Conditional deletion of β1-integrin from the developing lens leads to loss of the lens epithelial phenotype

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Abstract

β1-integrins are cell surface receptors that participate in sensing the cell's external environment. We used the Cre-lox system to delete β1-integrin in all lens cells as the lens vesicle transitions into the lens. Adult mice lacking β1-integrin in the lens are microopthalmic due to apoptosis of the lens epithelium and neonatal disintegration of the lens fibers. The first morphological alterations in β1-integrin null lenses are seen at 16.5 dpc when the epithelium becomes disorganized and begins to upregulate the fiber cell markers β- and γ-crystallin, the transcription factors cMaf and Prox1 and down regulate Pax6 levels demonstrating that β1-integrin is essential to maintain the lens epithelial phenotype. Further, β1-integrin null lens epithelial cells upregulate the expression of α-smooth muscle actin and nuclear Smad4 and downregulate Smad6 suggesting that β1-integrin may brake TGFβ family signaling leading to epithelial-mesenchymal transitions in the lens. In contrast, β1-integrin null lens epithelial cells show increased E-cadherin immunoreactivity which supports the proposed role of β1-integrins in mediating complete EMT in response to TGFβ family members. Thus, β1-integrin is required to maintain the lens epithelial phenotype and block inappropriate activation of some aspects of the lens fiber cell differentiation program.

Introduction

The ocular lens consists of two polarized cell types, the lens epithelial cells and lens fiber cells (Piatigorsky, 1981). The lens has been characterized as an inverted epithelium since lens epithelial and fiber cells make apical-apical contacts while the basal surfaces of both cell types contact the lens capsule (Zampighi et al., 2000), a thickened basement membrane that completely surrounds the cellular lens (Parmigiani and McAvoy, 1989). It is well established that contact between the lens epithelium and the capsule is crucial for lens cell survival (Coulombre and Coulombre, 1963; Wormstone et al., 1997) and the maintenance of its epithelial phenotype (Greenburg and Hay, 1982) although the molecular mechanisms involved in this process are not well understood.

Integrins are heterodimeric extracellular matrix (ECM) receptors consisting of one α- and one β-integrin subunit. These receptors allow cells to both attach to and detect the ECM, leading to cell signaling cascades that control such diverse processes as migration, proliferation, cell survival and cellular phenotype (Hynes, 2002; Longhurst and Jennings, 1998). The β1-integrin subunit is able to form functional receptors with the largest diversity of known α-integrins leading to the ability of cells to detect the composition of diverse ECM environments (Hynes, 2002). In the lens, β1-integrin is expressed by both epithelial and fiber cells (Duncan et al.,

2000; Menko and Philip, 1995; Wederell et al., 2005), and is a component of the basal fiber cell membrane complex (Bassnett et al., 1999). In cell cultures, function blocking antibodies to β1-integrin attenuate the ability of lens cells to bind to collagen and laminin suggesting that this molecule is important for lens cell-capsule communication (Nishi et al., 1997). However, β1-integrin is also found on the lateral and apical sides of lens cells in regions lacking lens capsule components (Duncan et al., 2000; Menko and Philip, 1995). In the equatorial zone, β1-integrin's partner, α6, interacts with the IGF receptor and regulates ERK phosphorylation suggesting that integrin-growth factor receptor cross talk is important for lens morphogenesis (Walker and Menko, 1999; Walker et al., 2002).

The expression of β1-integrin is upregulated during epithelial-mesenchymal transitions occurring as a consequence of lens epithelial cell suspension in collagen I gels (Zuk and Hay, 1994) and in response to TGFβ treatment. In fact, it has been proposed that interference with β1-integrin function may be clinically useful to block epithelial-mesenchymal transition of lens cells leading to posterior capsular opacification, a common side effect of modern cataract surgery (Kim et al., 2002; Mathew et al., 2003; Palmade et al., 1994). However, the function of β1-integrin in the lens in vivo is not well understood.

Complete deletion of the β1-integrin gene from mice leads to lethality at the blastocyst stage demonstrating its importance for development (Fassler and Meyer, 1995; Stephens et al., 1995). Conditional inactivation of the β1-integrin gene in embryonic skin keratinocytes leads to defective epithelial proliferation in the absence of increased apoptosis coincident with obvious defects in basement membrane assembly (Brakebusch et al., 2000; Raghavan et al., 2000). Inactivation of the β1-integrin gene in mammary epithelial cells results in defective alveolar morphogenesis associated with a downregulation of cell proliferation, apparently caused by the upregulated expression of p21^{cip} (Li et al., 2005). Further lobuloalveolar differentiation in response to prolactin is also defective in these glands due to defects in Stat5 activation (Naylor et al., 2005). Here we show that removal of the β1-integrin gene from the lens epithelium results in a distinctly different response consisting of the concurrent downregulation of the lens epithelial marker Pax6, upregulation of lens fiber cell transcription factors as well as α-smooth muscle actin in the embryo followed by apoptosis of these cells by birth. These data demonstrate that β1-integrin is important for the maintenance of the lens epithelial phenotype.

Materials and Methods

Animals

All animal experiments described in this article conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. B6;129-*Itgb1^{tm1Efu}*/J mice which harbor an allele of the β1-integrin gene in which exon 3 is flanked by LoxP sites (Raghavan et al., 2000) were obtained from The Jackson Laboratory (Bar Harbor, Maine). MLR10-cre mice which express Cre recombinase in all lens cells from the lens vesicle stage onward (Zhao et al., 2004) were obtained from Michael Robinson (Miami University, Oxford Ohio). All mice were maintained and bred in specific pathogen free conditions at the University of Delaware animal facility under a 14/10 hour light/dark cycle. Embryos are staged by designating the day that the vaginal plug was observed in the dam as 0.5 days post coitum (dpc).

DNA analysis

DNA was isolated from tail biopsies and lenses using the PureGene Tissue and Mouse Tail kit (Gentra Systems, Minneapolis, MN). Mice were genotyped for the presence of the floxed β1-integrin allele by using primers 5'-CGG CTC AAA GCA GAG TGT CAG TC -3' and 5'-CCA CAA CTT TCC CAG TTA GCT CTC -3' as recommended by the Jackson Laboratory. Mice

were genotyped for the presence of the MRL10-CRE transgene as previously described (Zhao et al., 2004). The extent of exon three deletion of the β1-integrin gene in lens cells was determined by PCR analysis of lens DNA using primers 5'- TGA ATA TGG GCT TGG CAG TTA -3' and 5'-CCA CAA CTT TCC CAG TTA GCT CTC -3'.

Histological analysis and immunohistochemistry

Animals were euthanized, tissue excised and immersion fixed in Pen-Fix (Richard Allan Scientific, Kalamazoo Michigan) for four hours prior to paraffin embedding. Six micrometer sections were cut and stained by hematoxylin and eosin by standard methods to visualized cellular morphology. These sections were also stained with Periodic Acid-Schiff (PAS) by standard methods to visualize the lens capsule basement membrane. The expression pattern of β-crystallin in the lens was determined by incubating deparafinized sections with rabbit anti-bovine β-crystallin or rabbit anti-bovine γ-crystallin (gifts of Samuel Zigler, National Eye Institute, Bethesda MD) followed by detection with an anti- rabbit Dako Envision horseradish peroxidase kit (Dako Laboratories, Carpinteria, CA) using diaminobenzidine as a substrate.

Immunofluorescence

All fluorescent immunolocalization studies were performed as previously described (Reed et al., 2001). Briefly, tissue was excised, embedded fresh in Optimum Cutting Temperature media (OCT, Tissue Tek, Torrance California), 16 μm thick sections prepared on a cryostat and mounted on ColorFrost plus slides (Fisher Scientific, Hampton New Hampshire). Sections were immersion fixed in ice cold 1:1 acetonemethanol for 10 minutes, blocked in 1% BSA and incubated for one hour at room temperature with the appropriate dilution of primary antibody (see below). Following two, 10 minute washes, unlabeled primary antibodies were detected with the appropriate Alexafluor 568 labeled secondary antibody (Molecular Probes, Eugene Oregon) diluted in blocking buffer containing a 1:2000 dilution of the nucleic acid stain Draq-5 (Biostatus Limited, Leicestershire, United Kingdom). Slides were visualized with a Zeiss LSM 510 Confocal Microscope configured with an Argon/Krypton laser (488 nm and 568 nm excitation lines) and Helium Neon laser (633 nm excitation line) (Carl Zeiss Inc, Göttingen, Germany). All comparisons of staining intensity between specimens were done on sections stained simultaneously and the imaging for each antibody was performed using identical laser power and software settings to ensure validity of intensity comparisons.

A rat monoclonal antibody against β1 integrin (clone MB1.2, MAB1997) was obtained from Chemicon (Temecula, California) and used at a concentration of 5 μg/ml. A FITC conjugated mouse monoclonal antibody to α-smooth muscle actin (clone 1A4, F3777) was obtained from Sigma-Aldrich (St. Louis, Missouri) and used at a concentration of 10 μg/ml. Rat monoclonal antibodies to laminin (clone A5, IgG2a), were obtained from Abcam and used at a concentration of 25 μg/ml. Rabbit polyclonal antibodies to collagen IV (ab6586) were obtained from Abcam and used at a concentration of 5 μg/ml. Rabbit polyclonal antibodies against Pax6 were obtained from Covance (Berkeley California) and used at a 1:150 dilution. Rabbit polyclonal antibodies to Smad6 (90-0400) were obtained from Zymed Laboratories and used at a concentration of 10 μg/ml. Rabbit polyclonal antibodies to Smad-4 (9515) were from Cell Signaling Technologies (Danvers, Massachusetts) and used at a 1:50 dilution. Rabbit polyclonal antibodies against E-cadherin (4065) and connexin 43 (3512) were purchased from Cell Signaling Technologies and used at a 1:50 dilution. Rabbit polyclonal antibodies against cMaf (sc7866) were obtained from Santa Cruz Biotechnology and used at a concentration of 2 μg/ml. Rabbit polyclonal antibodies against aquaporin 0 (AB3071) were obtained from Chemicon and used at a concentration of 1.7 μg/ml. Rabbit polyclonal antibodies against Prox1 were as previously described (Duncan et al., 2002). Rabbit polyclonal antibodies to cleaved caspase 3 (9661) were obtained from Cell Signaling Technology and were used at a 1:50 dilution with an overnight incubation at 4 C.

TUNEL labeling

Tissue was excised, fixed for two hours at room temperature in 4% paraformaldehyde and transferred to 70% ethanol prior to paraffin embedding. Six micrometer sections were prepared and nuclear DNA fragmentation detected by TUNEL staining using the ApopTag Peroxidase *in situ* Apoptosis Detection kit (Chemicon) following the manufacturer's directions. Slides were counterstained with 0.5% methyl green to visualize cell nuclei.

Proliferation Assays

Pregnant mice carrying 16.5 dpc embryos were injected intraperitoneally with 100 μg/g body weight of 5-bromo-2'-deoxyuridine (BrdU). Two hours later, the animals were sacrificed, ocular tissue embedded in OCT, and 16 μm thick sections generated on a cryostat. Sections are fixed for 15 minutes in 70% ethanol at -20 C, washed in PBS and treated for two hours with 2N hydrochloric acid at room temperature. The slides were washed twice in PBS, blocked in 1% BSA/PBS for 30 minutes at room temperature and the sections incubated with AlexaFluor 488 labeled monoclonal anti-bromodeoxyuridine (Molecular Probes) diluted to a concentration of 10 μg/ml in PBS containing a 1:1000 dilution of Draq-5 (Biostatus). The fluorescent signal was detected on a confocal microscope as above.

Results

β1 integrin is a major β-integrin subunit expressed in the chicken (Bassnett et al., 1999; Menko and Philip, 1995) and mouse (Duncan et al., 2000) lens. In order to evaluate the function of β1 integrin in the lens *in vivo*, we mated mice harboring a floxed β1 integrin gene (Raghavan et al., 2000) with mice (MLR10-CRE) which express CRE recombinase in all lens cells starting at 10.5 days post coitum (dpc) (Zhao et al., 2004).

β1-integrin is required for lens development

Adult mice homozygous for the floxed β1 integrin allele who also harbor the MLR10-CRE transgene were microphthalmic (Figure 1A), with little to no lens material present in the eye (data not shown). However, at birth, the eyes from these animals were of relatively normal size, although the lens was profoundly abnormal (Figure 1B). The central epithelium is missing, the lens fibers are highly vacuolated and disintegrating and the remaining equatorial epithelial cells are abnormally elongated (Figure 1B, 8A). The first abnormalities in lens structure are observed at 16.5 dpc and range from mild lens epithelial elongation (Figures 1D, 6B, 6D, 8D) to further elongation of lens epithelial cells associated with the beginning of epithelial cell loss and severe fiber cell abnormalities (Figure 1E,F). Prior to 16.5 dpc, lenses from β1flox/β1flox/ CRE mice were morphologically normal (Figure 1H; data not shown) as compared to wildtype (Figure 1G).

In order to establish when β1-integrin protein is lost from the lens, confocal immunofluorescent analysis of β1-integrin expression was performed. At 10.5 dpc, β1-integrin immunoreactivity was detected in lens vesicles from both wildtype (Figure 2A) and β1flox/β1flox/CRE mice (Figure 2B), however, β1-integrin staining was not detected in lens cells from 11.5 dpc β1flox/ β1flox/CRE mice (E,F) while β1-integrin protein was detected at both the basal and lateral cell membranes in wildtype lenses at this age (C,D). As expected, β1-integrin protein is detectable throughout the wildtype 12.5 dpc lens (G) while it is absent in both epithelial and fiber cells of the β1flox/β1flox/CRE lens (H) at this age. The efficiency of CRE mediated deletion of exon 3 of the β1-integrin gene in the lens was also assessed by PCR analysis (Figure 3A) of genomic DNA isolated from newborn lenses and found to be essentially complete (Figure 3B).

Loss of β1-integrin from the lens does not lead to loss of the lens capsule

The lens capsule is a thickened basement membrane synthesized by the lens cells that it encloses (Rafferty and Goossens, 1978). Since β1-integrins have been implicated in basement membrane synthesis and assembly (Li et al., 2002; Lohikangas et al., 2001), we investigated the integrity of this structure in the β1-integrin null lens. Immunostaining for collagen IV (Figure 4A,B) and laminin (Figure 4C,D), as well as periodic acid-Schiff staining (Figure 4E, F) demonstrated that the lens capsule is intact around the β1-integrin null lens (Figure 4B, D, F) and has a composition grossly similar to wildtype (Figure 4A, C, E). Notably, the anterior lens capsule is even detectable in regions that have already lost the lens epithelium (Figure 4F) suggesting that β1-integrins are not necessary for maintenance of the lens capsule.

β1-integrin null lens epithelial cells are lost from the lens by apoptosis

Since the epithelium is lost from β1flox/β1flox/CRE lenses, we next sought to determine whether this is a function of cell proliferation or apoptosis. BrDu incorporation assays revealed no obvious difference in the number of lens epithelial cells actively synthesizing DNA between wildtype (Figure 5A) and β1flox/β1flox/CRE (Figure 5B) lenses. In contrast, TUNEL labeling revealed a small number of β1-integrin null lens epithelial cells with fragmented DNA at 16.5 dpc (data not shown) while newborn β1flox/β1flox/CRE mice exhibited many apoptotic nuclei in the cells directly adjacent to the region of lens epithelial cell loss (Figure 5D). In contrast, few to no TUNEL positive cells are detected in the lens epithelium of wildtype mice at either age (Figure 5C, data not shown). In order to confirm that this reflects the activation of a classical apoptotic response, β1flox/β1flox/CRE lenses were stained for the cleaved form of caspase 3 responsible for apoptotic responses (Boatright and Salvesen, 2003). At 16.5 dpc, active caspase 3 was not detected in lenses lacking β1-integrin (data not shown), however, newborn lenses lacking β1-integrin exhibited appreciable cleaved caspase3 staining in both the remaining lens epithelial adjacent to the region of epithelial cell loss (Figure 5F) and fiber cells (data not shown), while no cleaved caspase 3 positive cells were found in the wildtype newborn lens (Figure 5E).

Some lens fiber cell markers are upregulated in β1-integrin null lens epithelial cells

Next, it was of interest to determine whether lenses lacking β1-integrin still maintain molecular markers associated with the lens lineage. First, we investigated the expression pattern of the lens fiber cell markers aquaporin 0/Mip (Shiels et al., 1991), β-crystallin and γ-crystallin (Chen et al., 2002). Aquaporin 0, a membrane protein important for lens function (Francis et al., 2000; Shiels et al., 1991), is normally found exclusively in lens fiber cells in the wildtype lens (Figure 6A) and this distribution was unchanged in the β1-integrin null lens (Figure 6B). In contrast, moderate amounts of ectopic β- and γ-crystallin protein were detected in β1-integrin null lens epithelial cells (Figure 6D, γ-crystallin data not shown) while these proteins were not detected in epithelial cells in wildtype lenses (Figure 6C, γ-crystallin data not shown).

Pax6 is downregulated while cMaf and Prox1 are upregulated in β1-integrin null lens epithelial cells

Pax6 is a paired/homeodomain transcription factor which is essential for early lens development (Ashery-Padan et al., 2000) and the maintenance of the lens epithelial phenotype (Collinson et al., 2001; Lovicu et al., 2004b). Further, Pax6 is a transcriptional repressor of the βB1-crystallin promoter (Duncan et al., 1998; Duncan et al., 2004) and β-crystallins are elevated in β1-integrin null lens epithelial cells (Figure 6D). Thus, we investigated Pax6 expression in the β1-integrin null lens. In the normal lens, Pax6 is found predominately in the lens epithelium, and its expression levels decrease during lens fiber cell differentiation (Figure 7A, (Duncan et al., 2000; Li et al., 1994)). In contrast, Pax6 levels are drastically decreased in the β1-integrin null lens epithelium (Figure 7B).

We next investigated the expression pattern of two transcription factors required for lens fiber cell differentiation, cMaf (Kawauchi et al., 1999) and Prox1 (Wigle et al., 1999), in the β1-integrin null lens. In the wildtype lens, cMaf (Figure 7C) and Prox1 (Figure 7E) are both found at relatively low levels in lens epithelial cells while their levels upregulate at the transition zone and are maintained at high levels in lens fiber cells. In contrast, intense cMaf (Figure 7D) and Prox1 (Figure 7E) immunostaining are detected in the β1-integrin null lens epithelium suggesting that they are responsible for the inappropriate activation of β-crystallin expression (Cui et al., 2004) in β1-integrin null lens epithelial cells.

Loss of β1-integrin from the lens results in upregulation of α-smooth muscle actin (αSMA) and E-cadherin expression

Since the inappropriate lens epithelial cell elongation (Figure 8A), upregulation of some fiber cell markers, followed by apoptosis was similar to that reported previously in transgenic mice overexpressing autoactivating TGFβ in lens fiber cells (Flugel-Koch et al., 2002; Lovicu et al., 2004b), we then investigated whether α-smooth muscle actin (αSMA), the most widely used marker of lens epithelial-mesenchymal transition (de Iongh et al., 2005), was upregulated in the β1-integrin null lens. In the wildtype newborn lens, αSMA was not detected under the conditions used (data not shown). In contrast, intense αSMA immunoreactivity was detected in the remaining lens epithelial cells of lenses lacking β1-integrin (Figure 8B). The elevated expression of αSMA is also detected throughout the lens epithelium at 16.5 dpc (Figure 8D) while αSMA protein was not detected in the wildtype lens under these conditions (Figure 8C). Elevations in αSMA protein levels were not detected in the lens at earlier developmental times (data not shown).

At 16.5 dpc, lens epithelial cells normally express the gap junction protein connexin 43, and this protein is found to be concentrated at the lateral membranes close to the apical end of the cells (Figure 8E, (Gao and Spray, 1998)). However, this distribution is lost from lens epithelial cells lacking β1-integrin and instead connexin 43 is found evenly distributed along the cell membrane (Figure 8F). In contrast, E-cadherin staining is more intense in 16.5 dpc lens epithelial cells lacking β1-integrin (Figure 8H) as compared to the wildtype lens (Figure 8G).

Smad6 protein is lost from the β1-integrin null lens and nuclear Smad4 levels increase

In the adult lens, TGFβ signaling is recognized as a major potentiator of epithelial-mesenchymal transition (de Iongh et al., 2005). The canonical TGFβ signaling pathway is mediated via R-Smad interaction with SMAD4 and translocation of this complex into the nucleus (ten Dijke and Hill, 2004). In other tissues, this pathway has been found to be antagonized by inhibitory SMADs which both block R-Smad activation by TGFβ receptors and target R-Smads for degradation (Park, 2005). In the wildtype 16.5 dpc lens, the inhibitory Smad, Smad 6, is found throughout the lens epithelium (Figure 9A) while only low levels of nuclear Smad4 (Figure 9E) are detected. In contrast, β1-integrin null lenses have nearly undetectable levels of Smad 6 in the lens epithelium (Figure 9C) while nuclear Smad4 levels are elevated (Figure 9F). These data indicate β1-integrin signaling is important to prevent inappropriate activation of Smad signaling pathways in the lens epithelium.

Discussion

This report investigates the phenotypic and molecular consequences of deleting exon 3 of the β1-integrin gene from the embryonic lens. Recombination of a floxed β1-integrin allele mediated by MLR10-Cre resulted in the loss of β1-integrin protein from the lens by 11.5 dpc coincident with primary lens fiber cell elongation. The first morphological consequences of this deletion are alterations in lens epithelial cell shape and fiber cell organization at 16.5 dpc associated with the downregulation of Pax6 expression, upregulation of fiber cell preferred

transcription factors, a redistribution of connexin 43 protein and increased E-cadherin and α-smooth muscle actin (SMA) staining. By birth, the central epithelium is absent due to increased amounts of apoptosis as measured by both TUNEL and cleaved caspase 3 staining. Investigation of pathways which could be controlling the increased amounts of SMA revealed that β1-integrin null lens epithelial cells have increased amounts of nuclear SMAD4 and reduced amounts of SMAD6. In aggregate, these results lead us to propose that β1-integrin cross talks with pathways controlling fiber cell differentiation, epithelial-mesenchymal transition and cell survival in the lens.

Late embryonic lens development is profoundly abnormal in the β1-integrin null lens

In the lens, β1-integrins have been implicated in the attachment of lens cells to the capsule (Bassnett et al., 1999; Nishi et al., 1997), and have been proposed to be essential for lens differentiation (Menko et al., 1998). Here we show that loss of β1-integrin protein from the lens vesicle coincident with primary fiber cell elongation does not result in obvious morphological defects in the embryonic-early fetal lens. As morphogenesis proceeds through fetal development into the postnatal period in mice, the expression profile of cellular receptors (Srinivasan et al., 1998), transcription factors (Min et al., 2004) and structural proteins (Carper et al., 1986; Kelley et al., 2002; Sinha et al., 2001) changes dramatically in the lens. Similarly, as lenses lacking β1-integrin protein enter late fetal development, the first morphological alterations are observed. At 16.5 dpc, the regular cuboidal organization of the lens epithelium is lost and by birth, most of the lens epithelium is absent and the remaining epithelial cells are elongated demonstrating an important role for β1-integrin in late fetal lens epithelial cells. Further defects are also detected in the lens fiber cells including cell disorganization, vacuole formation and finally postnatal liquefaction of the remaining lens material. While this phenotype may suggest that β1-integrin is also important for lens fiber cell function, it is also possible that these phenotypes are a secondary response to the loss of the lens epithelium since it is known that fiber cells communicate extensively with the lens epithelium in vivo (Bassnett et al., 1994; Rae et al., 1996). Definitive determination of β1-integrin function in lens fiber cells awaits the analysis of specific deletion of β1-integrin in lens fiber cells, leaving lens epithelial expression intact. It also should be noted that our data does not exclude the possibility that β1-integrin is essential for the early morphogenesis of the lens since β1-integrin expression is intact in the lens of homozygous flox β1-integrin/MLR10-cre mice during lens placode formation and lens vesicle closure. The role of β1-integrin in the lens placode and pit can be explored using a CRE strain active during early lens morphogenesis such as LE-CRE (Ashery-Padan et al., 2000).

Lens epithelial cells from β1-integrin null mice exhibit some but not all features of epithelial-mesenchyme transition and lens fiber cell differentiation

The morphology of lens epithelial cells lacking β1-integrin is reminiscent of the disruption of epithelial organization associated with epithelial-mesenchymal transition of these cells following injury (Saika et al., 2004) and/or exposure to inappropriate extracellular signals (de Iongh et al., 2005). Expression studies of the most common diagnostic marker for lens EMT (Marcantonio and Vrensen, 1999), α-smooth muscle actin, revealed that β1-integrin null lens epithelial cells upregulate the expression of this protein at 16.5dpc as the normal apical-lateral distribution of connexin43 (Gao and Spray, 1998) is lost, consistent with the proposition that these cells are undergoing epithelial-mesenchymal transition (de Iongh et al., 2005). Further, these lens epithelial cells inappropriately downregulate Pax6 expression, which has been proposed to be necessary for EMT in the lens epithelium (Lovicu et al., 2004b). However, the β1-integrin null lens does not have all molecular features of EMT since neither rt-PCR nor immunostaining detected increases in collagen I or fibronectin expression (data not shown) and β1-integrin null epithelial lens cells exhibited elevated, not reduced staining of the epithelial marker E-cadherin. Interestingly, it was recently reported that β1-integrin clustering

upon matrix binding decreases E-cadherin levels by activating MMP-9 and inducing the shedding of the E-cadherin ectodomain in ovarian cancer cells (Symowicz et al., 2007) which suggests that the enhanced E-cadherin staining observed in β1-integrin null lenses could result from reduced ectodomain shedding. However, it is still possible that the levels of intact E-cadherin are not being modulated in the β1-integrin null lens and instead the increased staining observed reflects a change in E-cadherin conformation resulting from the loss of β1-integrin.

These observations are consistent with two competing functions of β1-integrin in the lens. The upregulation of αSMA expression and the redistribution of connexin 43 in β1-integrin null lenses suggests that β1-integrin signaling serves as a block to these EMT responses under basal conditions in the lens epithelium. In contrast, the expression of several β1-binding α-integrins are upregulated in lens cells undergoing EMT (Barbour et al., 2004; Zuk and Hay, 1994) and EMT responses are blocked in lens cells treated with β1-integrin function blocking antibodies (Zuk and Hay, 1994) leading to the idea that integrins are necessary for a complete EMT response (de Iongh et al., 2005). This is consistent with the report that transfection of lens cells with active ILK, a mediator of β1-integrin signaling, leads to downregulation of E-cadherin (de Iongh et al., 2005), while E- cadherin levels appear enhanced in β1-integrin deficient lens epithelial cells. It is also possible that a portion of the phenotype observed results from an excess of α-integrin subunits that either can not find a β-integrin partner or which form inappropriate heterodimers due to changes in the ratio of α- to β-integrin subunits.

Loss of the lens epithelium from β1-integrin null lenses results from elevated levels of apoptosis, not decreased amounts of proliferation

Cell adhesion to extracellular matrices via β1-integrins has long been proposed to protect cells from apoptosis/anoikis by signaling to cell survival pathways (Frisch and Ruoslahti, 1997). However, conditional deletion of the β1-integrin gene from either skin keratinocytes , hair follicles (Brakebusch et al., 2000; Raghavan et al., 2000), or luminal mammary epithelial cells (Li et al., 2005; Naylor et al., 2005) does not result in increased apoptosis in vivo even following cell detachment from the underlying basement membrane. Instead, these cells have profound reductions in cell proliferation rates. In contrast, relatively normal amounts of cell proliferation are detected in β1-integrin null lens epithelial cells in vivo, instead, the loss of the lens epithelium at birth is mediated by increased amounts of apoptosis. Notably though, this increased apoptosis does not appear to be associated with classical anoikis since histological analyses found that β1-integrin null lens epithelial cells remain attached to the lens capsule.

In the lens, EMT associated with injury (Saika et al., 2002), anterior subcapsular cataract (Font and Brownstein, 1974) or TGFβ treatment (Maruno et al., 2002) are associated with significant levels of apoptosis which has been compared to the myofibroblast apoptosis that occurs as the granulation region of a healed epithelial wound resolves into a scar (Maruno et al., 2002). Since EMT markers are induced in the lens as a result of β1-integrin deletion prior to the onset of apoptosis, it is likely that the observed epithelial apoptosis is a secondary event related to the induction of EMT pathways in the lens instead of being a direct effect of β1-integrin loss.

β1-integrin null lens epithelial cells exhibit altered Smad pathway components

Adult lenses overexpressing an autoactivating form TGFβ1 in fiber cells under the control of a weak promoter develop anterior subcapsular cataracts associated with upregulation of α-SMA, β-crystallin and collagen I protein levels, downregulation of E-cadherin and connexin43 expression and increased cell proliferation (de Iongh et al., 2005; Lovicu et al., 2004a; Srinivasan et al., 1998). In contrast, lenses from mice expressing higher levels of autoactivating TGFβ1 begin expressing α-smooth muscle actin in the lens epithelium by 16.5 dpc and lose the lens epithelium by birth due to apoptosis (Flugel-Koch et al., 2002). This difference in response is probably attributable to the relative insensitivity of the fetal mouse lens to TGFβ

due to very low levels of TGFβ receptor expression as compared to the adult lens (Srinivasan et al., 1998). Notably, the morphology and dynamics of α-SMA expression in the β1-integrin null newborn lens is very similar to lenses expressing high levels of TGFβ leading to the hypothesis that β1-integrin deletion removes a block to TGFβ family member induced EMT responses in the embryonic lens.

Binding of TGFβ family members to their receptors leads to the phosphorylation of a receptor regulated Smad (R-Smad) leading to its dimerization with the signaling mediator, Smad4 and the translocation of this complex to the nucleus (ten Dijke and Hill, 2004). The activated Smad complex then interacts with other transcription factors which either results in the activation of genes involved in the EMT response such as α-smooth muscle actin (Hu et al., 2003) or the degradation of transcriptional repressors (Moustakas et al., 2001). This pathway can be negatively regulated by inhibitory Smads (Smad6 or 7) which can block R-Smad phosphorylation and lead to ubiquitin regulated degradation of both activated TGFβ receptors and R-Smads (Park, 2005). Our current data suggest that inappropriate activation of the Smad pathway is a likely explanation for the induction of SMA expression in the β1-integrin null lens since we detected increased amounts of nuclear Smad4 in β1-integrin null lens epithelial cells. While increased activation of TGFβ family member receptors is a possible explanation for this observation, our current data suggests that increased levels of nuclear Smad4 are a result of decreased amounts of Smad6 in the β1-integrin null lens. This hypothesis is consistent with prior reports of downregulated I-Smad expression in various fibrotic diseases (Dong et al., 2002; Yu et al., 2006).

Overall though, the function of β1-integrins in the lens is likely to be complex. Integrins serve as both structural linkers between the extracellular matrix and the cytoskeleton and cell signaling molecules that regulate cell behavior and identity (Belkin and Stepp, 2000; Heino, 2000; Wiesner et al., 2005). Notably, it was recently reported that short term disassembly of actin stress fibers in the lens epithelium was sufficient to induce lens fiber cell differentiation but sustained stress fiber disassembly results in lens epithelial cell apoptosis (Weber and Menko, 2006) suggesting a potential mechanism regulating the phenotypes in the β1-integrin null lens.. Alternatively, the loss of β1-integrin is expected to result in the alteration of many cell signaling cascades including those utilizing FAK, Rho GTPases, ERK, and ILK (Cohen and Guan, 2005; Grashoff et al., 2004; Longhurst and Jennings, 1998). Further experiments are necessary to determine the precise signaling cascades that β1-integrins control in the lens.

Conclusions

Our data demonstrate the β1-integrin is essential for the maintenance of the lens epithelium, preventing the expression of both lens fiber cell and myofibroblast markers and ensuring lens epithelial cell survival. Since prior work has shown that β1-integrin expression is upregulated by TGFβ in the lens and β1-integrin function is required for lens EMT in vitro, our data fits with proposed pathways in which increased β1-integrin engagement during EMT results in the loss of E-cadherin expression. However, the response of the lens to β1-integrin deletion is novel compared to the consequences of β1-integrin deletion in other epithelial tissues suggesting that the lens uses β1-integrin activated pathways in different ways than the skin or mammary epithelium.

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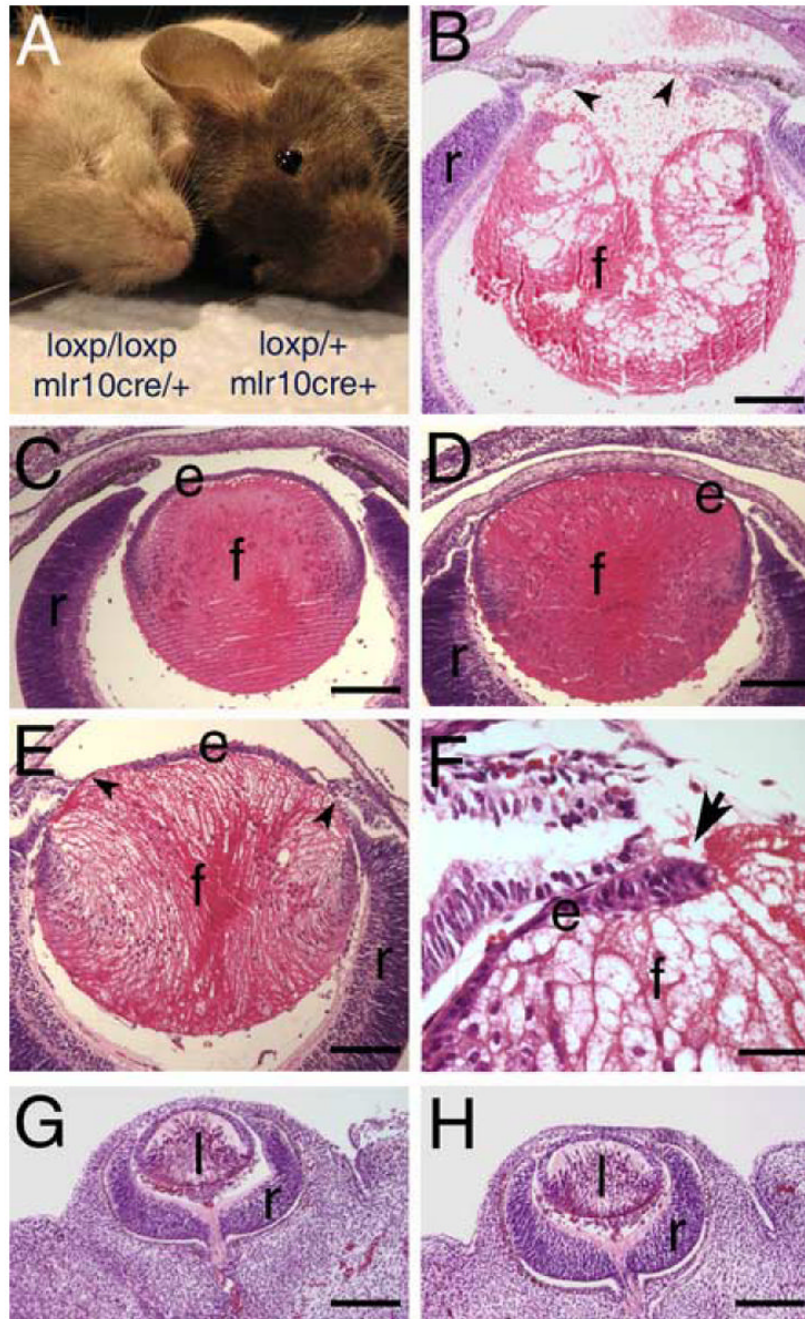
Figure 1.
Mice homozygous for a floxed β1-integrin allele carrying MLR10-cre are microophthalmic with lens defects first apparent in late embryonic development. A) Exterior appearance of littermates who are either homozygous or heterozygous for floxed β1-integrin and carry MLR10-cre. B-H) Hematoxylin and eosin stained paraffin sections B) Newborn eye from a mouse homozygous for the floxed β1-integrin allele and carrying MLR10-cre showing loss of the anterior epithelium (arrowheads) and vacuolation of the lens fiber cells. C) Lens from a 16.5 dpc mouse homozygous for the floxed β1-integrin allele but lacking MLR10-CRE showing the normal regular organization of the lens epithelium and fiber cells. D) Eye from a 16.5 dpc mouse homozygous for the floxed β1-integrin allele and carrying MLR10-cre showing

mild abnormalities in organization of the lens epithelium and fiber cells. E) Eye from 16.5 dpc mouse homozygous for the floxed β1-integrin allele and carrying MLR10-cre showing further elongation of the lens epithelial cells and regions of lens epithelial cell loss. F) High magnification image of a region of epithelial cell loss (arrow) observed in a 16.5 dpc lens. .Lens from 12.5 dpc mice either heterozygous (G) or homozygous (H) showing no abnormalities in lens morphology. Abbreviations; e, lens epithelium; f, lens fiber cells; r, retina; l- lens. Scale bars: Panels B, C, D, E, G, H-160 μm, Panel F- 40 μm
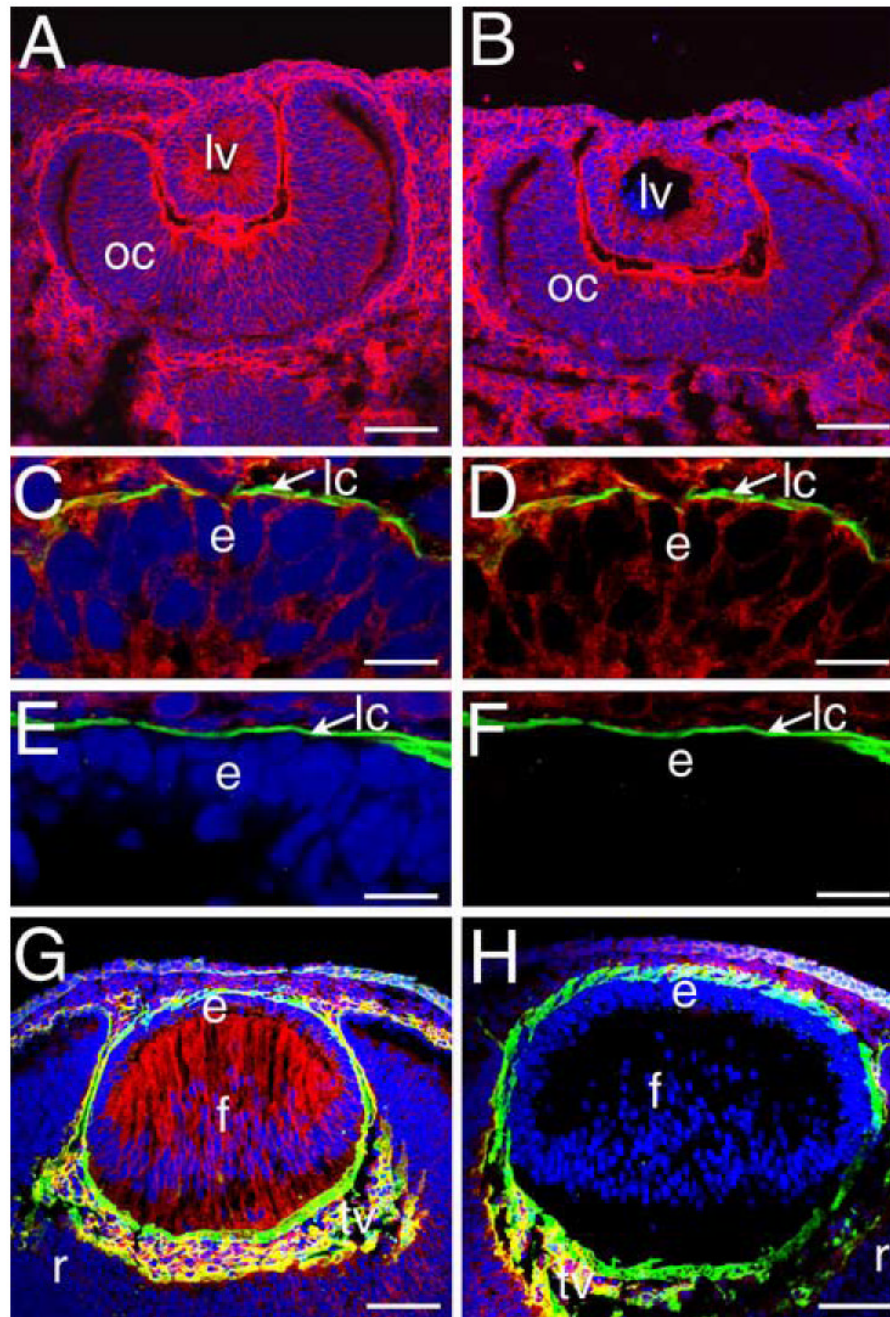
Figure 2.
Homozygous flox β1-integrin/MLR10-cre mice lose β1 integrin protein from the lens by 11.5 dpc. A, B) Eyes from 10.5 dpc mice either heterozygous (A) or homozygous (B) for the floxed β1-integrin allele carrying MLR10-cre showing no changes in β1-integrin protein distribution (red) at the lens vesicle stage. C) The anterior lens vesicle of a 11.5 dpc wildtype mouse embryo triple labeled for β1-integrin (red), collagen IV (green) and DNA (blue). Panel D shows the same image as panel C with the DNA channel deleted. E) Anterior lens vesicle from a 11.5 dpc mouse embryo carrying the floxed β1-integrin allele and MLR10-cre triple labeled for β1-integrin (red), collagen IV (green) and DNA (blue). Panel F shows the same image as E with the DNA channel deleted. G) Eye from a wildtype 12.5 dpc mouse embryo showing the

normal distribution of β1-integrin (red) in the lens at this stage. H) Eye from a 12.5 dpc mouse embryo homozygous for the floxed β1-integrin allele and carrying MLR10-cre showing the specific loss of β1-integrin protein from all lens cells. Red- β1-integrin, blue- DNA, green collagen IV; Panels A, B, G, H- Bar=77 μm; Panels C-F, Bar= 13 μm Abbreviations: lv- lens vesicle; oc- optic cup; e- lens epithelium; f- lens fibers; r- retina; lc-lens capsule; tv- tunica vasculosa.
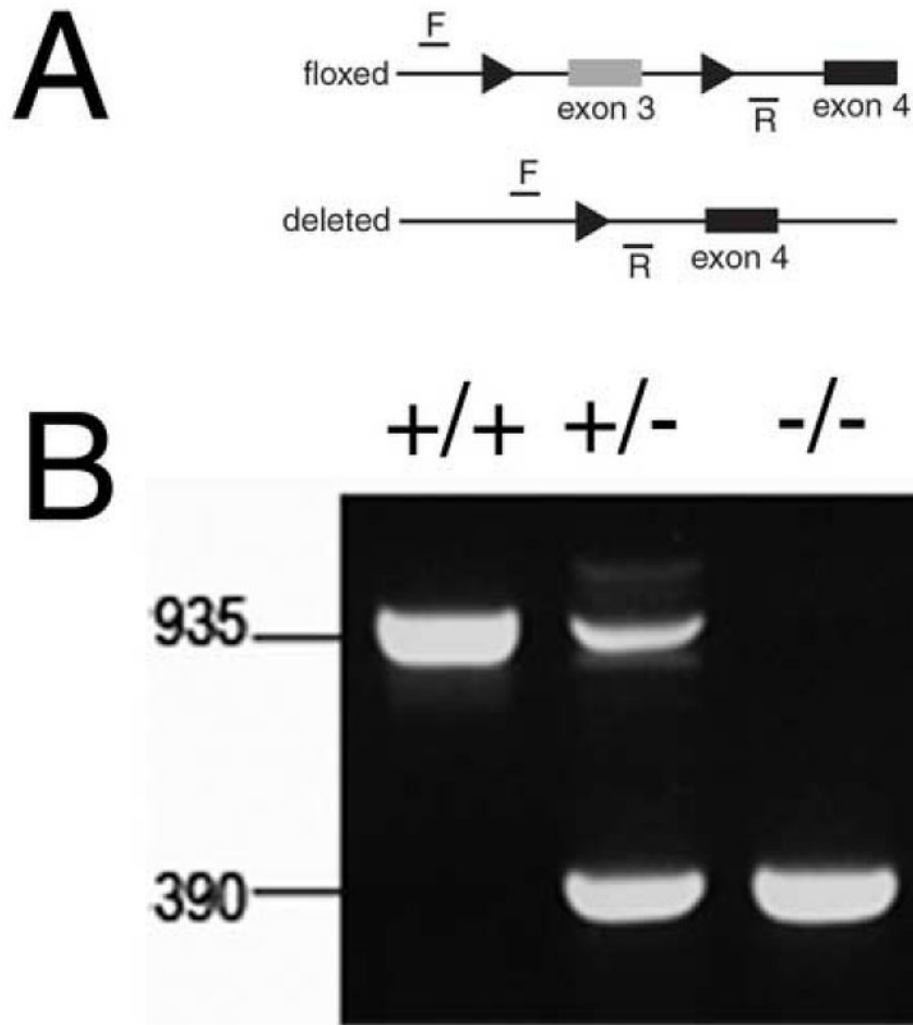
Figure 3.
Deletion of exon 3 from the β1-integrin gene using MLR10 CRE is efficient in the lens A) Diagram of the β1-integrin locus showing the postion of the PCR primers used to score the deletion and the presence of the loxP sites (arrowheads). B) PCR analysis of DNA obtained from newborn lenses either wildtype (+/+), heterozygous (+/-) or homozygous (-/-) for the floxed β1-integrin allele demonstrating that β1-integrin deletion is essentially complete.
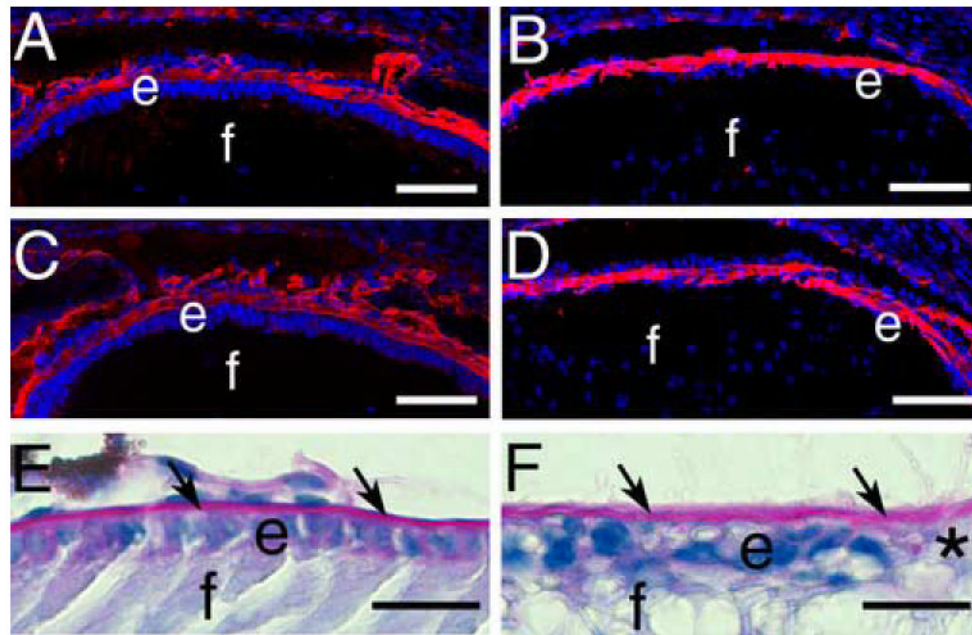
Figure 4.
β1-integrin null lenses retain the lens capsule A, B) Immunofluorescent staining of collagen IV (red) in the wildtype (A) and β1-integrin null (B) 16.5 dpc lens capsule C, D) Immunofluorescent staining of laminin (red) in the wildtype (C) and β1-integrin null (D) 16.5 dpc lens capsule. E, F) Periodic acid-Schiff staining (pink) of the newborn lens capsule (arrows) from wildtype (E) and β1-integrin null (F) mouse lenses showing that the lens capsule is retained even in areas denuded of lens epithelial cells (*). Abbreviations; e-lens epithelium; f- lens fiber cells. Panels A-D: scale bar= 77 μm. Panels E, F: scale bar =20 μm.

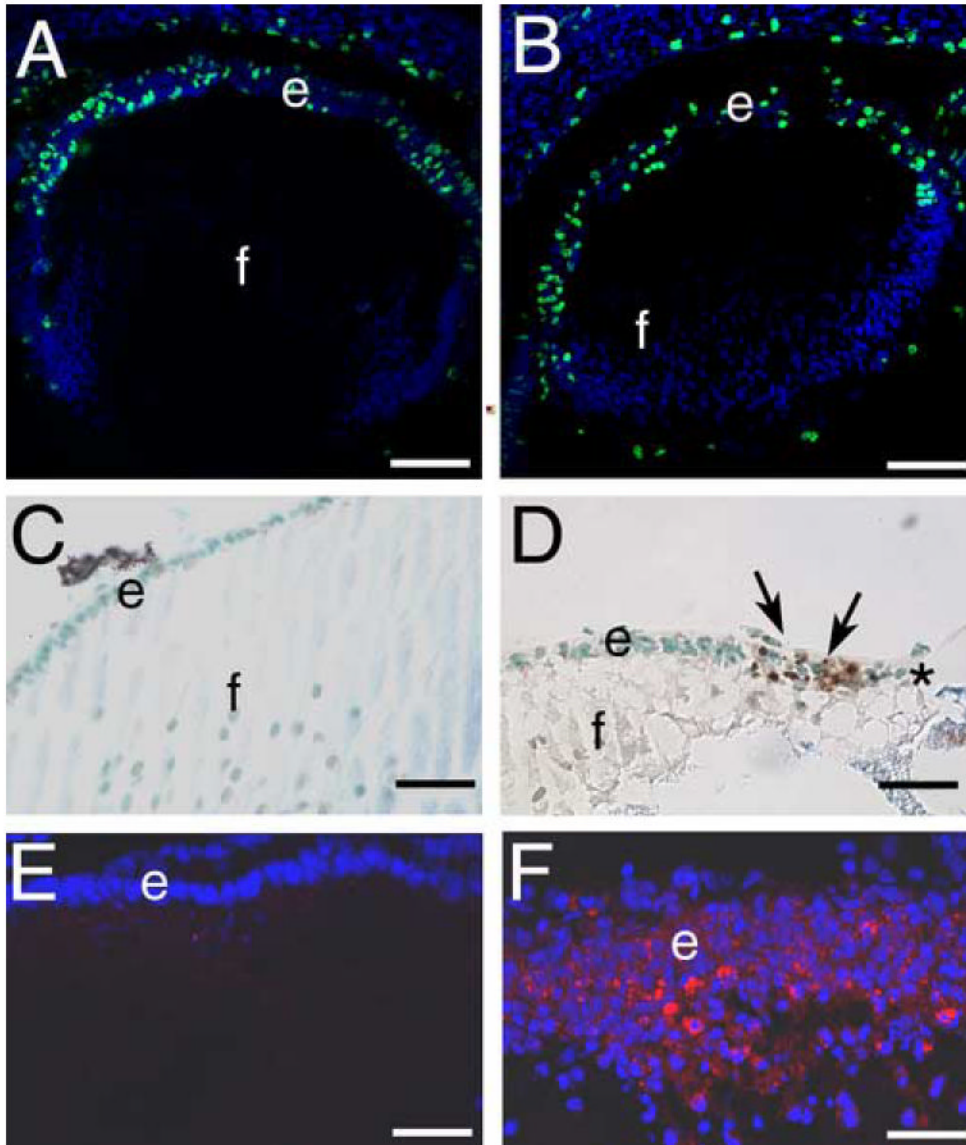**Figure 5.**
β1-integrin null lens epithelial cells are lost by birth due to elevated levels of apoptosis A, B) 16.5 dpc embryonic lenses stained for cells actively synthesizing DNA (green) using the 5-bromodeoxyuridine assay. Note that wildtype (A) and β1-integrin null (B) lens epithelia have qualitatively similar levels of cell proliferation. C, D) newborn lenses stained for apoptotic cells (brown) using TUNEL assay. Note that wildtype lenses (C) do not exhibit TUNEL positive lens epithelial cells while numerous TUNEL positive cells (arrows) are found in β1-integrin null lenses (D), especially in regions of lens epithelial cell loss (*). E, F) Newborn lens epithelial cells stained for cleaved caspase3 (Asp175). Note that wildtype lenses have little to no detectable cleaved caspase 3 (E) while numerous β1-integrin null lens epithelial cells are cleaved caspase 3 positive. Abbreviations: e- lens epithelium; f- lens fibers. Panels A,B: scale bar= 77 μm, C,D=30 μm, E,F=38 μm.
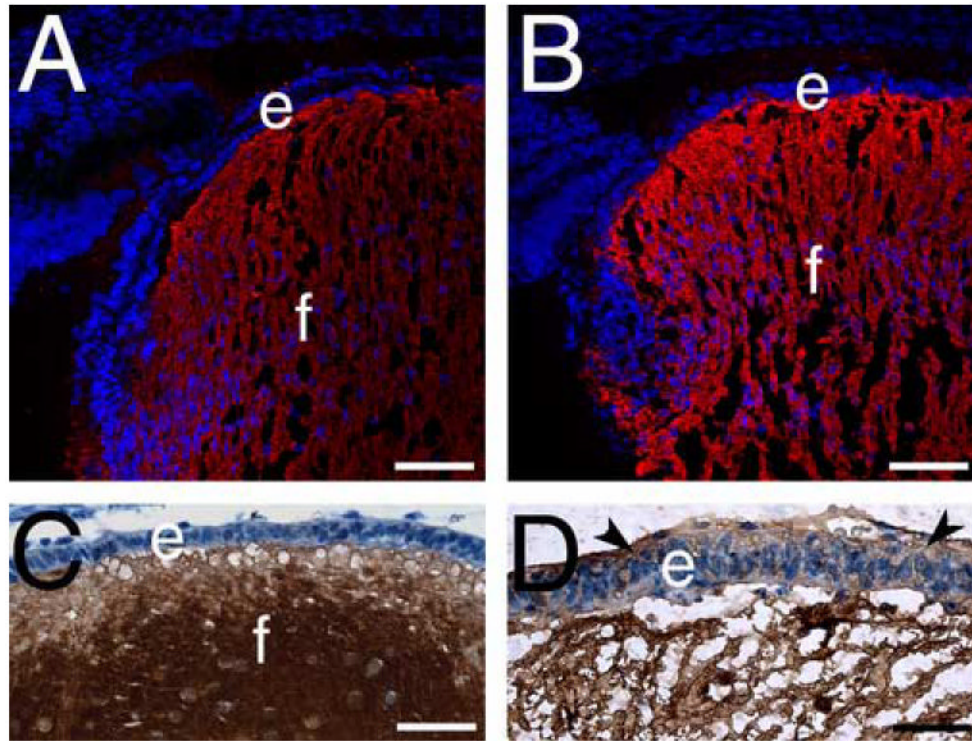
Figure 6.
β-crystallin but not aquaporin0 expression is upregulated in 16.5 dpc β1-integrin null lens epithelial cells A,B) Aquaporin 0 (red) immunolocalization in wildtype (A) and β1-integrin (B) null lenses. C, D) β-crystallin (brown) immunolocalization in wildtype (C) and β1-integrin (D) null lenses. Abbreviations. e- lens epithelium, f- lens fibers. Scale Bar: Panels A,B= 77 μm; Panels C,D= 30 μm

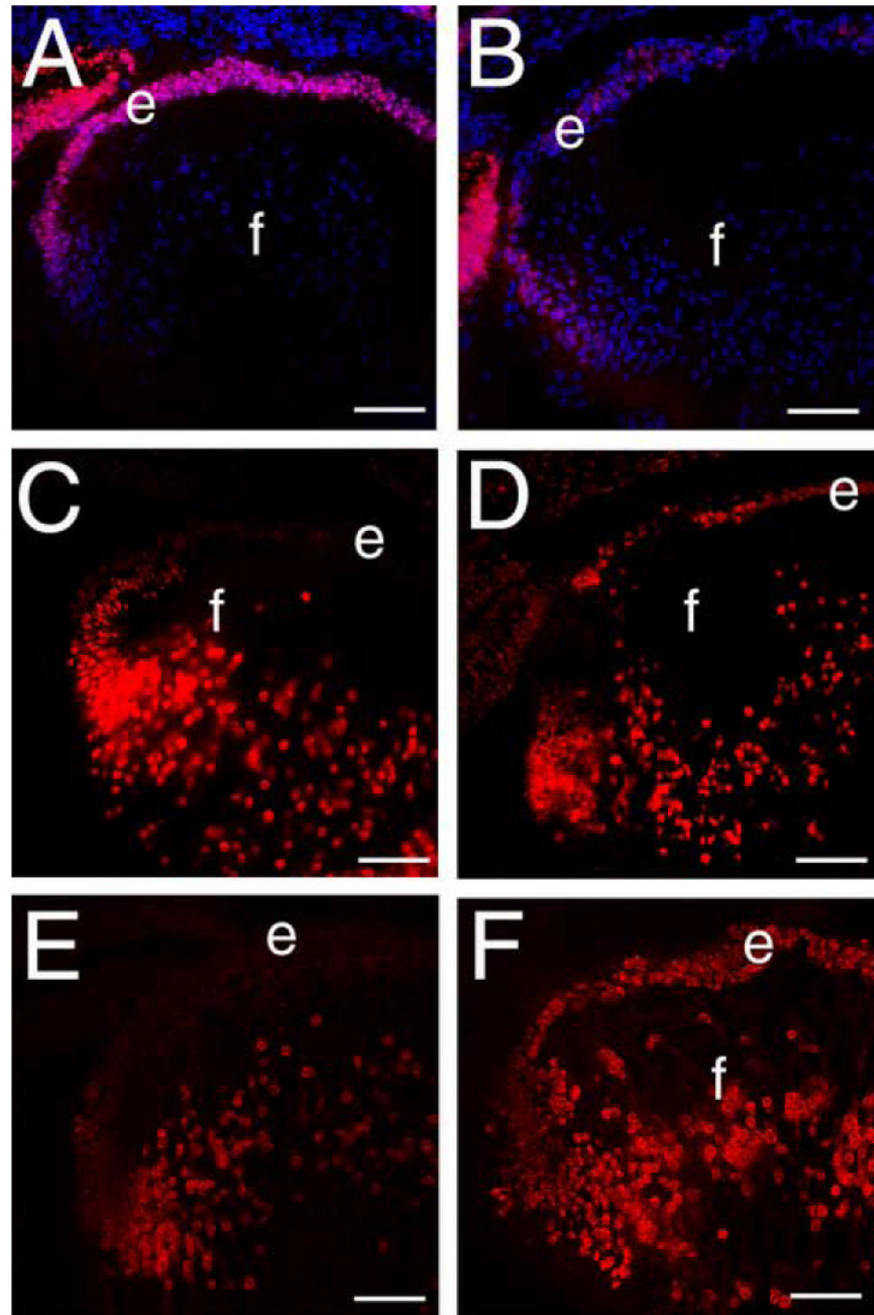**Figure 7.**
β1-integrin null lens epithelial cells show downregulated Pax6 and upregulated cMaf and Prox1 expression at 16.5 dpc A, B Pax6 (red) immunolocalization in wildtype (A) and β1-integrin (B) null lenses, C,D) cMaf (red) immunolocalization in wildtype (C) and β1-integrin (D) null lenses. E, F) Prox1 (red) immunolocalization in wildtype (E) and β1-integrin (F) null lenses. Abbreviations: e-lens epithelium; f- lens fibers. Blue in panel A,B is DNA staining All scale bars= 77 μm
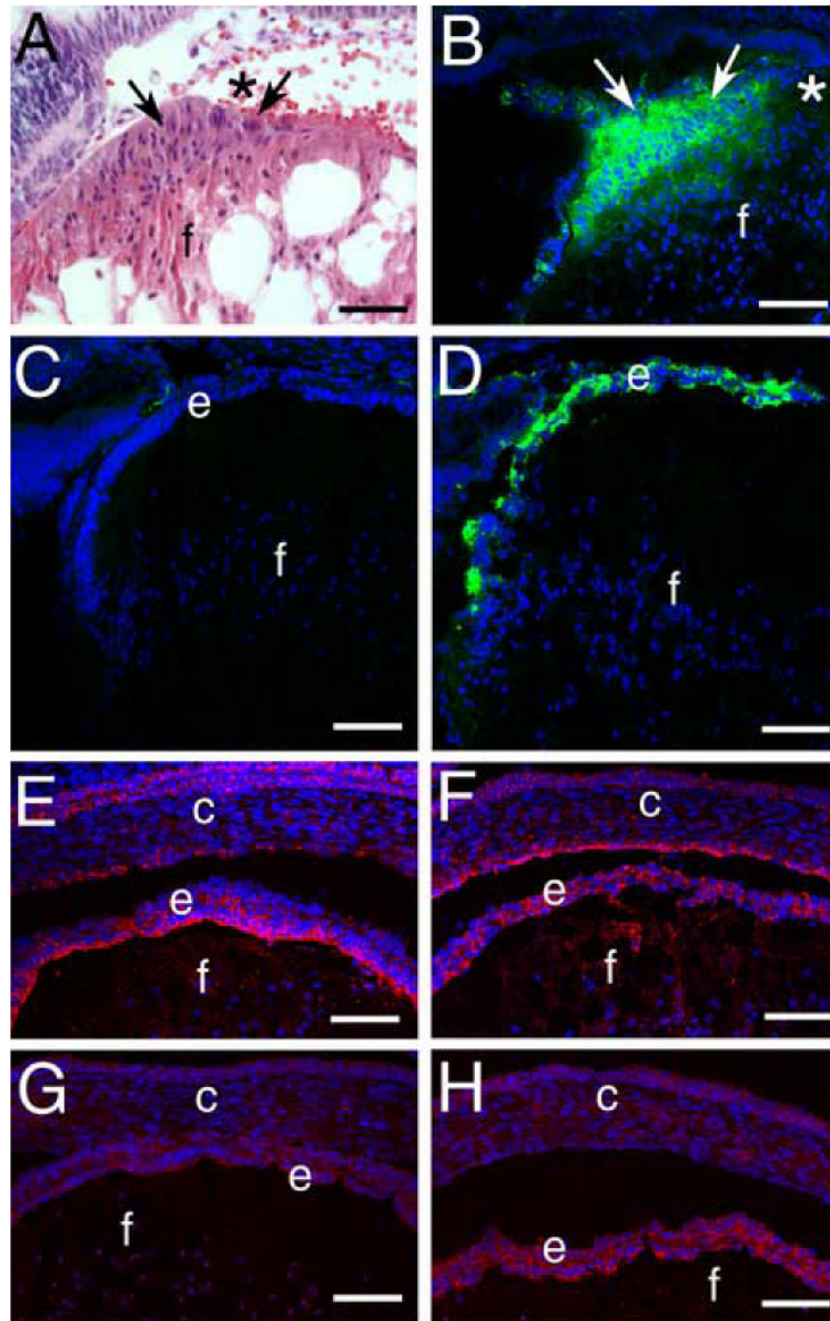
Figure 8.
β1-integrin null lens epithelial cells demonstrate some but not all features of epithelial-mesenchymal transition (EMT). A) Hematoxylin and eosin stained paraffin section of the β1-integrin null newborn lens epithelium showing the elongation of these cells (arrows) from their normal flattened cuboidal morphology and the edge of the region of central epithelial cell loss (*). B) Immunodetection of αSMA (green) in the β1-integrin null newborn lens epithelium showing strong staining in the abnormal lens epithelial cells. C) Immunolocalization of αSMA (green) in the 16.5 dpc wildtype lens . D) Immunolocalization of αSMA (green) in the 16.5 dpc β1-integrin null lens imaged under the same conditions as panel C showing αSMA upregulation in the disorganized lens epithelium. E) Immunodetection of connexin 43 (red) in

the wildtype 16.5 dpc lens demonstrating its preferred localization at the apical side of the lateral membranes of the lens epithelium. F) Immunodetection of connexin 43 (red) in the β1-integrin null 16.5 dpc lens demonstrating that its distribution is disorganized in the lens epithelium. G) Immunodetection of E-cadherin (red) in the wildtype 16.5 dpc lens demonstrating its expression in the lens epithelium. H) Immunodetection of E-cadherin (red) in the β1-integrin null 16.5 dpc lens demonstrating increased E-cadherin staining in the β1-integrin null lens epithelium. Abbreviations: c-cornea; e-lens epithelium; f-lens fiber cells scale bar: Panel A=30 μm, Panels B-H=77 μm
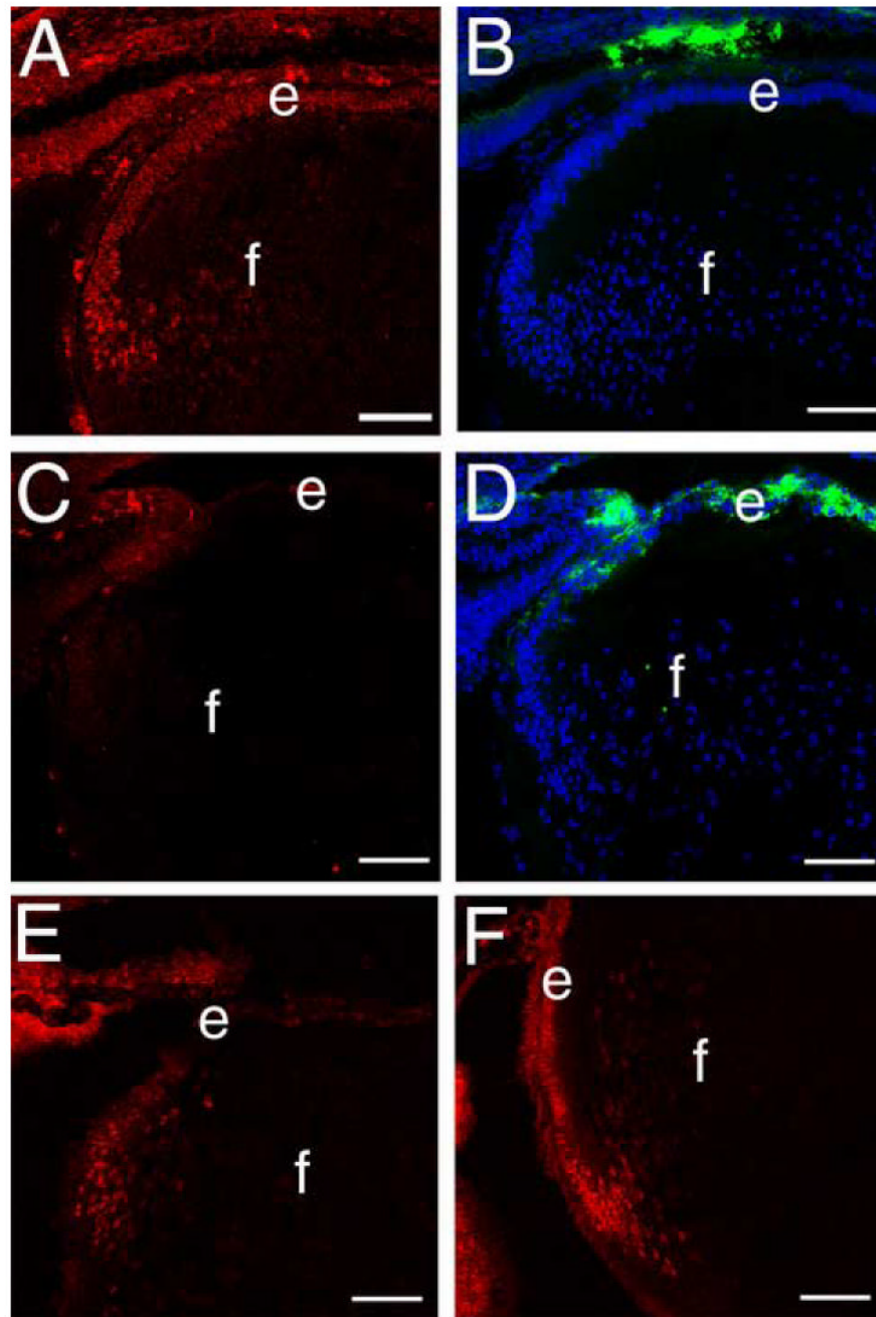
Figure 9.
Smad6 expression is downregulated and Smad4 levels are upregulated in β1-integrin null lens epithelial cells A) Distribution of Smad6 (red) in the wildtype 16.5 dpc lens. B) the same section shown in A stained for DNA (blue) and αSMA (green). C) Distribution of Smad6 (red) in the β1-integrin null 16.5 dpc lens showing the downregulation of Smad6 levels in the lens epithelium D) the same section shown in C stained for DNA (blue) and αSMA (green) showing the upregulation of αSMA levels in a lens with reduced Smad6 levels. E) the distribution of Smad4 (red) in the wildtype 16.5 dpc lens demonstrating that this protein is predominately located in the nuclei of lens cells undergoing the transition between epithelial and fiber cells. F) The distribution of Smad4 (red) in the β1-integrin null 16.5 dpc lens showing that nuclear

Smad4 levels are levated in β1-integrin null lens epithelial cells. Abbreviations: e-lens epithelium; f- lens fibers. All scale bars =77 μm