Absence of SPARC in Murine Lens Epithelium Leads to Increased Deposition of Laminin-1 in Lens Capsule

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Purpose. To investigate the role of SPARC in the regulation of the expression and deposition of extracellular matrix (ECM) proteins in the lens capsule.

Methods. Wild-type ($SP^{+/+}$) and SPARC-null ($SP^{-/-}$) mice of embryonic day (E)14 to 3 months of age were examined. Transcript levels of lens basement membrane (BM) components were analyzed by semiquantitative RT-PCR with mRNA from lens epithelia. Expression of ECM proteins in lens capsule and lens epithelium was analyzed by immunohistochemistry and Western blot analysis. Cell attachment was assessed by lens epithelial explant culture. Coimmunoprecipitation was performed to identify intracellular protein interactions.

Results. From postnatal day 5 to 3 months of age, SPARC-null lens capsules exhibited higher levels of laminin-1 deposition relative to their wild-type counterparts, as revealed by immunohistochemistry and immunoblot analysis. An uneven and aggregated distribution of laminin-1 protein was apparent in the anterior region of SPARC-null lens capsules. SPARC and laminin-1 were expressed abundantly in the endoplasmic reticulum (ER) of lens epithelial cells. Coimmunoprecipitation identified that SPARC associates with laminin-1 before laminin secretion. Furthermore, increased laminin-1 in lens capsule promoted the attachment of lens epithelial explants in culture.

Conclusions. SPARC affects the secretion and deposition of laminin-1 protein in lens epithelial cells. Because abnormal deposition of laminin-1 in the lens BM could influence lens epithelial cell adhesion and fiber cell differentiation, the authors propose that SPARC is important to lens homeostasis through its regulation of lens BM matrix organization. (*Invest Ophthalmol Vis Sci.* 2005;46:4652–4660) DOI:10.1167/iovs.05-0460

Lens capsule represents a specialized basement membrane (BM), an acellular and structurally complex extracellular matrix (ECM) that plays a critical role in the regulation of cell survival, adhesion, differentiation, permeability, epithelial polarity, and cell growth.[1,2] The components that occur in nearly all BM including lens BM are type IV collagen, laminin, perlecan, and entactin-nidogen.[3–5] Expression of these macromolecules must be precisely regulated for the maintenance of normal morphologic and functional properties of the organ and its homeostasis. The lens epithelium and newly differentiated fiber cells are responsible for the assembly, secretion, and deposition of the lens BM components,[6] and abnormal epithelial cell function is thought to lead to an altered lens BM.

Laminin is a noncollagenous glycoprotein that is a prerequisite of the production of the BM in vivo.[7] A major component of BM structure, laminin has been found to bind to type IV collagen, perlecan, and nidogen.[8,9] These multiple interactions are crucial for BM formation and stability. Laminin-1 heterotrimer ($\alpha1\beta1\gamma1$) is encoded by three different genes (LAMA1, LAMB1, and LAMC1) located on different chromosomes. Laminin-1 is localized to the epithelial BM of most organs including the lens[10] and plays central roles in lens development and morphogenesis.[11–13]

SPARC is a highly conserved, prototypic matricellular glycoprotein, shown to be essential in the regulation of morphogenesis, cell adhesion, migration, differentiation, and interaction with growth factors and ECM proteins.[14,15] The central role of SPARC in the lens function has been well demonstrated in SPARC-null ($SP^{-/-}$) mice, which exhibit cortical lens opacity 1 month after birth and mature cataract at 5 to 7 months.[16] One of the major alterations in the lens is the pathologic change in the capsule. The lens cells immediately beneath the capsule protrude into the lens BM and compromise its structural and functional integrity.[16–18] The molecular composition of the lens BM is essential for the correct organization of the matrix network, and disorganized ECM is likely to contribute to the cells' invasive projections into the lens BM in $SP^{-/-}$ mice. In this study, we asked whether the molecular composition of the SPARC-null lens capsule is aberrant, and/or whether SPARC regulates the expression or deposition of ECM proteins in the lens BM.

In this report, we characterize the expression of the major BM components in $SP^{-/-}$ lenses before and during the early stages of cataract formation (embryonic day [E]14 to 2 to 3 months of age). SPARC appeared not to regulate the levels of most lens BM mRNAs, but played a major role in determining the distribution of laminin-1 in the lens. Deposition of laminin-1 in the lens capsule was increased in the absence of SPARC. In lens epithelial cells, SPARC associated with laminin-1 in the endoplasmic reticulum (ER) before the secretion of laminin-1. This interaction is likely to affect the deposition of laminin-1 in the lens BM. The aberrant deposition of laminin-1 in the capsule affected BM matrix organization and changed lens epithelial cell adhesion.

Materials and Methods

Animals

129SvEv x C57BL/6j wild-type ($SP^{+/+}$) and SPARC-null ($SP^{-/-}$) mice were generated and maintained as described previously.[16,19] The chimeras were crossed with C57BL/6j mice to obtain homozygous mice. The treatment and use of the mice conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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TABLE 1. Antibodies

Antigen	Ig	Dilution (μg/mL) WB	Dilution (μg/mL) IHC	Source
Collagen IV	Rabbit polyclonal IgG	—	20	MP Biomedicals (Aurora, OH)
Fibronectin	Mouse monoclonal IgM	0.55	5.5	Sigma-Aldrich (St. Louis, MO)
GAPDH	Mouse monoclonal IgG	2	—	Ambion (Austin, TX)
Golgi 58K	Mouse monoclonal IgG	—	30	Sigma-Aldrich
Hsp47	Mouse monoclonal IgG	—	1	Stressgen (Victoria BC, Canada)
Laminin-1	Rabbit polyclonal IgG	1.2	12	Sigma-Aldrich
Nidogen-1	Rat monoclonal IgG	1	2	NeoMarkers (Fremont, CA)
Perlecan	Rat monoclonal IgG	1	2	NeoMarkers
SC1	Goat polyclonal IgG	2	4	Santa Cruz (Santa Cruz, CA)
SC1	Rat monoclonal IgG	1	—	Sage Lab (12–155)[24]
SPARC	Goat polyclonal IgG	1	10	R&D Systems (Minneapolis, MN)
Tenascin	Mouse monoclonal IgG	—	20	Sigma-Aldrich
β-Tubulin	Mouse monoclonal IgG	12	—	Sigma-Aldrich

WB, Western blot; IHC, immunohistochemistry; Ig, immunoglobulin.

Immunohistochemistry

Eyeballs from embryo to postnatal mice (E11 to 2 months of age) were fixed by immersion in methyl Carnoy's solution for 4 hours at room temperature. The eyeballs were dehydrated in a series of ethanol solutions and were embedded in paraffin.

Serial 5-μm sections were cut, deparaffinized, rehydrated, and washed in phosphate-buffered saline (PBS). The tissue sections were permeabilized (Auto/Zyme; Biomeda, Foster City, CA). Nonspecific binding sites were blocked by incubation in 20% blocking serum (AquaBlock; East Coast Biologics, Inc., North Berwick, MA) in PBS with 0.05% Tween-20 for 1 hour at room temperature. Sections were incubated for 2 hours at room temperature or overnight at 4°C with the primary antibodies (Table 1). Negative controls included replacement of primary antibodies by normal species isotype or PBS. Sections were washed in PBS with 0.05% Tween-20 and were incubated in fluorescein isothiocyanate or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Lens epithelial cells were grown on glass coverslips for immunocytochemistry. Cells were fixed with methyl Carnoy's solution for 10 minutes, followed by incubation in 20% blocking serum (AquaBlock, East Coast Biologics, Inc.) containing 0.1% Triton X-100 for 45 minutes. Antibodies were applied as described earlier.

Preparation and Detection of Lens Capsular Proteins

With the aid of a dissecting microscope, lens capsules with attached epithelium were removed from the fiber mass of the lens. For removal of the entire lens epithelium including the anterior and equatorial regions, the capsule was immersed in nonenzymatic cell dissociation solution (Sigma-Aldrich, St. Louis, MO) for 15 minutes to dissociate the epithelium from the lens capsule. Subsequently, also visualized with a dissecting microscope, the remaining epithelial cells were completely scraped off, and capsules were washed in PBS. The complete removal of epithelial cells was confirmed by microscopic inspection and by the lack of glyceraldehyde phosphate dehydrogenase (GAPDH; Ambion, Austin, TX) by immunoblot analysis (described later).

Lens capsules with attached lens epithelial cells were homogenized and incubated in extraction buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.2], and 20 mM EDTA) containing a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) overnight on ice. The extracts were diluted with lithium dodecyl sulfate (LDS) electrophoresis sample buffer (NuPAGE; Invitrogen, Carlsbad, CA) containing 10 mM dithiothreitol (DTT). Capsules were further homogenized and solubilized in sample buffer (Novex; Invitrogen), heated for 10 minutes at 70°C, and analyzed by SDS-PAGE on 3% to 8% precast polyacrylamide gradient gels (Novex; Invitrogen). Lens capsules without lens epithelium were incubated in the extraction buffer on ice overnight, and samples were homogenized and incubated on ice for another 4 hours. The extracts were centrifuged, and the supernates were collected as soluble fractions. They were further dissolved in sample buffer in the presence of DTT as described earlier.

Standard immunoblot analysis procedure was followed,[16] and the blots were exposed to rabbit anti-mouse laminin-1 IgG and anti-nidogen I IgG (Table 1). The same blot was stripped and reprobed with antibody against GAPDH to evaluate the elimination of lens epithelium from the capsular samples.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from lens epithelial cells of wild-type and SPARC-null lenses (RNeasy Mini Kit; Qiagen, Valencia, CA), as described previously.[17] The quality and yield of recovered RNA were evaluated by absorption at 260 and 280 nm. Total RNA was reverse transcribed into cDNA by the use of a reverse-transcription kit (Omniscript RT kit; Qiagen). Equal amounts of RNA were transcribed from each preparation. cDNA was amplified using the primer pairs listed in Table 2. The PCR program was 12 minutes at 94°C, followed by various cycles (Table 2) of 45 seconds at 95°C, 59 seconds at 65°C, 2 minutes at 72°C, followed by a final extension of 8 minutes at 72°C. GAPDH was used as an internal control for sample normalization. PCR products are analyzed within the linear range of amplification for the various genes examined. Products were separated on agarose gels and visualized by staining with ethidium bromide.

Attachment Assay of Lens Epithelial Explants In Vitro

Lenses (1–2 months of age) were dissected from the eyeballs, under a dissecting microscope, and were gently rolled on a sterile filter paper to remove any adhering iris and ciliary body tissues. Lens capsules with attached epithelium were peeled off by tearing the posterior side of the capsules using sharp forceps. Culture dishes (35-mm) were coated with fibronectin (25 μg/mL; Sigma-Aldrich) or fibronectin and laminin-1 (25 μg/mL; BD Biosciences, San Jose, CA) for 1 to 2 hours at 37°C. Five capsules with the epithelium downward were placed in one 35-mm dish with 1.2 mL of Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) containing 15% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin SO_4, and 2.5 μg/mL amphotericin-B (Sigma-Aldrich). Lens capsular explants were incubated for 4 days in a humidified atmosphere containing 5% CO_2 at 37°C, without disturbance. At 4 or 7 days of

TABLE 2. Primers Used for cDNA Amplification

Gene		Sequence (5′–3′)	Size (bp)	Accession No.
Collagen IV				
Alpha 1	F	GCTATTCCTTCGTGATGCACA	497	NM_009931
	R	CTCCGCTGCCTGTCCTTCT		
Alpha 2	F	CAACCCTGGTGATGTCTGCT	656	NM_009932
	R	GCCGGCTCACAGGTTCTT		
Alpha 5	F	ACAGCCAGGTGCCCGT	428	NM_007736
	R	ACAGCTGGCGCCTCAC		
Alpha 6	F	GCTGCTCTGTATGTGAGGCA	512	NM_053185
	R	GGATGACCATTGTCCAGTAGTCC		
Fibronectin	F	ACCCTGGGTATGACACCGAAA	452	NM_010233
	R	GTGCCTCCACTATGATGTTGTAGG		
GAPDH	F	GACCCCTTCATTGACCTCAACT	310	M32599
	R	TGGTTCACACCCATCACAAAC		
Laminin				
LAM A1	F	GATGCCATTGGCCTAGAGATTG	509	NM_008480
	R	GGATGGGAATGGGAGCTGA		
LAM B1	F	CTATGGGTACTACCGCGATGCTC	631	M15525
	R	CAGAGTCCACGGTCTCTCGGTA		
LAM C1	F	ACCTGGACCGTCTGATTGACC	522	NM_010683
	R	AGCTGCCTCAGCATACCGTT		
Nidogen 1	F	GATTGGAAGTCCTGCTACCGC	187	NM_010917
	R	CATTGTGTGGGTCAGGGTATCC		
Nidogen 2	F	CTTTCCTTACGGGTCGTG	611	NM_008695
	R	TCTCTCTTCAGGTCGTCCGCT		
SC1	F	CCAGCATGAAGGCTGTGCTT	425	NM_010097
	R	AGGAAGTGGACACGGTCGC		
SPARC	F	ATGAGGGCCTGGATCTTCTTTC	365	NM_009242
	R	GGAAGAGTCGAAGGTCTTGTTGTC		

incubation, primary cultures were examined and photographed under a phase-contrast microscope, to evaluate the attachment of the lens epithelial explants to the substrates.

Cultured lens epithelial cells ($SP^{+/+}$ and $SP^{-/-}$ at passage 3) formed a monolayer on the 35-mm dishes. Cells were incubated with 0.05% trypsin/PBS for 5 minutes at 37°C. Responses of cells to this treatment were photographed under a phase-contrast microscope.

Coimmunoprecipitation

Cultured lens epithelial cells were washed twice with ice-cold Ca^{2+}/Mg^{2+}-free PBS, followed by exposure to 0.05% trypsin-0.02% EDTA (Invitrogen-Gibco). Detached cells were collected and washed twice with PBS. The cell pellets were solubilized in lysis buffer (M-PER; Pierce Biotechnology, Rockford, IL), containing a complete protease inhibitor cocktail (Roche Diagnostics). Cell lysates were incubated with immobilized rabbit anti-laminin-1 IgG or nonimmune rabbit IgG (control), according to the manufacturer's instructions (ProFound Co-IP kit 23600; Pierce Biotechnology). The coprecipitated protein complexes were eluted (total 100 μL), 16 μL of which was solubilized in LDS sample buffer containing DTT and analyzed by Western blot analysis to probe for SPARC, laminin-1, or β-tubulin, as described earlier. Control experiments were conducted with (1) $SP^{-/-}$ lens epithelial cell lysates, (2) nonimmune rabbit IgG control, and (3) quenched amino-link gel controls included in the kit (Pierce Biotechnology).

RESULTS

Expression of ECM Molecules in Lens BM

A schematic of a mammalian mouse lens is shown in Figure 1. Steady state levels of mRNA of major lens BM components were determined by RT-PCR, and mRNAs for collagen IV, nidogens 1 and 2, and laminin-1 did not differ significantly between $SP^{+/+}$ and $SP^{-/-}$ lens epithelia in mice 3 months of age or younger (Fig. 2). Expression of collagen type IV, perlecan, nidogen, and fibronectin proteins in $SP^{+/+}$ and $SP^{-/-}$ lenses were examined by immunohistochemistry (Fig. 3A). Collagen type IV is a major structural protein of the lens capsule, and we have reported its abnormal expression in $SP^{-/-}$ lenses.[16,17] Immunohistochemical staining of collagen type IV revealed a faint spotted appearance in the anterior capsule of this 1-month-old $SP^{-/-}$ lens, possibly as a result of lens cell protrusion into the capsule[17] (Fig. 3A, Col IV, arrowhead). However, the intensity of the collagen

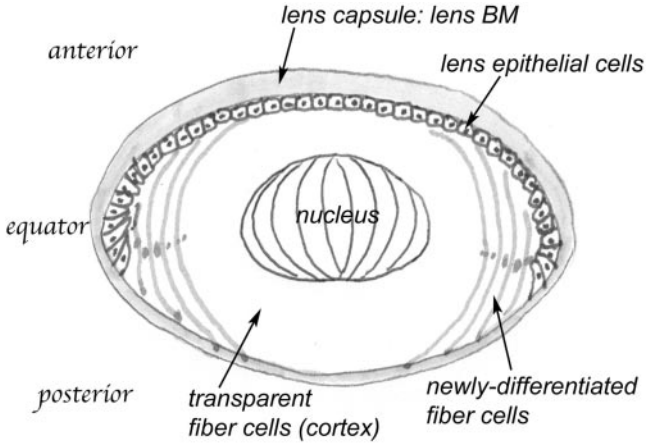

FIGURE 1. The mammalian lens. The lens is completely surrounded by lens capsule (BM). Lens epithelial cells are underneath the anterior capsule, in close contact with it. The newly differentiated fibers (bow region) containing nuclei and organelles can produce ECM proteins in the posterior capsule. The well-differentiated fibers, lacking organelles and nuclei, are transparent and no longer capable of protein synthesis. The ECM proteins in the capsule interact with lens cells to influence their migration, adhesion, polarization, shape, and differentiation (lens components are not drawn to scale).

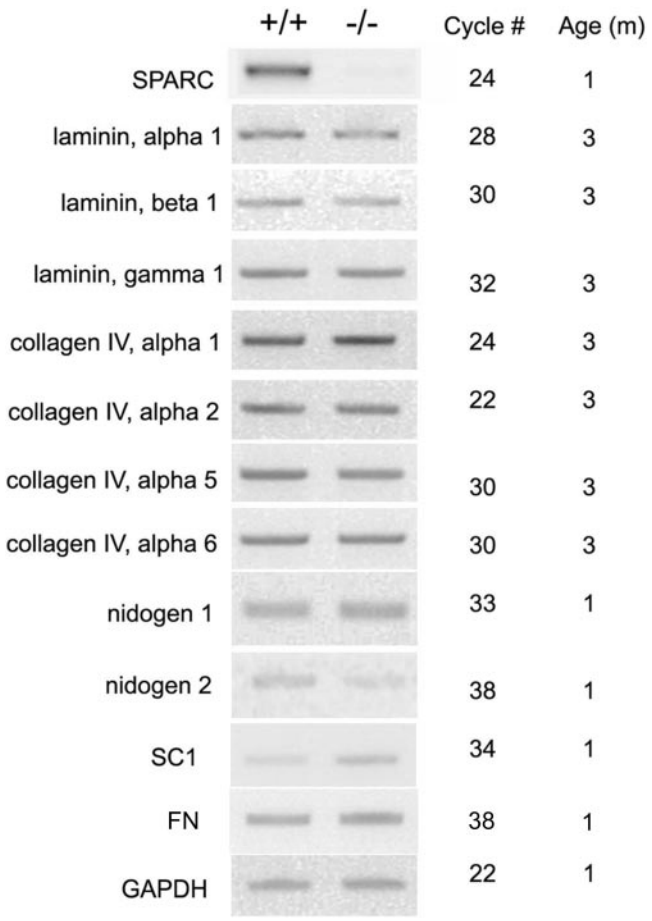

FIGURE 2. RT-PCR of lens BM components in $SP^{+/+}$ and $SP^{-/-}$ mice. RNA was extracted from lens epithelium, and semiquantitative RT-PCR was performed with specific primers for laminin-1 (three chains), collagen IV (four chains), SC1, nidogen 1 and 2, and SPARC, concurrently with GAPDH primers as an internal control. PCR cycle numbers and lens ages are indicated.

IV staining was not significantly different between $SP^{+/+}$ and $SP^{-/-}$ lenses up to 2 to 3 months of age, suggesting that the production of collagen IV by $SP^{-/-}$ lens epithelial cells may not have altered significantly at this age.

Fibronectin has been shown to be a component of embryonic lens capsule, but it is significantly diminished or absent in adult rat lens.[20,21] Although a strong staining was found in lens epithelium and lens capsule in embryos (data not shown), fibronectin was not detected in the lens epithelium (Fig. 3A, FN, arrow) or within the lens capsule (arrowhead) in the adult murine lens. Labeling on the outer surface of the capsule was seen that may have resulted from absorbed components of the aqueous humor. Lens fibers showed nonspecific staining by the IgM (Fig. 3A, FN, double arrows), because the fiber cells exhibited no immunoreactivity with anti-fibronectin IgM by immunoblot analysis (data not shown). One to 2-month-old lens capsules showed essentially no signal for fibronectin (Fig. 3B, FN). These data confirm that the amount of fibronectin in adult mouse lens capsule is significantly diminished. Nidogen-1 was immunostained in the lens capsule, and immunoblot analysis indicated levels that were similar between $SP^{+/+}$ and $SP^{-/-}$ lenses (Fig. 3B, Nid I). Nidogen-2 and tenascin C were not detected by either immunohistochemistry or immunoblot (data not shown). Perlecan was evident in the lens capsule, with a similar labeling intensity between $SP^{+/+}$ and $SP^{-/-}$ lenses (Fig. 3A, Per). SC1 (also termed hevin) is a SPARC family protein that exhibits the highest similarity to SPARC.[22,23] To test the possibility that SC1 could be upregulated to compensate for the absence of SPARC, we examined the abundance of SC1 in the lens. The SC1 mRNA level was low in $SP^{+/+}$, and was slightly higher in $SP^{-/-}$ lenses (Fig. 2). However, SC1 protein was below the level of detection in $SP^{+/+}$ and $SP^{-/-}$ lenses by immunoblot analysis, with two different anti-SC1 antibodies[24] (Table 1; Fig. 3B, SC1).

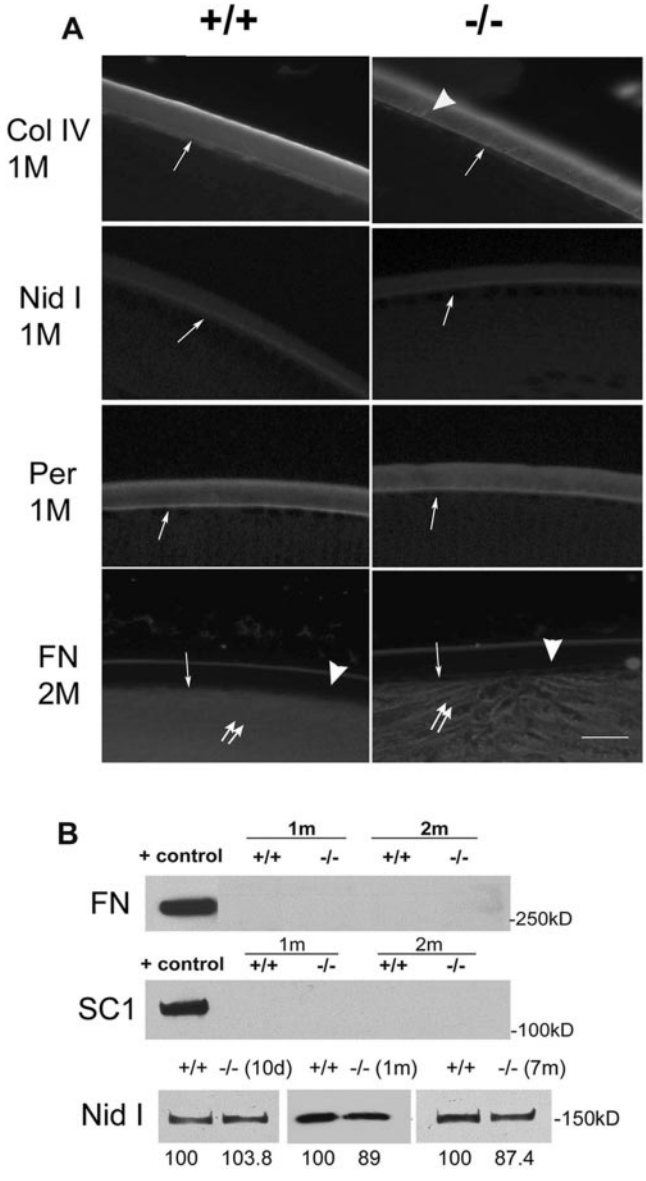

FIGURE 3. Expression of collagen IV, fibronectin, nidogen, perlecan, and SC1 in lens capsules. (**A**) Sections of lenses from 1-month-old mice were exposed to antibodies against collagen IV (Col IV), nidogen-1 (Nid I), and perlecan (Per). Sections of lenses from 2-month-old mice were exposed to anti-fibronectin (FN) IgM. This IgM antibody labeled lens fibers nonspecifically (*double arrows*). Scale bar, 40 μm. (**B**) Lens capsular proteins were immunoblotted with anti-fibronectin IgM (FN), anti-SC1 IgG (SC1), or anti-nidogen I IgG (Nid I). Positive controls for the antibodies included cultured epithelial cell lysate (for FN) or recombinant human SC1 (for SC1). Levels of nidogen I in null capsules representing three age groups are shown with percentage change in comparison to $SP^{+/+}$ samples of the same age (set at 100% for each $SP^{+/+}$ sample).

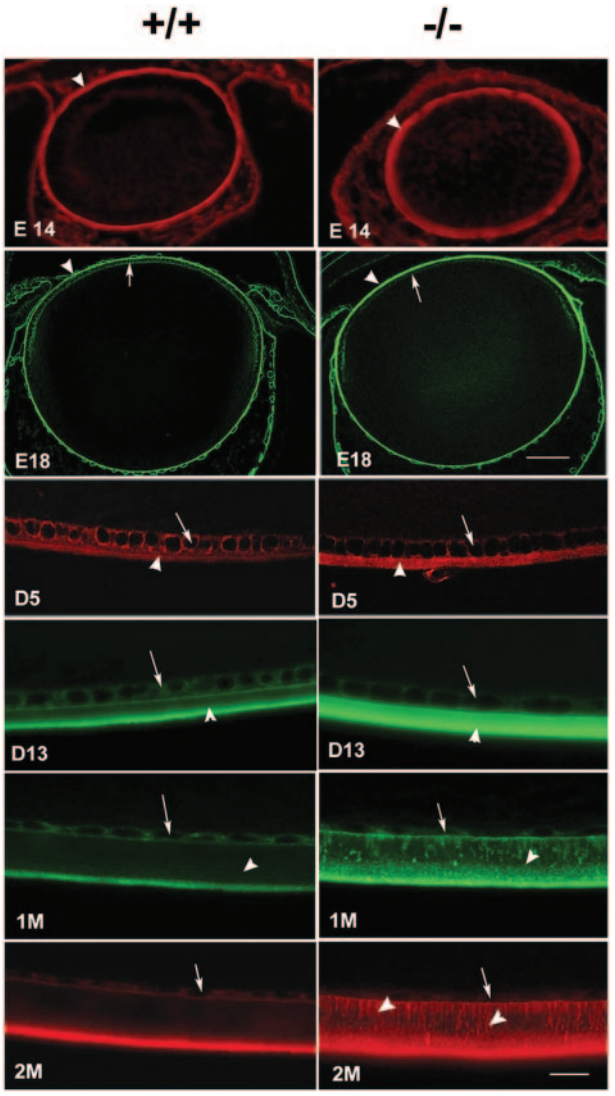

FIGURE 4. Immunofluorescent analysis of laminin-1 in $SP^{+/+}$ and $SP^{-/-}$ lens capsules at the ages indicated. Sections of lenses of various ages were exposed to anti-laminin-1 IgG, followed by secondary antibody conjugated to fluorescein isothiocyanate or Texas red. *Arrows*: lens epithelial cells; *arrowheads*: lens capsules. Scale bar: (E14–E18), 160 μm; (D5–2M) 10 μm.

Increased Soluble Laminin-1 Protein in Lens BM in SPARC-Null Lenses

Among all the lens BM components that we examined in young mice, the distribution of laminin-1 in the lens BM displayed a discrepancy between $SP^{+/+}$ and $SP^{-/-}$ lenses. Expression and localization of laminin-1 were revealed by a polyclonal antibody that recognizes all three chains of laminin-1. Laminin-1 was expressed in E14 to E18 lens capsules, whereas the anterior and equatorial epithelia showed less intensity relative to the capsules (Fig. 4, E14 and E18). At postnatal days 5 and 13, the intensity of laminin-1 staining in $SP^{+/+}$ lens BM was reduced relative to that of the embryonic stages; however, $SP^{-/-}$ lens BM continued to show strong reactivity, whereas the lens epithelium displayed a slightly weaker reactivity than its wild-type counterpart (Fig. 4, D5 and D13). Increased immunostaining for laminin-1 in lens BM, showing an uneven and aggregated appearance, was detected in 1-or 2-month-old $SP^{-/-}$ lenses (Fig. 4, 1M and 2M, arrowheads). However, by 1 to 2 months of age, the null lens cells started to protrude into the lens capsules,[16] which might have increased the antibody's accessibility to the lens capsule and contributed to the abnormal staining at this age.

To further characterize and quantify the expression of capsular laminin-1 between $SP^{+/+}$ and $SP^{-/-}$ lenses, we subjected proteins extracted from the capsules with or without lens epithelium to SDS-PAGE. The extraction buffer has been used previously for efficient extraction of laminin protein.[25] The heterotrimeric chains were revealed by immunoblot analysis with anti-laminin-1 IgG. Laminin-1 from $SP^{-/-}$ capsules of different ages consistently showed slightly higher levels in comparison to their $SP^{+/+}$ counterparts (Figs. 5A, 5B). This differential expression was more pronounced in the soluble fractions of the capsules without attached epithelium (Figs. 5C, 5D). There were no other immunoreactive bands of lower electrophoretic mobility recognized by this polyclonal antibody. In contrast, nidogen 1 exhibited similar levels between $SP^{+/+}$ and $SP^{-/-}$ lens capsules (Fig. 3B), and there was little nidogen detected in the soluble fractions of the capsules (Fig. 5E). We did not observe increased nidogen expression in $SP^{-/-}$ capsules by immunohistochemistry and immunoblot analysis. In summary, soluble laminin-1 exhibited an increased deposition in the $SP^{-/-}$ lens BM by Western blot analysis.

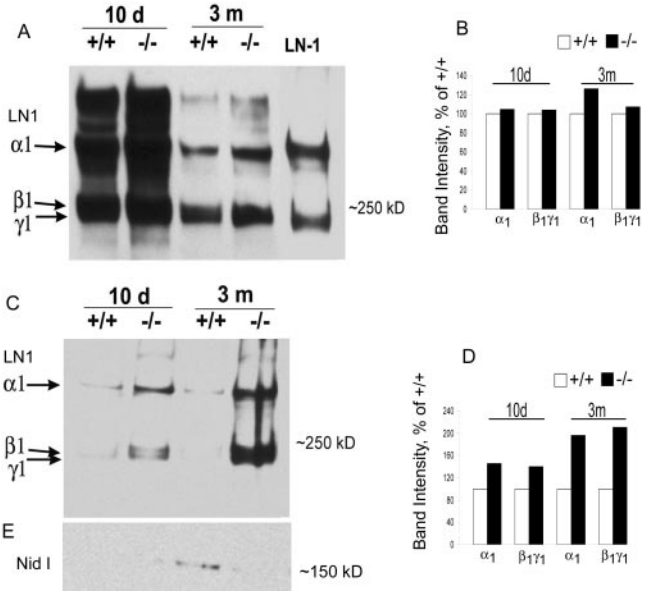

FIGURE 5. Western blot analysis of laminin-1 in $SP^{+/+}$ and $SP^{-/-}$ lens capsules. Proteins extracted from equal numbers of lens capsules of $SP^{+/+}$ and $SP^{-/-}$ lenses were fractionated by electrophoresis on 3% to 8% polyacrylamide gradient gels, followed by immunoblot for the detection of laminin-1. (**A**, **B**) Total lens capsular proteins (with lens epithelium) extracted from the same number of $SP^{+/+}$ or $SP^{-/-}$ lens capsules at 10 days and 3 months. (**C**, **D**) Soluble lens capsular proteins (without lens epithelium), extracted from the same numbers of $SP^{+/+}$ or $SP^{-/-}$ lens capsules, were examined at 10 days and 3 months. The soluble fraction blot was probed with anti-nidogen I IgG (**E**). The data shown are representative of five experiments. The EHS laminin-1 protein was used as a control (LN-1) for the polyclonal antibody. *Arrows*: positions of the α1, β1, and γ1 chains. Loading was controlled by an equal number of capsules between wild-type and null samples at each time point. (**B**, **C**) Data are the results of scanning densitometry of laminin α1 chain and β1γ1 chains with the percentage change in comparison to $SP^{+/+}$ samples of the same age (set at 100% for each $SP^{+/+}$ sample).

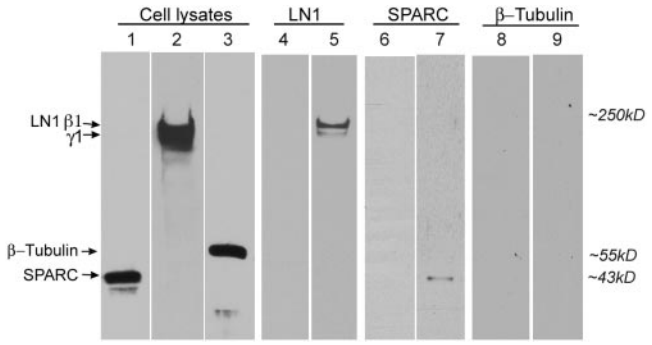

FIGURE 6. Interaction of SPARC and laminin-1 in lens epithelial cells by co-IP. Cell lysates extracted from cultured lens epithelial cells were resolved by reducing SDS-PAGE and were immunoblotted against anti-SPARC IgG (*lane 1*), anti-laminin-1 IgG (*lane 2*), or anti-β tubulin IgG (*lane 3*), respectively. Cell lysates were immunoprecipitated with immobilized rabbit anti-laminin-1 IgG (*lanes 5*, *7*, and *9*) or nonimmune rabbit IgG controls (*lanes 4*, *6*, and *8*). Immunoprecipitated proteins were eluted (total, 100 μL), and 16 μL of eluted proteins was analyzed by Western blot against anti-laminin-1 IgG (LN1), anti-SPARC IgG (SPARC), and anti-β tubulin IgG (β-tubulin).

Intracellular Interaction between SPARC and Laminin-1

How does SPARC regulate the secretion and deposition of laminin-1 in lens epithelial cells? One possibility is that SPARC interacts with laminin-1 before its secretion from the lens epithelial cells. An intracellular protein interaction (by binding to or dissociation from SPARC in the ER or Golgi apparatus) could modulate, at least partially, the amount and/or quality of laminin secreted and deposited into the lens BM. A coimmunoprecipitation (co-IP) assay was thus performed in cultured lens epithelial cells to test this hypothesis. Cell lysates were positive for SPARC (Fig. 6, lane 1), laminin-1 (lane 2), and β-tubulin (lane 3), detected by antibodies against SPARC, laminin-1, and β-tubulin, respectively. Most of the extracellular proteins deposited on the culture dishes were excluded from the cell lysates, because cells were collected after trypsin treatment and washes, as opposed to scraping of the dishes (see the Methods section). The cell lysates were immunoprecipitated with immobilized rabbit anti-laminin-1 IgG or immobilized rabbit IgG controls. The immunoprecipitated immune complexes were collected, and an aliquot was analyzed by Western blot analysis with anti-SPARC IgG. The presence of a 43-kDa reactive band in the laminin-1 immunoprecipitates (lane 7), but not in the nonimmune IgG controls (lane 6) or $SP^{-/-}$ cell lysates controls (data not shown), indicates an association of SPARC with laminin-1 in lens epithelial cells. The immunoprecipitated complexes were also immunoblotted with anti-β-tubulin IgG or anti-GAPDH IgG, and there was no detection of these proteins (Fig. 6; lane 9, GAPDH data not shown), which supports the specific association of SPARC and laminin-1 in lens epithelial cells.

Double immunofluorescence in lens epithelial cells was performed with antibodies against SPARC and laminin-1 (Fig. 7), and both exhibited an abundant cytoplasmic distribution (Figs. 7D, 7E). Double labeling of Hsp47 (an ER marker) or Golgi 58 (a Golgi marker) and SPARC revealed that SPARC was heavily distributed in the Golgi and ER (Fig. 7B), whereas laminin-1 was preferentially stained in ER, relative to the Golgi (Fig. 7C). The abundant distribution of two proteins in the ER further supports the data in Figure 6 that their association occurred before the secretion of these ECM proteins.

Effect of Increased Laminin on Attachment of Lens Epithelial Explants in Culture

We asked whether the increased production of laminin-1 in the capsule affects the attachment and adherence of lens epithelial cells. The attachment of lens explants to the culture dish is permissive for the growth of epithelial cells from the explants. We observed that $SP^{-/-}$ capsules consistently attached to fibronectin-coated dishes earlier than their wild-type counter-

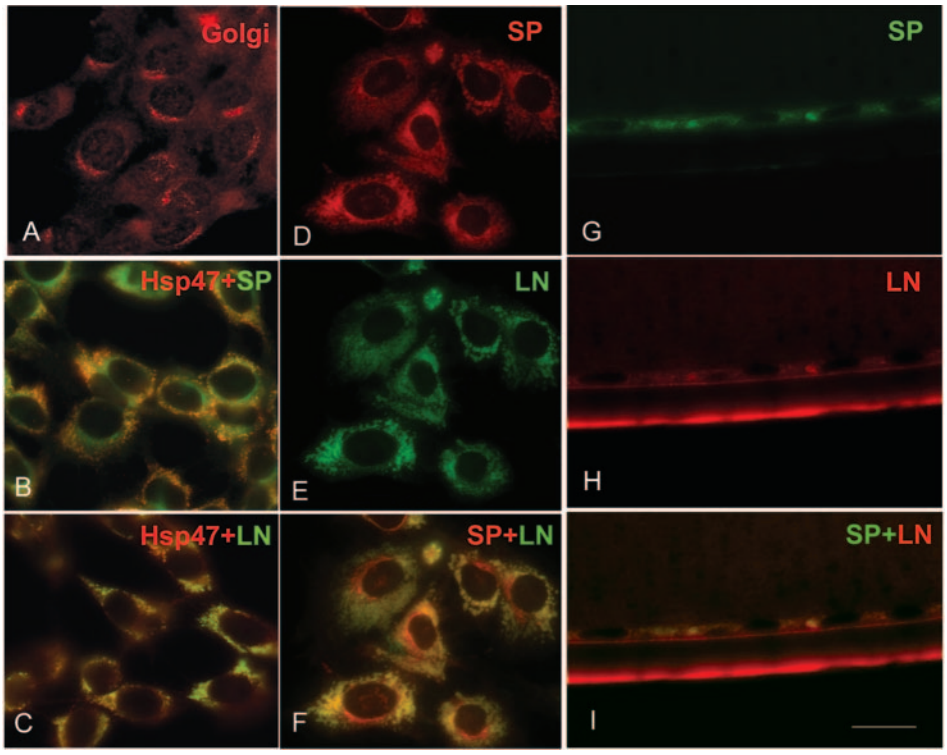

FIGURE 7. Distribution of SPARC and laminin-1 in lens epithelial cells by immunofluorescence. Lens epithelial cells were grown on glass coverslips for immunocytochemistry. The Golgi apparatus showed a perinuclear labeling (**A**). Double immunofluorescent staining of Hsp47 and SPARC (**B**) or of Hsp47 and laminin (**C**) were merged. SPARC was observed in the ER and Golgi, whereas laminin-1 was located predominantly in the ER. Double immunofluorescent staining of SPARC and laminin-1 in cultured lens epithelial cells (**D–F**) or lens sections (**G–I**) is shown. Secondary antibodies were fluorescein isothiocyanate- or rhodamine-conjugated donkey anti-goat IgG (for SPARC) and fluorescein isothiocyanate- or rhodamine-conjugated donkey anti-rabbit IgG (for laminin-1). SPARC labeling coincided with laminin-1 labeling in the ER regions in lens epithelial cells (**F**, **I**, merged, *yellow*). Scale bar, 10 μm.

TABLE 3. Attachment of Lens Epithelial Explants In Vitro

	1 Month Old		2 Months Old	
	+/+	−/−	+/+	−/−
Total capsules (*n*)	20	18	24	27
Attached capsules (4 d)*	6	13	5	18
% attached (4 d)	30	72.2	20.8	66.7

* Cellular outgrowth from the explants after 4 days.

parts (Table 3). Because of their earlier attachment, the area of the outgrowth of the epithelial monolayer was significantly larger from $SP^{-/-}$ lens explants than from that of $SP^{+/+}$ explants after 7 days (Figs. 8A, 8B). When dishes were simultaneously coated with fibronectin and laminin-1 together (see the Methods section), attachment of $SP^{+/+}$ capsular explants was improved significantly (data not shown). Furthermore, the cultured $SP^{-/-}$ cells also demonstrated an enhanced adhesion to the substratum relative to their $SP^{+/+}$ cells by their differential response to trypsin treatment (Figs. 8C, 8D).

DISCUSSION

In this report, we have characterized the expression of ECM components in the BM between $SP^{+/+}$ and $SP^{-/-}$ lenses of E14 to 3-month-old mice. SPARC has been claimed to regulate cell-ECM interaction, as well as the production and the degradation of ECM proteins.[26–29] Altered composition, production, or structure of ECM has also been identified in SPARC-null mice.[30–32] The lens is advantageous for studying epithelial cell-ECM protein synthesis and deposition because the lens epithelium is a cellular monolayer adjacent to the lens BM (Fig. 1). ECM proteins produced by lens epithelial cells are distributed largely in the lens capsule. In addition, adult lens is avascular without innervation, and has no other type of cells. The lens epithelial cells and lens BM perform collaborative functions.

SPARC appears to affect the deposition of laminin-1 in lens epithelium. Increased levels of laminin-1 in $SP^{-/-}$ capsules of various ages were detected by immunohistochemistry and immunoblot analysis. Although the anterior lens capsules at 1 or 2 months of age show minor protrusions of the lens cells,[16,18] the following data indicate that this structural compromise did not contribute significantly to the immunostaining of laminin-1 shown in Figure 4: (1) The protrusion of epithelial cells into the anterior capsule began at approximately 1 month and was minimal. Moreover, increased laminin-1 staining was detected before this age; (2) immunostaining with other antibodies to detect ECM proteins on the same null lens capsules did not increase as did that of laminin-1 (Fig. 3; and data not shown), suggesting that the $SP^{-/-}$ lens capsule may not have increased accessibility to antibodies; and (3) the differential expression of laminin was confirmed in $SP^{+/+}$ and $SP^{-/-}$ capsules by immunoblot analysis. Although matrix proteins, especially the insoluble, complex proteins are difficult to extract from tissues, our biochemical analyses showed that laminin-1 was increased in the EDTA-soluble material derived from $SP^{-/-}$ lens capsules of different ages in comparison to $SP^{+/+}$ samples (Fig. 5). The enhanced production of laminin-1 was also observed in the conditioned media of cultured $SP^{-/-}$ lens epithelial cells (unpublished data). These data are in good agreement with the observation that lens epithelial cells lacking SPARC secrete more laminin-1. The differential release of laminin into the conditioned medium was further observed in our laboratory in $SP^{-/-}$ lens epithelial cells transfected with SPARC cDNA, and this characteristic contributed to a differential adhesion of lens epithelial cells in vitro (Weaver M et al., manuscript submitted). The adhesive protein laminin-1 and the counter-adhesive protein SPARC exert apparently opposite effects on cell adhesion. The enhanced adhesion of $SP^{-/-}$ lens epithelial cells relative to their $SP^{+/+}$ cells could be due to the increased secretion of laminin-1, because inclusion of laminin-1 as a coating of the culture dish diminished the difference in attachment observed in Figures 8A and 8B. SPARC influences lens epithe-

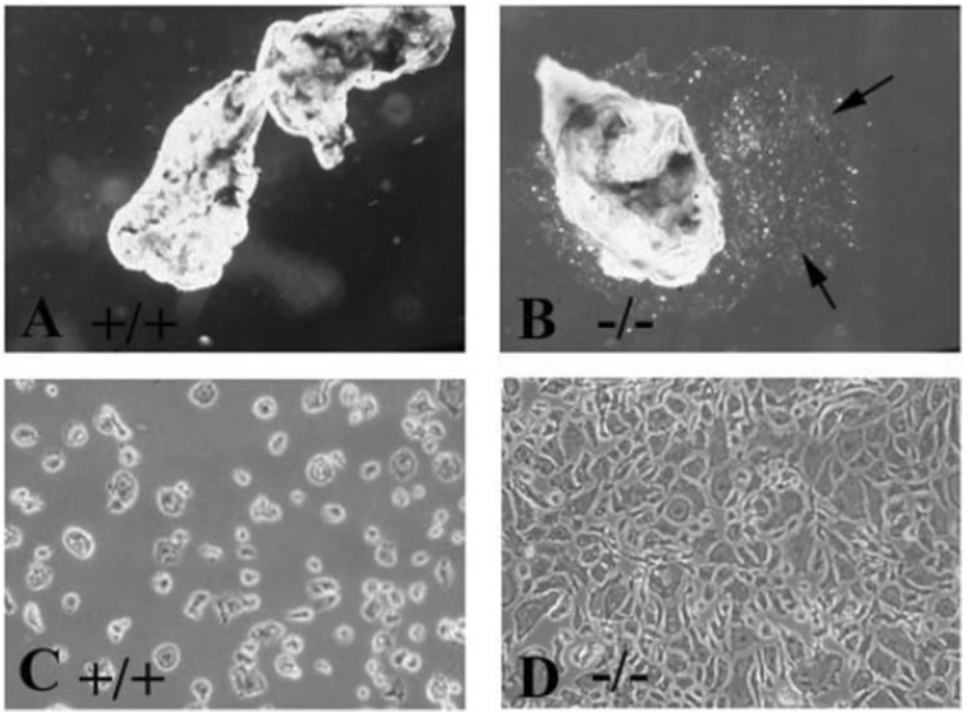

FIGURE 8. $SP^{-/-}$ lens epithelial explants exhibited enhanced attachment in vitro. Lens epithelial capsules from 2-month-old mice were explanted in 15% FBS/DMEM on a fibronectin-coated dish for 7 days (**A**, **B**). $SP^{-/-}$ explants attached to the substrate much earlier than $SP^{+/+}$, and a monolayer of cells was apparent at day 7 (**B**, *arrows*). (**C**, **D**) The primary cells were passed to culture dishes. Shown are passage-3 cells treated with 0.05% trypsin/PBS for 5 minutes at 37°C. The $SP^{+/+}$ cells (**C**) were rounded, whereas $SP^{-/-}$ cells were less responsive to the same treatment.

lial cell adhesion, at least in part, by its regulation of laminin distribution in the lens BM.

To understand how SPARC affects the secretion of laminin-1 in lens epithelium, we investigated their potential interaction by co-IP. Our data demonstrated that SPARC and laminin-1 are associated intracellularly (Figs. 6, 7). The interaction most likely takes place in the ER, and it could be important in controlling the rate and amount, or perhaps the quality, of the laminin-1 secreted into the BM. It has been proposed that *Drosophila* SPARC, and type IV collagen may form heterotypic complexes in the ER, and this interaction appeared to be involved in determining the assembly and production of SPARC in the basal lamina.[33] In addition, SPARC can be induced by stresses such as endotoxin,[34] sparse plating,[34] and heat shock[35,36] in some types of cells. Because almost all heat shock proteins or stress proteins are intracellular, it is possible that SPARC is involved in an intracellular function (e.g., the interaction between SPARC and laminin-1 observed in lens epithelial cells). In addition, there is evidence that SPARC has an intracellular role (e.g., SPARC was found to be translocated into the nuclei of lens epithelial cells under certain conditions),[37] it was associated with microtubule arrays of cilia,[38] and it was identified in the nuclear matrix of urothelial cells[39] and embryonic chicken cells.[40]

The consequence of the increased production of laminin-1 in $SP^{-/-}$ lens capsule could affect lens epithelial cell–ECM interaction; integrin activation; and intracellular signaling, adhesion, polarization, and differentiation, all of which are essential for the maintenance of normal epithelial cell viability and identity and for the continuous formation of normal lens fibers. In addition, the abnormal deposition of laminin-1 could cause disorganization of the ECM proteins in the lens capsule, as the role of laminin-1 in the assembly of BM has been well established.[10,41]

In summary, we have identified a relationship between SPARC and laminin-1 in the mouse lens. Our data emphasize a role for SPARC in the regulation of ECM protein deposition and/or assembly in the lens BM and in the maintenance of the structural integrity of the lens BM.

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