenuation of membrane protrusions and formation of “furrowed membranes” on their broad faces which further increase fiber cell packing by

decreasing the extracellular space (Al-Ghoul et al., 2003, Costello et al., 1989, Kuwabara, 1975, Lo and Harding, 1984). Notably, both “clear” and “cataractous” cKO lenses have similar morphological abnormalities in fiber cell organization including defects in fiber cell packing, a loss of membrane protrusions and the expansion of the furrowed membrane network, although this is more severe in cataractous lenses. Overall, these data demonstrate that $\beta 1$ -integrin expression in lens fibers is required for their morphology, although the fiber cell phenotype of even the cataractous cKO lenses is much less severe than that reported for lenses in which $\beta 1$ -integrin was deleted from all lens cells (Samuelsson, Belvindrah, 2007, Simirskii, Wang, 2007), suggesting that those phenotypes were, in part, driven by indirect effects caused by lens epithelial cell dysfunction.

The present data strongly suggest that $\beta 1$ -integrins located on the lateral lens fiber cell membrane play a major role in the long term maintenance of the cortical actin network associated with the lens fiber cell lateral membranes as the F-actin network is absent shortly after $\beta 1$ -integrin protein levels drop after gene deletion (Figure 6J–L). This function is consistent with the known roles of integrins as organizing centers for the actin cytoskeleton (Wiesner et al., 2005). It is also likely that the morphological abnormalities seen in the cKO fibers are directly related to the observed defects in F-actin. Lens fiber cells have an extensive cortical actin cytoskeleton that is reinforced by actin bundles that are found at vertices of the hexagonal fiber cell (Lo et al., 1997b). Further, membrane protrusions, which increase the membrane surface area between fiber cells, and help to minimize light scatter (Bassnett et al., 2011), are stabilized by an F-actin network within each protrusion (Zhou and Lo, 2003). Thus, the disruption of the normal hexagonal geometry of lens fiber cells (Figure 7 and supplemental figures 3–5) and the loss of membrane protrusions (Figure 3) seen in cKO lens fiber cells is likely directly due to the destabilization of the F-actin cytoskeleton.

Numerous intracellular signaling pathways are influenced by integrins leading to cell differentiation, adhesion complex remodeling, and migration via actin cytoskeleton regulation (Hynes, 1987). However downstream proteins, which interact with the cytoplasmic integrin tails, are needed to propagate these signal transduction pathways and to direct actin organization (Hynes, 1987, Wederell and de Jongh, 2006). ILK interacts with $\beta 1$ -integrin and plays numerous roles in its function. In the lens; ILK is required for cell survival, as well as migration of the lens fiber tips to form the sutures (Cammass, Wolfe, 2012, Samuelsson, Belvindrah, 2007). In the normal lens, ILK is found in all lens cells, but at higher levels in the LECs and early differentiating fibers (Figure 8A) (Cammass, Wolfe, 2012, Weaver, Toida, 2007). Using super resolution SIM, we find that ILK is predominately localized near the fiber cell membrane (Figure 8D, d), in a pattern similar to $\beta 1$ -integrin (Figure 5B, E), while in lenses lacking $\beta 1$ -integrin, ILK immunoreactivity is also detected away from the cell membrane, suggesting that the correct localization of ILK, at least in part, requires $\beta 1$ -integrin (Figure 8B, G, g). Notably though, while ILK has been found to play important roles in $\beta 1$ -integrin mediated F-actin organization in other situations (Hynes et al., 2002), ILK null lenses still stained robustly with phalloidin (Cammass, Wolfe, 2012), suggesting that the F-actin defects observed in the $\beta 1$ -integrin cKO mice in this study are mediated by other $\beta 1$ -integrin effectors.

Overall, our observation that $\beta 1^{\text{flox/flox}}$ MLR39 Cre+ lenses could remain grossly transparent for extended periods of time (up to 12 months or older) while at the same time exhibiting severe ultrastructural defects in fiber cell packing and morphology associated with the loss of the F-actin cytoskeleton was unexpected. Thus, we investigated how the loss of $\beta 1$ -integrins from the lens fibers affected physiological parameters important to maintain lens transparency that are often disturbed in cataractous lenses (Gao et al., 2004). This analysis revealed that gap junctional coupling between LFCs, which is essential for the maintenance of the correct ion balance and transparency of the avascular lens (Gao et al 2004), is increased in $\beta 1^{\text{flox/flox}}$ MLR39 Cre+ lenses, consistent with the observation that lenses with increases of similar magnitude maintained transparency (Gao et al, 2004). This suggests that one possible reason for the maintained transparency of cKO lenses is that this high level of coupling allows these lenses to maintain calcium homeostasis despite the morphological defects. This would prevent the activation of the lens specific calpain3 splice variant LP82 which drives cataract associated proteolysis and light scatter in many rodent cataract models (Baruch et al., 2001, Shearer et al., 2000, Shearer et al., 1998). However, it is also possible that this abnormally high gap junctional coupling contributes to the defects observed in the cKO lenses since regulation of the lens circulation is crucial to maintain a normal lens (Mathias et al., 2007) and mutations in the major gap junctional proteins of the lens can disturb normal lens fiber cell differentiation (Xia et al., 2006). The observed decrease in lens hydrostatic pressure in $\beta 1$ MLR39 lenses is likely directly due to the observed increase in gap junctional coupling as the intracellular hydrostatic pressure measured in the lens is known to vary inversely with the number of open gap junction channels (Gao, Sun, 2011).

The connection between the loss of $\beta 1$ -integrin from the lens and the observed increase in gap junctional coupling is less clear. Integrins have been shown to affect gap junctional coupling between cells in a variety of ways. Integrins, particularly $\alpha 3\beta 1$ and $\alpha 5\beta 1$ -integrin, has been reported to mediate channel opening upon either their interaction with ligand (Lampe et al., 1998) or mechanical stress (Batra et al., 2012). Further, loss of Cx43 from neural crest cells results in a loss of $\beta 1$ -integrin immunoreactivity and a reduction in focal contacts (Xu et al., 2006). In contrast, gap junctional communication is reduced in embryonic stem cells (ESC) upon the activation of $\beta 1$ -integrins by laminin binding (Suh et al., 2012) while loss of $\beta 1$ -integrin from ESC derived cardiomyocytes results in the upregulation of connexin expression (Czyz, Guan, 2005). Here we find that loss of $\beta 1$ -integrin from the lens fibers results in an apparent redistribution of Cx50 from the broad faces of the fiber cell to all fiber cell membranes, perhaps explaining the increase in gap junctional coupling observed in cKO lenses. The molecular mechanisms underlying this observation are less certain, but could include altered cell signaling leading to a dysregulation of connexin gating or a function for $\beta 1$ -integrin in connexin localization. Overall, these data imply that one function of the $\beta 1$ -integrin found on the lens fiber cell lateral membrane is to modulate the gap junctional coupling necessary for lens physiology. It is unclear whether this function is related or not to the role of $\beta 1$ -integrin in maintaining the F-actin network of the lens fiber cell.

Alternatively, the increase in gap junctional coupling may be more related to the observed increase in the abundance of furrowed membranes in the lens nucleus. Furrowed membranes

are normally found on the broad faces of nuclear lens fiber cells (Al-Ghoul et al., 2001, Kuszak, Ennesser, 1988) and appear to form as aquaporin0, the most abundant protein in the lens fiber cell membrane (Bassnett et al., 2009), is packed into crystalline arrays during fiber cell differentiation (Grey et al., 2009, Lo and Harding, 1984) leading to an increase in cell adhesion and a reduction in the extracellular space between lens fiber cells (Gonen et al., 2004). Notably, Aquaporin 0 also can bind directly to Cx50 in the lens increasing the coupling of gap junctions by pulling the membranes closer together as furrowed membranes form (Figure 4) (Liu et al., 2011). Thus, the increases in gap junctional coupling, may also, at least in part, be related to the increase in furrowed membranes observed in cKO lenses.

In conclusion, we show that $\beta 1$ -integrin has a large role in maintaining/specifying the structure of the lens fiber cell membrane during differentiation of the LFCs, presumably due to its function in maintaining the F-actin cytoskeleton underlying the lateral membrane, as opposed to its better-known functions mediated via its interactions with ECM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LECs	lens epithelial cells
LFCs	lens fiber cells
ECM	extracellular matrix
IGF	insulin-like growth factor
OCFs	outer cortical fibers
ICFs	inner cortical fibers
F-actin	filamentous actin
cKO	conditional knockout
ILK	integrin linked kinase
G_{DF}	coupling conductance of differentiating fibers
G_{MF}	coupling conductance of mature fibers
G_m	membrane conductance
G_s	surface cell membrane conductance
R_e	extracellular resistivity
ESC	embryonic stem cells

Cx	connexin
SIM	structured illumination microscopy

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Highlights

- Lenses lacking $\beta 1$ -integrin are cataractous or transparent with refractive defects.
- $\beta 1$ -integrin F/F MLR 39 CRE+ lenses have abnormal fiber cell morphology.
- Lenses fibers without $\beta 1$ -integrin lack a F-actin network on the lateral membranes.
- Fibers lacking $\beta 1$ -integrin have abnormal gap junction function and localization.
- $\beta 1$ -integrin plays a role in lens fiber structure and physiology

Figure 1.

$\beta 1$ integrin F/F MLR39 CRE⁺ mice have varying lens phenotypes. A schematic of the $\beta 1$ integrin allele used to produce cKO mice (A) (Raghavan, Bauer, 2000). Dark field view of a three month old cataractous $\beta 1$ -integrin cKO lens (B) and dark field view of a clear three month old $\beta 1$ -integrin cKO lens (C). Hematoxylin (purple) and eosin (pink) stained section obtained from a three month old adult cataractous $\beta 1$ -integrin cKO lens (D) showing disruption of fiber cell packing and vacuoles. Hematoxylin (purple) and eosin (pink) stained section obtained from a three month old adult clear $\beta 1$ -integrin cKO lens (E) showing normal lens morphology. Bright field view of a seven month old wild type (C57Bl/6) lens (F) photographed on top of a hexagonal EM microscopy grid showing an undistorted image indicative of normal refraction. The apparent peripheral haze in this image arises from focusing the microscope on the underlying grid. Bright field view of a seven month old “clear” $\beta 1$ -integrin cKO lens (G) photographed on top of a hexagonal EM microscopy grid showing a distortion of the grid image indicating a refractive defect. In the WT three month old adult lens (H), $\beta 1$ -integrin protein is prominent in the lens epithelium with lesser amounts detected on the outer cortical fibers. This protein continues to decrease as lens fibers denude and even less protein is detected in the lens nucleus. In the three month old adult cKO (I) lens, $\beta 1$ -integrin protein is still detected in the lens epithelium, but little to no protein is seen in either the cortical or nuclear fiber cells. The sections shown in panels H and I were stained simultaneously and imaged on the same day using identical settings to ensure validity of comparisons. Panels H, I- Red- $\beta 1$ -integrin, blue- DNA; Scale bars for D, E = 143 μ m; Scale bars for F, G = 1mm; Scale bars for H, I=71 μ m; e – epithelial cells, f – fiber cells, tz – transition zone.

Figure 2.

Developmental time course of $\beta 1$ -integrin protein localization in embryonic wild type (WT, C57Bl/6) and $\beta 1$ -integrin cKO lenses. At E12.5, $\beta 1$ -integrin protein is detected at similar levels in all cells of the WT (A) lens and a similar distribution is seen in the E12.5 cKO (B) lens. At E13.5, $\beta 1$ -integrin protein is still evenly distributed in all cells of WT (C) lenses but starts to decrease in the central fibers of cKO lenses (D). At E16.5, $\beta 1$ -integrin protein is more abundant in the outer most cortical fibers compared to the lens nucleus of WT (E and E') lenses. In contrast, $\beta 1$ -integrin protein is largely absent from the nuclear fibers of cKO (F and F') lenses and is found in fewer cortical fibers compared to WT (E, E'). The sections shown in panels A–F were stained and imaged simultaneously to ensure validity of comparison. Panels E' and F' show the same images as E and F, however, the brightness is increased to oversaturate the signal in the lens epithelium and outer cortical fibers so that the expression of $\beta 1$ -integrin in the inner cortical and nuclear fibers can be better seen in the wildtype (E') and its absence in these cells seen in the cKO lens (F'). Red – $\beta 1$ -integrin, Blue – DNA; Scale bars for all panels= 71 μ m; e – epithelial cells, f – fiber cells, tz – transition zone, r – retina.

Figure 3.

Scanning electron micrographs of cortical lens fiber cell morphology from eight month old mouse lenses. Wild type (WT, C57Bl/6) lens (A, D, G), cKO lenses lacking an obvious cataract (B, E, H), and cKO lenses exhibiting a frank cataract (C, F, I). In cortical fiber cells, WT (A) lenses have organized fiber shells, while β 1-integrin cKO (B, C) lenses show abnormal fiber cell organization. Parallel fibers of WT (D) lens fiber cells are more organized than β 1-integrin cKOs (E, F). WT (G) lenses have distinct membrane protrusions (arrowheads), which are indistinct in cKO (H) lenses lacking an obvious cataract and are completely disorganized in cKO lenses exhibiting a frank cataract (I). Scale bars for A, B, C = 500 μ m; Scale bars for D, E, F = 10 μ m; Scale bars for G, H, I = 5 μ m; s = socket.

Figure 4.

Scanning electron micrographs of nuclear lens fiber cell morphology from eight month old mouse lenses. Wild type (WT, C57Bl/6) lens (A, D, G), cKO lenses lacking an obvious cataract (B, E, H), and cKO lenses exhibiting a frank cataract (C, F, I). In nuclear fiber cells, WT (A) lenses have organized fiber shells, while $\beta 1$ -integrin cKO (B, C) lenses show disrupted fiber shells. WT (D, G) nuclear fibers have membrane protrusions and a random pattern of membrane furrows on the broad face of the fibers. It is difficult to distinguish individual fibers in cKO (E) lenses lacking an obvious cataract. The cKO fibers that exhibit a frank cataract (F) are similarly disorganized but can be distinguished since they appear to have a larger extracellular space. Both clear and cataractous $\beta 1$ -integrin (E, F, H, I) cKO fibers lack membrane protrusions. WT (G) nuclear fiber cells at high magnification showing membrane furrows along the broad surface. $\beta 1$ -integrin cKO nuclear fibers (H, I) show a highly organized lattice like geometric pattern. Scale bars for A, B, C = 500 μ m; Scale bars for D, E, F = 5 μ m; Scale bars for G, H, I = 2.5 μ m.

Figure 5.

$\beta 1$ integrin is localized to the entire lateral membrane of young lens fiber cells. (A) Wheat germ agglutinin (WGA) staining showing that WT (FVB/N) lens fiber cells are organized into radial cell columns. (B) $\beta 1$ integrin protein is detectable in the outer cortical fiber cells while its levels start to decrease as the cells mature. (C) The merged image of the WGA and $\beta 1$ integrin staining shown in panels A and B (the overlap is yellow). (D) The boxed area shown in panel A imaged at a higher magnification showing organized radial fiber cell columns by WGA staining. (E) The boxed area shown in panel B imaged at a higher magnification showing $\beta 1$ -integrin protein on both the broad and short sides of the fiber cell cross section (arrowheads). (F) The boxed area shown in panel C imaged at a higher magnification showing colocalization of the WGA and $\beta 1$ -integrin staining with the colocalization on both the long and short sides of the cells noted by arrowheads. Red - $\beta 1$ integrin, Green - wheat germ agglutinin, Blue - DNA; Scale bars for A–C = 11 μm ; Scale bars for D – F = 9 μm ; e – epithelium; OCF- outer cortical fibers; ICF- inner cortical fibers

Figure 6.

The loss of $\beta 1$ -integrin causes disruption of the actin cytoskeletal network along lateral membranes. Intact ten week old lenses were stained and visualized at different depths from the anterior surface of the lens. At a depth of 20 μ m, WT (A) (*Itgb1tmt1EFu/J*) lenses have a cortical F- actin ring in the epithelium and an F-actin network seen along the lateral fiber cell membranes. This F-actin network along the lateral membranes continues to be seen at 100 μ m (B) and 150 μ m (C) into this WT lens. In the $\beta 1$ -integrin cKO lens (D) there is still a cortical F-actin network visible in the epithelium, but the F-actin network on the lateral fiber cell membranes is lost at 20 μ m. At 100 μ m (E) and 150 μ m (F), the $\beta 1$ integrin cKO lenses exhibits fiber cell disruption although there are patches of F-actin staining detected. (G–L) A comparison between the timing of $\beta 1$ -integrin deletion and the loss of F actin from lens fiber cells. Three month old WT (FVB/N) lens sections (G) show that $\beta 1$ integrin is most predominately found in the outer cortical fiber cells while F-actin (H) is seen throughout the lens. The merged image (I) shows the overlap of $\beta 1$ integrin (G) and F-actin (H) in the outer cortical fiber cells (shown in yellow). This pattern is not seen in the three month old $\beta 1$ integrin cKO lens sections where $\beta 1$ integrin is seen only in the epithelium and in the very youngest early fiber cells (J). Phalloidin staining of the $\beta 1$ integrin cKO (K) revealed that F-actin first drops off shortly after $\beta 1$ -integrin is lost from outer cortical lens fiber cells (solid arrowhead), then drops off further in the inner cortical fibers (open arrowhead). The merged image (L) show no overlap in the fiber cells of of F-actin (K) or $\beta 1$ integrin (J). Red – F-actin(phalloidin), Green - $\beta 1$ integrin, Blue – DNA; Scale bars for A–L = 71 μ m e- lens epithelium; f- lens fibers

Figure 7.

Three month old equatorial sections of WT (FVB/N; A) inner cortical fiber cells show a distinct organized hexagonal pattern of the radial cell columns. The WT (FVB/N; B) inner cortical fiber cells have a distinct F-actin organization along the short sides and vertices. (C) Merge of panels A and B showing the overlap of F-actin and wheat germ agglutinin (yellow) at the short sides of WT fiber cells. In $\beta 1$ -integrin cKO inner cortical fiber cells (D), the packing organization of the radial cell columns is disrupted. In $\beta 1$ -integrin cKO inner cortical fiber cells (E), the F-actin network is disorganized/lost. (F) Merged images from panels D and E showing that the $\beta 1$ -integrin cKO inner cortical fiber cells exhibit little to no overlap of the F-actin and wheat germ agglutinin staining. F-actin intensity profiles of WT (FVB/N; G) fiber cells versus the $\beta 1$ -integrin cKO (H). (I) Quantitation shows that cKO lenses have a significant decrease in the inner cortical fibers (ICF) F-actin cytoskeleton compared to WT lenses (N=10). Red - F-actin (phalloidin), Green - wheat germ agglutinin, Blue - DNA, Scale bars for A–F = 7.5μm; Error bars = $\pm$ SEM. *** p < 0.0001; e- lens epithelium; f- lens fibers

Figure 8.

The localization of integrin linked kinase (ILK) changes in $\beta 1$ -integrin cKO. Three month old WT (FVB/N) (A) and $\beta 1$ -integrin cKO lenses (B) show no obvious changes in the overall levels of ILK protein, but the staining pattern is more diffuse in the cytoplasm in the $\beta 1$ -integrin cKO by conventional confocal microscopy. In structured illumination images taken of three month old WT (FVB/N) equatorial sections (C, c), the cortical fibers show an organized hexagonal arrangement of radial cell columns and ILK is localized near the membrane at both the short and long sides of cortical fiber cells (D, d, E, e). In $\beta 1$ -integrin cKO cortical fiber cells (F, f), the radial cell columns are less organized with malformed vertices and short sides depicted by the intense wheat germ agglutinin staining. In $\beta 1$ -integrin cKO cortical fiber cells (G, g, H, h), ILK is localized to both the membranes and the cytoplasm (arrows). Green - Integrin linked kinase (A, B), Blue = DNA (A, B); Green - wheat germ agglutinin (C, c, E, e, F, f, H, h), Red - Integrin linked kinase (D, d, E, e, G, g, H, h); Scale bar = 71 μ m (A, B); Scale bars C–h = 10 μ m, e- lens epithelium; tz- transition zone; OCF- outer cortical fibers; ICF- inner cortical fibers

Figure 9.

Two month old $\beta 1$ -integrin cKO lenses have altered physiology. (A) The intracellular series resistance (R_s) between the normalized radial location (r/a) and the lens surface ($r/a = 1$) is lower in the cKO compared to WT, indicating more gap junctional coupling in the cKO lenses. Green dots – wildtype (FVB/N), Red dots – $\beta 1$ -integrin cKO. (B) The hydrostatic pressure is lower in the cKO lenses, consistent with higher gap junctional coupling. Green dots – wildtype (FVB/N), Red dots – $\beta 1$ -integrin cKO. By structured illumination microscopy, WT (FVB/N) equatorial sections of three month old cortical fibers (C,c) show an organized hexagonal arrangement of radial cell columns. WT (FVB/N) equatorial sections of cortical fibers stained for Cx46 (D, d, E, e) show that Cx46 is localized predominately to the broad sides with lesser staining at the vertices of fiber cells. In $\beta 1$ -integrin cKO lenses, the three month old cortical fiber cells show some minor fiber cell packing abnormalities although this was variable between samples. The $\beta 1$ -integrin cKO equatorial lens sections stained for Cx46 (G, g, H, h) show that Cx46 is still seen on both the broad sides and vertices, but may have an increase in the size of gap junctional plaques. WT (FVB/N) equatorial sections (N, n) of three month old cortical fibers show an organized hexagonal arrangement of radial cell columns. In WT (FVB/N) equatorial sections of cortical fibers, Cx50 is localized to the broad sides of fiber cells (J, j, K, k). In $\beta 1$ -integrin cKO equatorial sections of three month old cortical fibers (L, l), the hexagonal arrangement of the radial cell columns is disorganized. In $\beta 1$ -integrin cKO equatorial sections of cortical fibers (M, m, N, n), Cx50 is localized to all sides of the membrane and the gap junctional plaques may be larger in size. Red - Cx 46 D, d, E, e, G, g, H, h; Red - Cx50 J, j, K, k, M, m, N, n; Green - wheat germ agglutinin C, c, E, e, F, f, H, h, I, i, K, k, L, l, N, n; Scale bars for C-n = $10\mu m$.

Table 1

Antibodies, stains, and dyes product and usage information.

Antibodies/Stains/Dyes	Source	Catalogue #	Fixative	Buffer	Dilution
Beta-1 integrin	Millipore, Billerica, MA	MAB 1997	4% PFA or Acetone:Methanol	1XPBS	1:100
Wheat germ agglutinin	Invitrogen, Grand Island, NY	W11261	4% PFA or Acetone:Methanol	1XPBS or 1XTBS	1:200
Phalloidin	Invitrogen, Grand Island, NY	A12380	4% PFA	1XPBS	1:200
Integrin Linked Kinase	Abeam, Cambridge, MA	ab2283	4% PFA	1XTBS	1:200
Connexin 46	Invitrogen, Grand Island, NY	700384	Acetone:Methanol	1XPBS	1:200
Connexin 50	Santa Cruz Biotechnology, Dallas, TX	sc-20876	Acetone:Methanol	1XPBS	1:200
Aquaporin-0	Millipore, Billerica, MA	AB3071	Acetone:Methanol	1XPBS	1:200
DRAQ 5	Biostatus Limited, Shepshed, Leicestershire, UK	DR50200	4% PFA or Acetone:Methanol	1XPBS or 1XTBS	1:2000

Table 2

Gap junction coupling of $\beta 1$ -integrin cKO lenses is two fold increased. The membrane conductances (G_m and G_s) show no significant difference between WT and cKO lenses however the effective extracellular resistivity (R_e) is significantly lower in the cKO lenses ($P = 0.05$).

	WT	cKO
G_{DF} (S/cm ²)	0.48	0.75
G_{MF} (S/cm ²)	0.23	0.62
G_m (μ S/cm ²)	6.9 ± 6.0 (N= 12)	4.7 ± 3.0 (N = 23)
G_s (mS/cm ²)	0.69 ± 0.45 (N= 12)	0.49 ± 0.27 (N = 23)
R_e (K Ω -cm)	34.1 ± 17.5 (N= 12)	21.9 ± 14.5 (N = 23)