Mice deficient for the secreted glycoprotein SPARC/osteonectin/BM40 develop normally but show severe age-onset cataract formation and disruption of the lens

Darren T.Gilmour[1], Gholson J.Lyon[2], Mark B.L.Carlton, Joshua R.Sanes[3], Jeanette M.Cunningham[3], Janice R.Anderson[4], Brigid L.M.Hogan[5], Martin J.Evans and William H.Colledge[6,7]

Wellcome/CRC Institute of Cancer and Developmental Biology and Department of Genetics, University of Cambridge, Tennis Court Rd, Cambridge CB2 3QR, [4]Department of Histopathology, Addenbrooke's Hospital, Hills Rd, Cambridge CB2 2QQ, [6]Physiological Laboratory, University of Cambridge, Downing St, Cambridge CB2 3EG, UK, [3]Department of Anatomy and Neurobiology, Room 469 McDonnell Bldg, Washington University Medical School, 660 South Euclid Ave, St. Louis, MO 63110, and [5]Howard Hughes Medical Institute and Vanderbilt University Medical Center, Nashville, TN 37232, USA

[1]Present address: Max Planck Institut für Entwicklungsbiologie, Spemannstrasse 35/III, Tübingen D-72076, Germany
[2]Present address: Cornell/Rockefeller/Memorial Sloan-Kettering Tri-Institutional MD/PhD Program, New York, NY 10021, USA

[7]Corresponding author
e-mail whc23@cus.cam.ac.uk

SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin/BM40) is a secreted Ca^{2+}-binding glycoprotein that interacts with a range of extracellular matrix molecules, including collagen IV. It is widely expressed during embryogenesis, and *in vitro* studies have suggested roles in the regulation of cell adhesion and proliferation, and in the modulation of cytokine activity. In order to analyse the function of this protein *in vivo*, the endogenous *Sparc* locus was disrupted by homologous recombination in murine embryonic stem cells. SPARC-deficient mice (*Sparc^{tm1Cam}*) appear normal and fertile until around 6 months of age, when they develop severe eye pathology characterized by cataract formation and rupture of the lens capsule. The first sign of lens pathology occurs in the equatorial bow region where vacuoles gradually form within differentiating epithelial cells and fibre cells. The lens capsule, however, shows no qualitative changes in the major basal lamina proteins laminin, collagen IV, perlecan or entactin. These mice are an excellent resource for further studies on how SPARC affects cell behaviour *in vivo*.

Keywords: BM40/cataract/gene targeting/osteonectin/SPARC

Introduction

SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin/BM40) is a secreted Ca^{2+}-binding glycoprotein (M_r 43 kDa) which can interact with several extracellular matrix (ECM) molecules and cytokines (for review, see Lane and Sage, 1994). Ca^{2+} is bound by two regions within the protein; an acidic N-terminal domain and a C-terminal domain containing two EF hand motifs (Hohenester *et al.*, 1996). The protein was first purified as a major non-collagenous component of bovine bone, with binding affinity to collagen I (Termine *et al.*, 1981), and was cloned independently as a major product of mouse parietal endoderm cells (Mason *et al.*, 1986a,b). SPARC is widely expressed in many different tissues and cell types in both embryonic and adult mice, often at high levels. The high sequence conservation between species, with mammalian (Termine *et al.*, 1981; Mason *et al.*, 1986b), amphibian (Damjanovski *et al.*, 1992) and avian (Iruela-Arispe *et al.*, 1995) SPARC showing >70% amino acid identity, suggests that the protein has an important physiological role. SPARC expression appears to be restricted to sites of high cell proliferation, matrix remodelling and epithelial–mesenchymal interactions, suggesting a role in these processes (Holland *et al.*, 1987; Howe *et al.*, 1988; Sage *et al.*, 1989a; Tremble *et al.*, 1993). Indeed, a variety of *in vitro* studies have shown that secretion of SPARC, or specific structural domains of the protein, affects cell morphology by reducing the number of focal contacts and lowering adhesion to the substratum and to neighbouring cells (Sage *et al.*, 1984; Lane and Sage, 1990). This type of 'anti-adhesive' function has also been described for the secreted proteins tenascin and thrombospondin, and purified SPARC protein binds to many ECM components such as thrombospondin and collagens I and IV (Sage *et al.*, 1989b).

Sparc expression is up-regulated in transformed cells (Mason *et al.*, 1986b), and increased expression is also associated with the neoplastic progression of several human tumour types (Bellahcene and Castronovo, 1995; Porter *et al.*, 1995). Moreover, a recent study shows that suppression of *Sparc* expression using antisense RNA significantly decreases the tumorigenicity of human melanoma cells by reducing their invasive and adhesive properties (Ledda *et al.*, 1997). In addition to its anti-adhesive effect, SPARC may also have a role in modulating polypeptide signalling. SPARC contains a follistatin-related structural motif which is thought to be involved in growth factor binding (Patthy and Nickolics, 1993). In addition, *in vitro* studies have shown that SPARC binds to specific dimeric forms of platelet-derived growth factor (PDGF) and prevents them from interacting with their receptors (Raines *et al.*, 1992). Since SPARC and the PDGF-B chain are co-expressed in both platelet α granules and at sites of arterial injury, SPARC may regulate the activity of at least one cytokine *in vivo*.

To examine the role of SPARC in embryonic development, we have inactivated the murine *Sparc* locus by homologous recombination in embryonic stem (ES) cells. Although previous reports have suggested an important role for SPARC homologues or SPARC-related proteins in the embryonic development of other species (Purcell
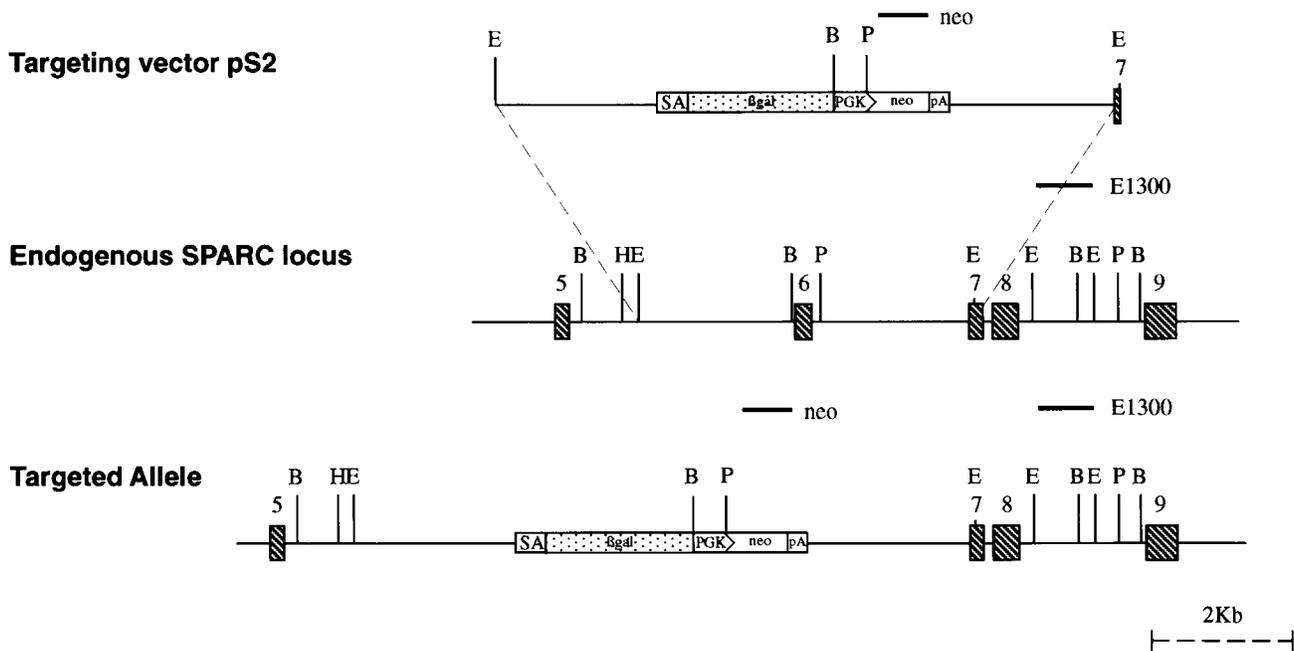
Fig. 1. *Sparc* targeting strategy. The top line shows the targeting vector pS2. The marker SA β-gal/PGK*neo* is shown as a box, where the β-gal coding sequence is stippled and the PGK*neo* marker cassette and splice acceptor sequences are unfilled. The middle line shows the region of the wild-type *Sparc* gene covering exons 5–9 (striped boxes). Finally, the bottom line shows the disrupted *Sparc* allele resulting from targeted integration of vector pS2. The positions of the probes used to verify targeting are indicated by thick horizontal bars. Targeting leads to the replacement of exon 6 with the disruptive marker and a concomitant size increase in the *Bam*HI and *Pst*I fragments which hybridize to the E1300 external probe. Restriction enzyme sites: B, *Bam*HI; P, *Pst*I; E, *Eco*RI; H, *Hin*dIII.

et al., 1993; Schwarzbauer and Spencer, 1993), SPARC-deficient mice (*Sparc^{tm1Cam}*) develop normally, are fertile and initially are indistinguishable from wild-type littermates. Histological analysis of mice older than 6 months of age, however, reveals abnormal vacuolation of lens fibre cells, followed by extensive disorganization of the lens structure including posterior migration of epithelial cells and possibly delayed epithelial cell differentiation into lens fibres. These changes are accompanied by late stage posterior rupture of the lens capsule. This suggests that SPARC plays an important role in maintaining lens homeostasis and capsule integrity, which may account for the high degree of evolutionary conservation of this protein among vertebrates.

Results

Generation of SPARC-deficient mice

The approach used to disrupt the *Sparc* gene is outlined in Figure 1. Homologous recombination with the targeting vector pS2 results in the replacement of exon 6 with a splice accepting marker, SA β-*gal*, and a neomycin resistance cassette (Friedrich and Soriano, 1991). This event deletes half of the follistatin module (Patthy and Nickolics, 1993) and causes premature termination of transcription and translation, resulting in the removal of downstream domains which are known to be required for proper folding and secretion of the protein (Pottgiesser *et al.*, 1994). Targeted ES clones were identified using a 3′ probe external to the targeting vector (Figure 2A), and targeting was confirmed by hybridization to an internal *neo* probe. Chimaeras derived from targeted ES cells transmitted the disrupted *Sparc* allele through the germline.

SPARC is not essential for normal development

Heterozygous *Sparc^{tm1Cam}* mice appeared normal, and intercrossing of these animals produced viable homozygous offspring (Figure 2B). Statistical analysis of 95 offspring indicated no significant deviation from the expected Mendelian ratio of genotypes (20 +/+, 54 +/−, 21 −/−). Southern analysis of DNA from these animals with an exon 6 probe, as well as with the external E1300 probe and internal *neo* probe, confirmed that exon 6 had been replaced by the targeting vector (Figure 2C). In addition, an exon 6-specific PCR designed for genotyping *Sparc^{tm1Cam}* mutant mice consistently gave a PCR product for heterozygous and wild-type mice, but never for *Sparc^{tm1Cam}* homozygous null mice (data not shown). This confirms that exon 6 is deleted from the genome of *Sparc^{tm1Cam}* mutant mice.

To prove that transcription of the *Sparc* gene was disrupted in the homozygous mutant animals, RNA was made from 15 d.p.c. embryos of all three genotypes, and these samples were analysed by Northern blotting (Figure 3A). Probing with cDNA fragments representing sequences downstream (probe EP290) and upstream (probe C33i) of the replacement site showed that although there are high levels of *Sparc* transcription in the wild-type and heterozygous embryos, no *Sparc* mRNA could be detected in homozygous mutant embryos. The absence of a truncated transcript upstream of the site of marker integration was confirmed by probing the RNA samples with the complete *Sparc* cDNA sequence (data not shown). It is likely that the fusion transcript resulting from insertion of the marker construct is unstable. In addition, Western blot analysis of protein from the testes of *Sparc^{tm1Cam}* mutant mice confirmed that no SPARC is produced (Figure 3B).
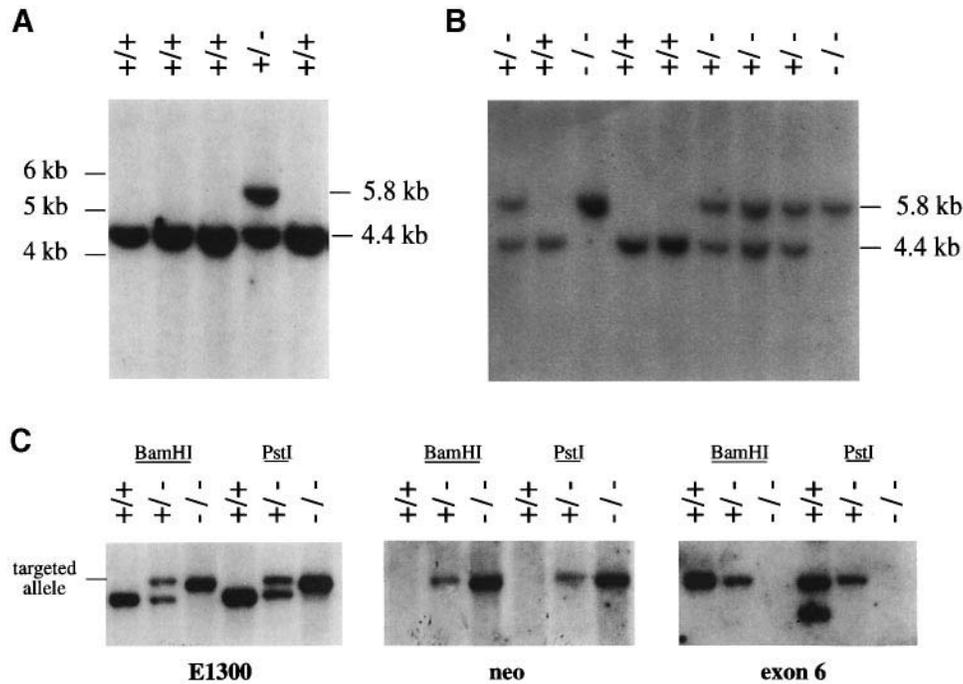
Fig. 2. Generation of SPARC-deficient mice. (**A**) Identification of ES cells carrying a mutated *Sparc* allele. ES cell DNA was digested with *Bam*HI and probed with the external fragment E1300. Shown here is an example of an ES cell clone revealing the fragment size increase characteristic of a targeting event. (**B**) Southern blot of tail DNA from 2-week-old offspring derived from the crosses of *Sparc^tm1Cam* heterozygous mice (+/–). Genomic DNA was digested with *Bam*HI and probed with the external fragment E1300. This blot shows the presence of viable pups which are homozygous for the targeted *Sparc* allele (–/–). (**C**) Confirmation that exon 6 has been deleted in the homozygous mutants. The left hand blot shows tail DNA from wild-type (+/+), heterozygous (+/–) and homozygous (–/–) offspring digested with *Bam*HI or *Pst*I and probed with E1300. This blot was stripped and probed with marker-specific probe *neo* (middle panel). As expected, this gives a band only in the heterozygous and homozygous lanes, the latter being more intense. Finally, probing of this blot with the *Sparc* exon 6 gives a strong band in the wild-type lane, a band of roughly half this intensity in heterozygotes and a complete lack of the band in homozygotes, proving deletion of this exon in the mutant mice. The presence of the second band when the wild-type *Pst*I digest is probed with exon 6 was shown to be due a restriction fragment length polymorphism between the strains of mice used to generate these animals (129/SvEv and MF1).

Although SPARC has been implicated in the morphogenesis of many tissues and organs such as bone, muscle, blood vessels, skin, kidney, testes, ovary and heart (Holland *et al.*, 1987; Sage *et al.*, 1989a; Engelmann, 1993; Nakase *et al.*, 1994), histological examination of these tissues from adult mutant animals did not reveal obvious defects (not shown). In addition, alizarin red staining of bone preparations from adults showed that the cranial, axial and appendicular skeletons of mutant animals were indistinguishable from those of non-mutant littermates (Figure 4). *Sparc^tm1Cam* mutant males are fully fertile, even though SPARC is secreted by Leydig and Sertoli cells in the adult mouse testis (Vernon and Sage, 1989).

SPARC-deficient mice develop cataracts

Beginning around 6 months of age, many of the *Sparc^tm1Cam* homozygous mice on a mixed genetic background (129/SvEv×MF1) began to develop visible cataracts. This was first observed in albino mice where it was easier to see the initial eye opacity characteristic of cataract formation (Figure 5A). Initial histological analysis revealed that the lenses from *Sparc^tm1Cam* mutant mice had undergone major architectural disorganization to form sub-capsular cataracts (Figure 5B). The majority of the SPARC-deficient mice began to develop cataracts after 6 months of age. These results are summarized in Table I. A total of 75 *Sparc^tm1Cam* null mice, ranging in age from 3 to 18 months, were analysed either histologically or by visual

inspection post-mortem after pupil dilation. Of 51 *Sparc^tm1Cam* null mice aged 10–20 months, all showed severe bilateral cataracts. In the age range of 6–9 months, 17/18 *Sparc^tm1Cam* null mice had developed more subtle but clear bilateral cataracts (usually without posterior lens rupture but including extensive lens fibre vacuolation). Mutant animals <6 months old showed lens structures indistinguishable from wild-type mice. In contrast, out of a total of 30 heterozygous or wild-type littermates or age-matched controls of similar genetic background, ranging from 2 to 18 months of age, none showed bilateral cataract development.

The gross appearance of the eyes from 1-year-old albino wild-type and *Sparc^tm1Cam* mutant mice is shown in Figure 5A. The opacity in the eyes of *Sparc^tm1Cam* mutant mice was obvious by visual inspection, especially post-mortem after pupil dilation. Histological analysis of eyes from wild-type (Figure 5B, panels a, c and e) and *Sparc^tm1Cam* mutant mice (Figure 5B, panels b, d and f) showed a dramatic difference in lens structure due to the development of a sub-capsular cataract with subsequent posterior rupture of the lens in mutant mice. In Figure 5B, panel c, the normal equatorial bow of epithelial cells migrating inward away from the capsule region is shown in a wild-type mouse lens. In Figure 5B, panel d, the bow region from a *Sparc^tm1Cam* mutant mouse shows severe disorganization, with cell vacuolation and a lack of coherent bowing of epithelial cells. In Figure 5B, panels e and f, the posterior
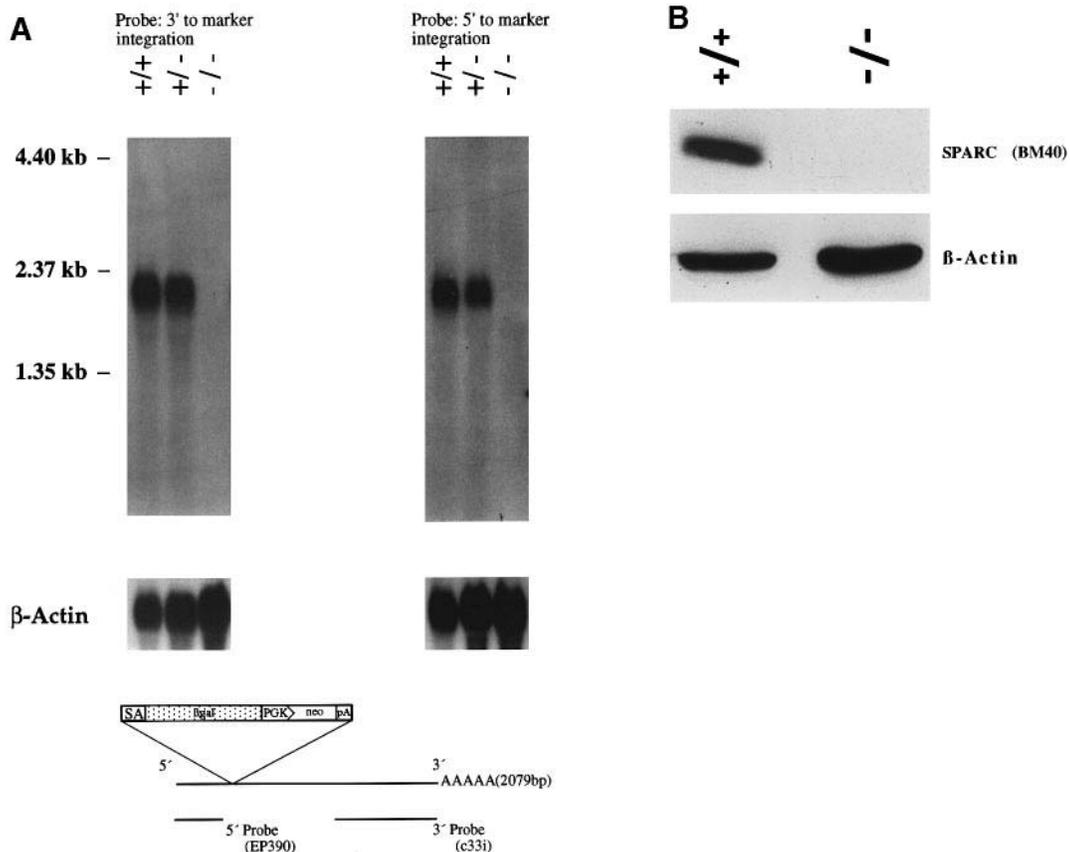
Fig. 3. Confirmation of null phenotype by Northern and Western blotting. (**A**) Northern analysis confirming the absence of *Sparc* mRNA. Total embryonic RNA from 15 d.p.c. embryos of all three genotypes was analysed using probes 5′ (EP390) or 3′ (c33i) to the insertion point. Blots were re-probed with β-actin to confirm equal RNA loadings between lanes. (**B**) Western analysis confirming the absence of SPARC protein. SPARC protein was detected in the testes of wild-type (+/+) but not mutant animals (−/−) using a purified polyclonal rabbit antibody (1064 +E). Equivalent protein loading in each lane was confirmed by visualization with a monoclonal β-actin antibody.

region of wild-type and *Sparc^tm1Cam* mutant mouse lenses are shown. In the mature wild-type lens, there are no epithelial cells present in this region. However, this is not the case in the *Sparc^tm1Cam* mutant mouse lens, as this region is infiltrated with epithelial cells (arrowed, Figure 5B, panel f).

The lenses from one *Sparc^tm1Cam* mutant mouse at 18 months with different cataract severity in each eye were examined to gain an insight into the time course of cataract formation (Figure 6). In the least severely affected eye, there was extensive vacuolation both in differentiating epithelial cells and in lens fibre cells (Figure 6a and b). The normal arrangement of epithelial cells at the equatorial bow region was disrupted by this vacuolation (Figure 6a). In the more severely affected eye, vacuolation and degeneration of lens fibres along with rupture of the basement membrane was seen (Figure 6c, arrowed). This eye also showed severe disruption of the retinal layer, which is secondary to the dramatic disorganization and deterioration of the lens (Figure 6d, arrowed).

Electron microscopy studies revealed striking abnormalities in the posterior and equatorial regions of lenses from *Sparc^tm1Cam* mutant mice (Figure 7). Abnormal protrusions of electron-dense material, possibly cellular debris, in the posterior capsule of *Sparc^tm1Cam* mutant mice were observed (Figure 7b). In addition, the normal concentric arrangement of the lens fibres was often dis-

organized close to the capsule region (not shown). In the anterior part of the lens, epithelial cells showed abnormal rounding (Figure 7d, arrowed) compared with their usual elongated appearance (Figure 7c, arrowed). Vacuolation was particularly pronounced in the bow region (Figure 7f), and this region was also characterized by extensive cellular debris (Figure 7e), perhaps from ruptured lens cells.

No major qualitative changes are found in capsular extracellular matrix proteins

To understand the mechanism of lens degeneration in SPARC-deficient mice, capsules were assessed for changes in the deposition and expression of major ECM proteins by immunohistochemistry (Figure 8). No qualitative differences were found between *Sparc^tm1Cam* mutant and wild-type mice using polyclonal antibodies to laminin 1 or collagen α1,2(IV), or monoclonal antibodies to perlecan or entactin. These are the major components of basal laminae throughout the body. In addition, no differences were observed between mutant and control animals for these ECM components in other basal laminae in the eye (cornea, retina, extra-ocular muscle, Figure 8f and l and data not shown). Further studies with antibodies for laminin subunits also failed to reveal differences: both control and mutant lens capsules were rich in the α1, α5,
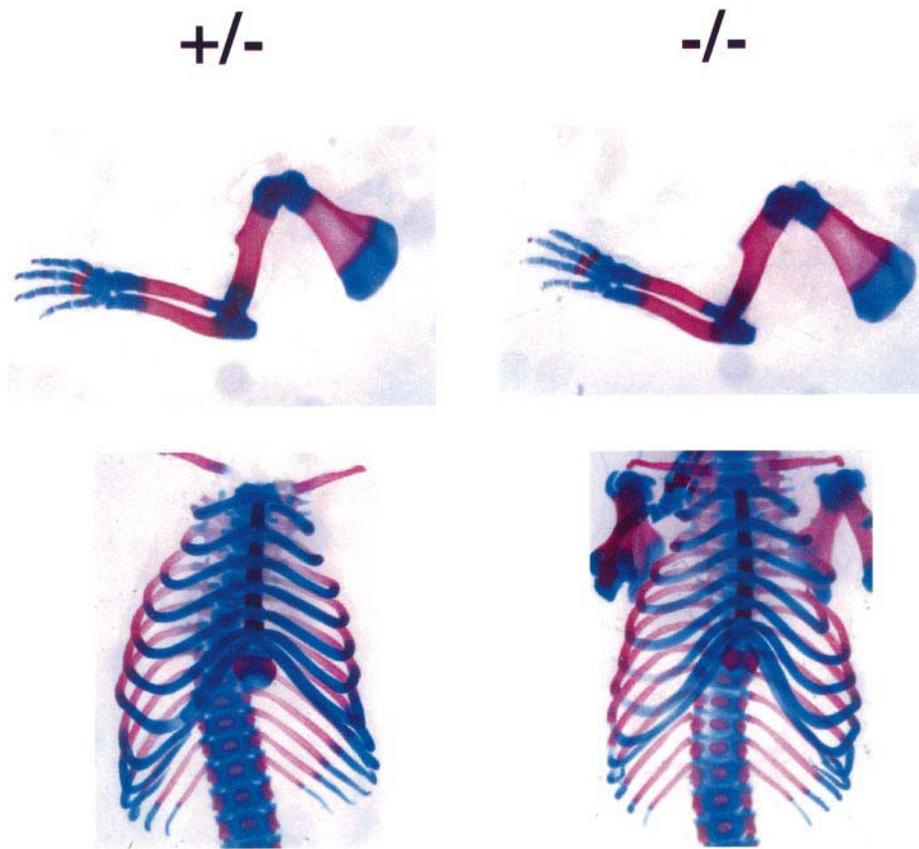
Fig. 4. Skeletal analysis. Alizarin red and Alcian blue staining of heterozygous (+/–) and *Sparc^{tm1Cam}* mutant (–/–) mice showing no obvious differences in skeletal structure.

β1 and γ1 chains, and contained low but detectable levels of α2 and β2 (data not shown).

Discussion

### Abnormal lens morphology in mutant mice

We have shown that SPARC-deficient mice appear normal and fertile until around 6 months of age, when they develop severe eye pathology characterized by cataract formation and rupture of the lens. Prior to this age, lenses appear histologically normal, suggesting that the initial morphogenesis of the lens and the organization of the epithelial cells in relation to the capsule are not compromised. The first distinguishable signs of lens pathology are observed around 6 months of age in the equatorial bow region of the eye, where vacuoles gradually form within differentiating epithelial cells and fibre cells. This vacuolation process might reflect abnormal differentiation of sub-capsular epithelial cells. In wild-type mice, anterior lens epithelial cells differentiate into secondary fibre cells at the equatorial bow region, losing their nuclei during this process. In the *Sparc^{tm1Cam}* mutant mice, this differentiation process is abnormal, with the nuclei appearing more rounded and the cells in the bow region more disorganized. Interestingly, quail retina protein (QR1), which has strong C-terminal homology to SPARC, has been implicated in regulating cell differentiation pathways (Casado *et al.*, 1996). Eventually, rupture of the lens capsule occurs which has possibly been weakened by proteases released from degenerating lens fibre cells.

The precise mechanism by which loss of SPARC results in rupture of the lens is not clear. SPARC/BM40 has been detected in the mouse and bovine lens capsule (Dziadek *et al.*, 1986; Sawhney, 1995) and is secreted by bovine lens epithelial cells (Sawhney, 1995). The level of SPARC protein in the mouse lens capsule is only ~2.5 pmol/mg protein, making it extremely difficult to detect by indirect immunofluorescence (Dziadek *et al.*, 1986). SPARC binds to type IV collagen which is a major structural component of the lens capsule (Iwata *et al.*, 1995). It has been suggested that SPARC, through its binding to type IV collagen, may play an important role in the assembly and/or stabilization of the ECM. Although we found no qualitative changes in collagen α1,2(IV) or other ECM proteins in mutant lens capsules, it is still possible that the absence of SPARC can weaken the capsule sufficiently to contribute to its eventual rupture.

Absence of a major developmental phenotype in homozygous mutants

The phenotype of the mutant mice was somewhat unexpected based on the widespread expression pattern of SPARC during mouse embryonic development and in adult tissues (Holland *et al.*, 1987; Howe *et al.*, 1988; Sage *et al.*, 1989a; for a review, see Lane and Sage 1994). Alizarin red-stained skeletons from mutant embryos and adults were indistinguishable from wild-type, even though SPARC can account for up to 3% of total bone protein (Termine *et al.*, 1984). It is possible that subtle phenotypic differences will be identified following physiological perturbations.
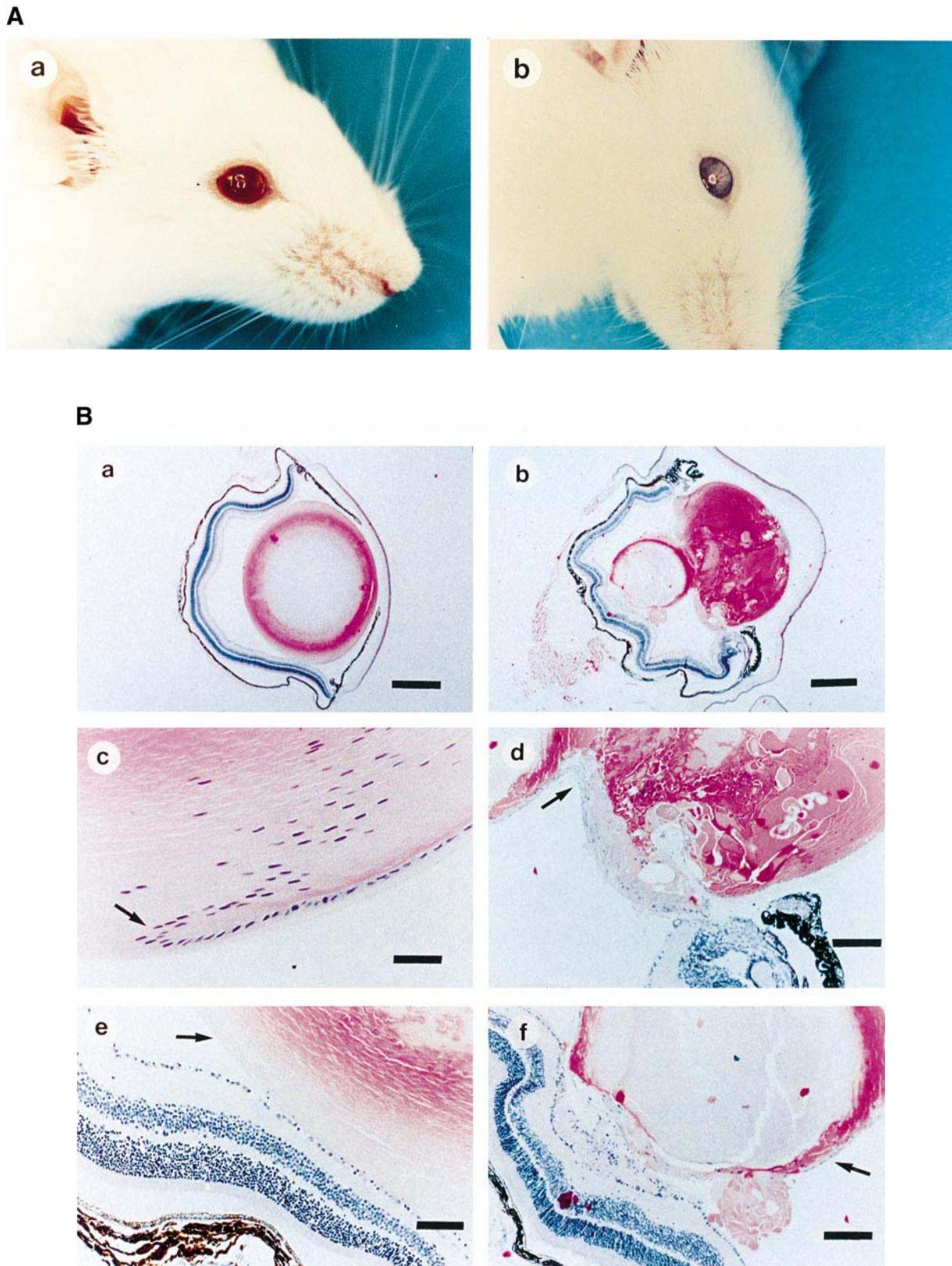
Fig. 5. Development of cataracts in *Sparc^tm1Cam* mutant mice. (**A**) Gross appearance of lens cataract. (a) The lens of a wild-type MF1 mouse at 1 year of age. (b) The lens of a *Sparc^tm1Cam* mutant mouse also at 1 year of age with opacity of the lens. (**B**) Histological appearance of the lens in mutant mice. Sections at increasing magnification of lenses from wild-type MF1 (a, c and e) and *Sparc^tm1Cam* mutant mice (b, d and f), both at age 10.5 months. (a) Wild-type mouse lens, scale bar = 600 μm. (b) *Sparc^tm1Cam* mutant mouse lens also with an obvious sub-capsular cataract. Scale bar = 600 μm. (c) Wild-type mouse lens at higher magnification showing the equatorial bow of epithelial cells differentiating into fibre cells. Scale bar = 75 μm. (d) Similar region from a *Sparc^tm1Cam* mutant mouse, with vacuolation and lack of a coherent bowing of epithelial cells (arrowed). Scale bar = 150 μm. (e) Posterior region of a wild-type lens, demonstrating that there are normally no epithelial cells present in this region. Scale bar = 75 μm. (f) Similar region from a *Sparc^tm1Cam* mutant mouse, showing epithelial cells in the posterior region (arrowed). Scale bar = 150 μm.

Thus, studies to assess whether wound healing or bone strength and fracture repair are affected in *Sparc*^{tm1Cam} mutant mice are underway. Perhaps SPARC plays a role in protection against infection, for example enhancing cellular responses to pathological stimuli. Alternatively, it may be that SPARC does not serve a major role during embryogenesis but its sequence has been conserved during

evolution due to a selective advantage conferred upon the fitness and survival of the adult. This possibility is strengthened by the eye phenotype observed in the SPARC-deficient mice, which would be a serious impediment to survival in the wild, and would be strongly selected against, although this would not explain why *Sparc* expression is widely observed during embryogenesis in many different phyla.

Alternatively, it is possible that the lack of a lethal phenotype in *Sparc*^{tm1Cam} mutant animals is due to functional rescue by a related protein or proteins. The concept of functional redundancy implies that different proteins have overlapping expression patterns and share similar functional domains. The C-terminus of SPARC, which contains the Ca^{2+}-binding EC domain (Maurer *et al.*, 1995), is also present in at least four other extracellular proteins which also contain a nearby follistatin-like (FS) domain. These proteins include SC1 (Johnston *et al.*, 1990; McKinnon *et al.*, 1996), the human testicular proteoglycan TESTICAN (Alliel *et al.*, 1993), transforming growth factor-β-induced protein TSC36 (Shibanuma *et al.*, 1993) and QR1 (Casado *et al.*, 1996). Preliminary data also indicate the existence of at least three additional members of this family (C.Vannahme and P.Maurer, personal communication). SC1 is a good

Table I. Age of onset of cataract formation

Age (months)	No. of mice with cataracts of each genotype		
	+/+	+/–	–/–
10–20	0/5*	ND	51/51$^{\alpha}$
6–9	0/7	0/5	17/18$^{\beta}$
	0/2*		
<6	0/8	0/3	0/6

All mice of similar age were littermate controls apart from the group marked * which consisted of age-matched controls of similar genetic background. Mice in the α group showed the most dramatic lens degeneration that was visually obvious and/or histologically determined, while mice in the β group showed more subtle but clear bilateral cataract formation as revealed by histological analysis. All other mice had normal histology that could not be distinguished from wild-type. No sex-related differences were observed.
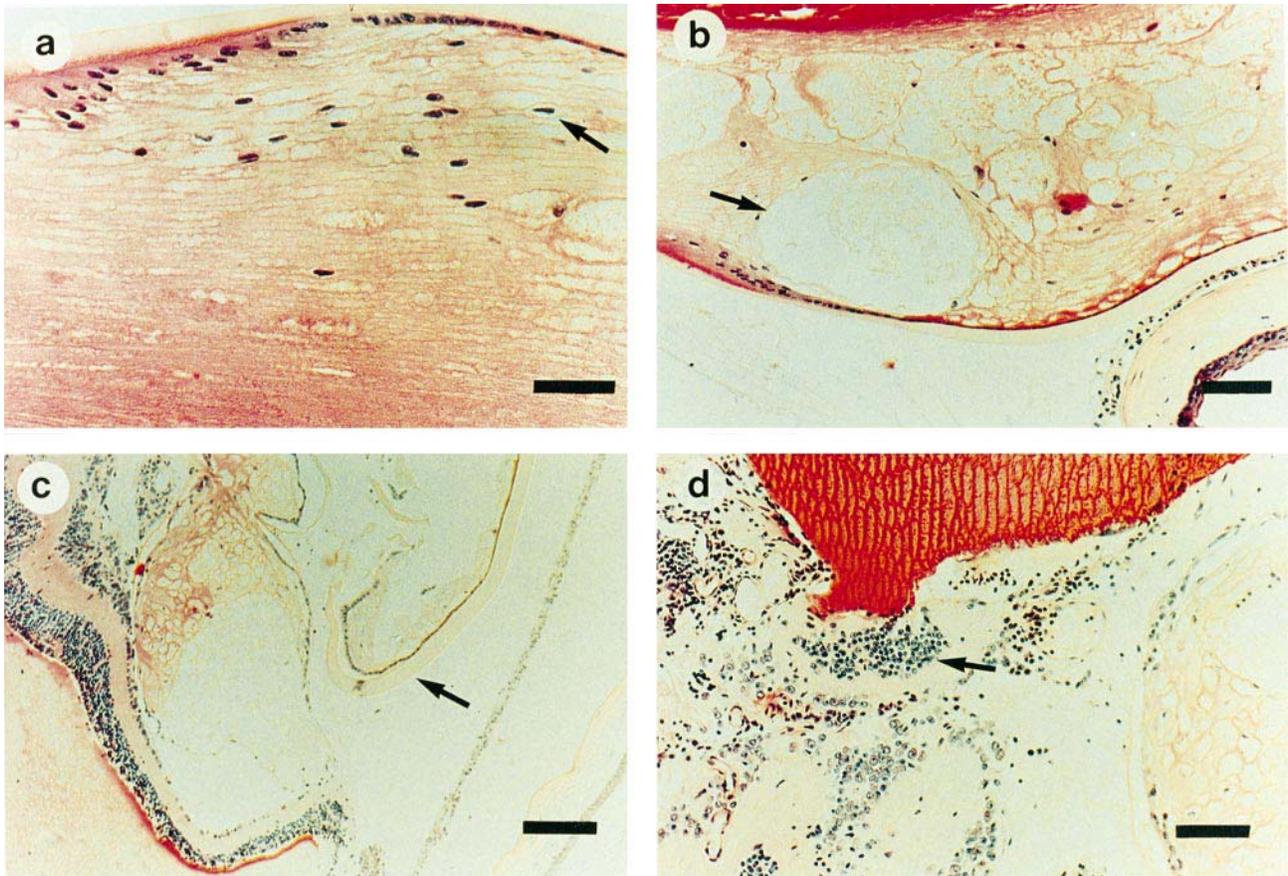

Fig. 6. Progression of cataract formation. Both lenses from a *Sparc*^{tm1Cam} mutant mouse at 18 months of age with differing severity of cataract development in each eye. (**a**) Lens with moderate signs of cataract development showing the initiation of vacuolation. The arrow points to intracellular vacuoles forming within a differentiating epithelial cell in the equatorial region. Scale bar = 37.5 μm. (**b**) A different section of the same lens showing more extensive vacuolation (arrowed). Apart from this portion of the lens, there were no other signs of cataract formation. Scale bar = 75 μm. (**c**) The other lens from the same mouse shows much more dramatic vacuolation and posterior rupture of the lens capsule (arrowed). Scale bar = 150 μm. (**d**) The posterior region of the same lens as in (c) is shown along with the retinal layer. The distortion of the retina (arrow) is secondary to the massive disruption of lens structure. Scale bar = 75 μm.
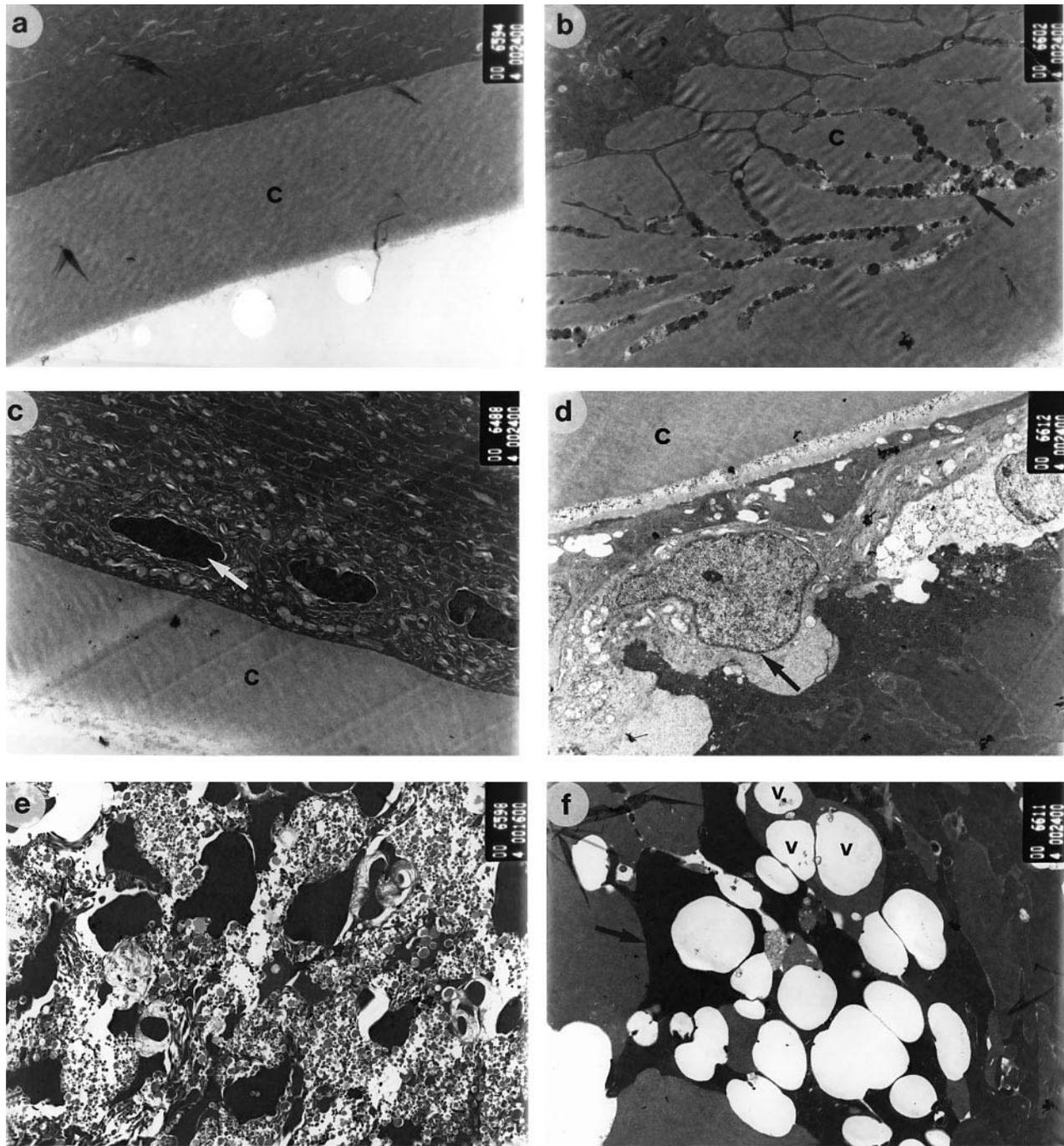
Fig. 7. Electron micrographs of the lens capsule in *Sparc^tm1Cam* mutant mice. Electron microscopy studies of lenses from wild-type (a and c) and *Sparc^tm1Cam* mutant mice (b, d, e and f) revealed striking differences. (**a**) The posterior capsule region from a 9-month-old MF1 wild-type mouse. (**b**) The posterior capsule region from a 7-month-old mutant mouse showing protrusion of material into the capsule (arrowed). (**c**) The anterior region of a lens from a 5-month-old wild-type littermate showing epithelial cells with typical flattened nuclei (white arrow) adjacent to the capsule. (**d**) The anterior region from an 8-month-old mutant mouse shows epithelial cells with more rounded nuclei (arrowed) and the initiation of vacuole formation. (**e**) The equatorial bow region from a 7-month-old mutant shows extensive cellular debris probably originating from rupture of differentiating epithelial and fibre cells. (**f**) The equatorial bow region from an 8-month-old mutant shows intracellular vacuolation in differentiating epithelial cells that appears to displace the nuclei within these cells (arrowed). All micrographs, 2400× magnification, except (e) 1600×. c = capsule; v = vacuole.

candidate to compensate for loss of SPARC since it is co-expressed in a range of tissues (Girard and Springer, 1996; McKinnon *et al.*, 1996) and has an identical intron–exon boundary for the EC- and FS-coding domains, indicating that the two genes may be derived from a common ancestor

(McKinnon *et al.*, 1996). Mice carrying disruptions in both the *Sparc* and *Sc1* genes are being generated to assess the possibility of functional overlap. Functional rescue may also account for the lack of a developmental phenotype in mice with null mutations in other ECM
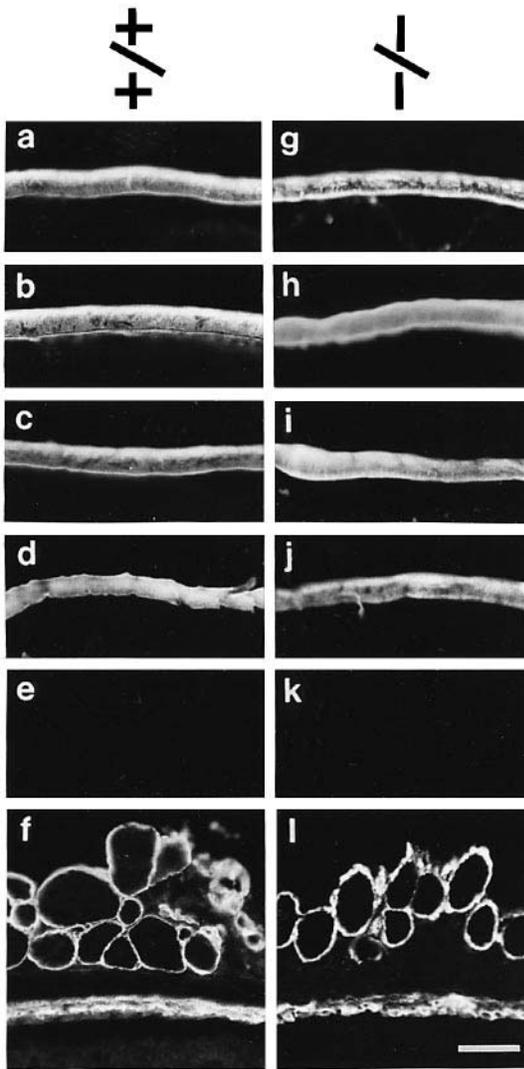
Fig. 8. Immunohistochemical localization of extracellular matrix proteins in the lens capsule from wild-type and mutant mice. Cryostat sections were analysed for the following proteins: (**a** and **g**) laminin 1; (**b** and **h**) collagen α1/2(IV); (**c** and **i**) entactin; (**d** and **j**) perlecan; (**e** and **k**) pre-immune negative control; (**f** and **l**) extra-ocular muscles stained with laminin 1. (a–f) +/+, wild-type mice; (g–l) –/–, *Sparc^{tm1Cam}* mutant mice. Bar = 50 μm.

proteins, such as tenascin-C (Saga *et al.*, 1992), thrombospondin 1 (Kyriakides *et al.*, 1998), vitronectin (Zheng *et al.*, 1995) and osteopontin (Liaw *et al.*, 1998). Thus, there seems to be considerable developmental plasticity associated with loss of ECM molecules, perhaps caused by functional overlap.

Materials and methods

Construction of the Sparc targeting vector, pS2

A 14.5 kb fragment containing *Sparc* exons 6–10 was released from the genomic cosmid p30g3 (McVey *et al.*, 1988) by *Hin*dIII digestion. A 5.5 kb *Eco*RI fragment containing exon 6 was subcloned from this *Hin*dIII fragment into a modified pBluescript KS+ (Stratagene) vector with the *Pst*I and *Xma*I sites removed from the polylinker. Digestion with *Xma*I and *Pst*I allowed removal of the *Sparc* exon 6. This was replaced with a 4.4 kb *Xma*I–*Sal*I fragment from the plasmid ROSA β-gal (kind gift from Dr P.Soriano) which has a splice acceptor placed upstream of a promoterless β-galactosidase gene and a PGK promoter-

driven neomycin^r sequence (Friedrich and Soriano, 1991). The *LacZ* gene is not in-frame with the *Sparc* coding sequence.

Generation of targeted ES cell clones and chimeric mice

CCB ES cells (Robertson *et al.*, 1986) were cultured on neomycin-resistant primary embryonic fibroblast feeder layers in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Sera-labs), 10% newborn calf serum (NBCS, Sigma), 2 mM glutamine (Gibco-BRL) and 0.1 mM β-mercaptoethanol (Sigma) in a humidified incubator (Gallenkamp) at 37°C in 5% CO$_2$. A total of 1×10^7 cells were electroporated with 25 μg of *Not*I-linearized targeting construct using a Bio-Rad Gene Pulser (250 μF, 250 V). After electroporation, the ES cells were plated onto mitotically inactivated feeder layers and neomycin-resistant clones were selected in the presence of 150 μg/ml G418 (Sigma) with daily replacement of medium for 12 days. Resistant colonies were isolated and plated on fresh feeder layers in individual wells of a 24-well dish (Nunc) and grown to confluency. Genomic DNA was isolated from individual clones, digested with *Bam*HI and Southern blotted (Sambrook *et al.*, 1989). These blots were hybridized with the external probe E1300, a 1.3 kb *Eco*RI fragment located 800 bp external to the 3′ flank of the targeting vector. Southern screening of 123 G418-resistant colonies led to the identification of 14 independently targeted clones. Targeting was confirmed by *Bam*HI and *Pst*I digestions and hybridization with an internal *neo* probe and an exon 6-specific probe. The targeted *Sparc* allele is defined as *Sparc^{tm1Cam}* according to the nomenclature guidelines issued by the Jackson Laboratory.

Two independently targeted clones were used to generate chimeric mice as previously described (Bradley, 1987). Male chimeras were mated to non-agouti MF1 (Harlan Olac) mice and agouti offspring screened for transmission of the disrupted *Sparc* allele. Germline chimeras were obtained from one of the targeted ES clones.

Northern analysis

Embryos from timed matings were collected at 15.5 d.p.c. (days post-coitum). Half of each embryo was used to prepare DNA for genotyping, and total RNA was extracted from the remainder using the single step guanidinium thiocyanate–phenol technique (Chomczynski and Sacchi, 1987). Twenty μg of this RNA was analysed on a formaldehyde gel and blotted as described by Sambrook *et al.* (1989).

Western analysis

Protein was extracted from testes by homogenization in 50 mM Tris–HCl pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol (DTT) and proteinase inhibitor cocktail [0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin, 1 μg/ml trypsin inhibitor, 0.5 μg/ml aprotinin, 40 μg/ml bestatin]. Protein concentrations were determined using the Coomassie Protein Assay Reagents from Pierce, and 100 μg of each sample were separated by SDS–PAGE as described by Laemmli (1970). Following electrophoresis, proteins were transferred to nitrocellulose as described by Towbin *et al.* (1979). The filter was blocked with 10% (w/v) dried milk in TBST (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20) and incubated with primary antibody for at least 1 h. The affinity-purified rabbit antibody (1064 +E) against recombinant mouse SPARC/BM40 was a kind gift from Dr Rupert Timpl and was used at a dilution of 1:200 in TBST. The monoclonal β-actin antibody (Sigma) was used at 1:2000 in TBST. Proteins were visualized using the ECL detection system from Amersham after addition of the horseradish peroxidase-conjugated secondary antibody (Sigma)

Histology and skeletal analysis

Mice were killed by CO$_2$ asphyxiation and organs fixed in 4% formaldehyde in phosphate-buffered saline. Organs were embedded in paraffin wax and 5 μm sections cut and stained with haematoxylin and eosin. Skeletons were prepared and stained with Alizarin red as described by Hogan *et al.* (1994). Eyes were removed with tweezers post-mortem and initially fixed for several days in 70% ethanol, followed by 30, 50 and 70% alcohol fixations for 2 h each (with the 70% alcohol fixation usually proceeding overnight). This was followed by three changes of methanol on the second day, followed by an overnight wash in chloroform. On the third day, the eyes were placed in three changes of molten paraffin wax (BDH) at 62°C and cooled in ice-cold water to preserve the lens. Sections were cut at 3–4 μm onto glass slides coated with 0.25% gelatin, placed on a hot plate between 35 and 40°C for 30 min, and then dried overnight at 37°C.

Electron microscopy

Eyes were fixed by immersion for 24 h at 23°C in a solution of 2.5% glutaraldehyde and 6% sucrose, buffered to pH 7.2 with 50 mM sodium cacodylate. Specimens were post-fixed in OsO_4 buffered with 150 mM sodium–potassium phosphate (pH 7.4), embedded in Spurr resin (Spurr, 1969) and sectioned with a glass knife for electron microscopy. Sections were examined using a JEM 100B electron microscope (JEOL).

Immunohistochemistry

Mouse eyes were snap-frozen in liquid nitrogen in OCT medium and cryostat sectioned. The indirect immunofluorescence method and source of the antibodies are as described in Miner and Sanes (1996) and Miner et al. (1997).

Acknowledgements

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