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Type I Collagen-Deficient Mov-13 Mice Do Not Retain SPARC in the Extracellular Matrix: Implications for Fibroblast Function

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ABSTRACT **The *Mov*-13 strain of mice was created by the insertion of the murine Moloney leukemia virus into the first intron of the α1(I) collagen gene. Consequently, *Mov*-13 embryos do not transcribe α_1 (I) collagen mRNA and lack type I collagen protein in the extracellular matrix (ECM). Homozygotes die within 12–14 days of embryonic development, in part from the rupture of large blood vessels, and also exhibit deficiencies in hematopoesis and assembly of the ECM (Löhler et al. [1984] Cell 38:597–607). Several matricellular proteins, proteoglycans, and growth factors bind to type I collagen, e.g., fibronectin, secreted protein acidic and rich in cysteine (SPARC), decorin, and transforming growth factor-β. Here we investigate the expression and function of SPARC in the absence of type I collagen. We show that fibroblasts isolated from *Mov*-13 homozygous, heterozygous, and wild-type embryos transcribed and translated SPARC mRNA in vitro. However, accumulation of extracellular SPARC was severely affected in the tissues of *Mov*-13 homozygotes, whereas extracellular deposition of the secreted glycoproteins fibronectin and type III collagen was not altered. Since SPARC has been shown to be a regulator of cell shape, the functional consequences of the absence of extracellular SPARC were evaluated in collagen gel contraction assays. Fibroblasts isolated from homozygous *Mov*-13 mice did not contract native type I collagen gels as efficiently as fibroblasts from heterozygous littermates; however, addition of exogenous SPARC enhanced the contraction of collagen by homozygous *Mov*-13 fibroblasts. The stimulatory effect of SPARC was blocked by antibodies specific for the amino terminus of the protein. These results provide evidence that type I collagen is one of the major extracellular proteins that binds SPARC in vivo. Furthermore, the capacity of fibroblasts to contract ECM in vitro is enhanced by extracellular SPARC. We therefore propose that the remodeling of ECM by cells in vivo is regulated in part by a specific interaction between SPARC and type I collagen.**

Key words: Development, Extracellular matrix, Fibroblast function

INTRODUCTION

Extracellular matrix (ECM) macromolecules contribute significantly to the organization of tissues, the distribution and stability of growth factors, the control of nutrient diffusion, and the regulation of cellular differentiation (Bissell and Barcellos-Hoff, 1987; Blau and Baltimore, 1991; Flaumenhaft and Rifkin, 1991, 1992; Adams and Watts, 1993). Although interactions among proteins/proteoglycans of the ECM have been characterized in vitro, the complexity of the extracellular environment has made it difficult to study binary (or higher-order) associations between certain secreted proteins in vivo. Recently, mice that lack a given protein have been produced by targeted disruption of specific genes. With these animals one can begin to analyze the contributions of individual molecules to integrated physiological processes.

In the *Mov*-13 mouse, the production of type I collagen has been disrupted by the insertion of a murine Moloney leukemia virus into the first intron of the α1(I) collagen gene (Jaenisch et al., 1983; Harbers et al., 1984; Hartung et al., 1986; Kratochwil, 1988). Since cells from these mice do not synthesize α1(I) collagen mRNA, there is no type I collagen protein secreted into the ECM. An exception to this rule is seen in long bones and teeth, in which type I collagen is transcribed in *Mov*-13 homozygotes (Kratochwil et al., 1989; Schwartz et al., 1990). Unlike fibroblasts, osteoblasts and odontoblasts appear not to utilize the first intron of the α1(I) collagen gene for transcriptional regulation (Slack et al., 1993; Liska et al., 1994; Rossert et al., 1995); therefore, disruption of the first intron affects neither initiation nor progression of transcription in these cell types. Nevertheless, most homozygous *Mov*-13 animals die in utero prior to osteogenesis and odontogenesis, i.e., between d11 and d13, although

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d16 embryos have been reported (Kratochwil et al., 1989). The cause of death has been attributed to the rupture of major blood vessels, which are abnormally fragile. Other developmental anomalies involve the liver, hematopoetic system, and general mesenchyme (Schnieke et al., 1983; Löhler et al., 1984). Abnormal development of *Mov*-13 homozygous embryos can be circumvented in part by reintroduction of the human or mouse α1(I) collagen gene (Wu et al., 1990).

The presence of type I collagen affects other components of the ECM. For example, Dzamba et al. (1993) have shown that fibrillogenesis of fibronectin is compromised in *Mov*-13 mice. Moreover, type I collagen functions as a binding protein for growth factors and other structural components of the ECM, such as TGF-β, decorin, fibronectin, vitronectin, and secreted protein acidic and rich in cysteine (SPARC) (Gebb et al., 1986; Ingham et al., 1988; Brown and Vogel, 1989; Pringle and Dodd, 1990).

Several proteins, recently denoted as matricellular components, associate transiently with the ECM but do not in themselves provide a structural framework to this compartment (Bornstein, 1995). One of these proteins is SPARC, a collagen-binding glycoprotein synthesized at high levels by a variety of cells, often concomitantly with the production of type I collagen (Lane and Sage, 1994). During development, SPARC is expressed by cells of connective tissue, endothelial cells, smooth and skeletal muscle, and chondrocytes (Holland et al., 1987; Sage et al., 1992). SPARC is also expressed at high levels by osteoblasts, megakaryocytes, and Leydig and Sertoli cells (Termine, et al., 1981; Holland et al., 1987; Vernon and Sage, 1989; Vernon et al., 1991). Although SPARC has been identified in the extracellular spaces of bone, in granulation tissue, and in specialized basement membranes (Mann et al., 1987), the mechanism of its retention by ECM could only be surmised by reference to its molecular interactions in vitro. Studies in vitro have shown that SPARC binds to a number of proteins that comprise the ECM, e.g., collagens (particularly types I, III, and IV) and thrombospondin-1 (Clezardin et al., 1988; Sage et al., 1989). The association of SPARC with specific components of ECM in vivo has yet to be substantiated, and the relevance of extracellular accumulation of SPARC in vivo is not known.

We have investigated the composition of ECM in embryonic tissues of type I collagen-deficient *Mov*-13 mice. We found that the synthesis of SPARC was unaffected in cells of *Mov*-13 homozygotes, both in vivo and in vitro; however, the extracellular accumulation of SPARC in vivo was significantly compromised in these mice. Other components of ECM, such as type III collagen and fibronectin, exhibited normal distributions. Moreover, fibroblasts from *Mov*-13 homozygous embryos did not incorporate SPARC into the insoluble ECM deposited in vitro, in contrast to the significant accumulation of SPARC in the ECM of cultured cells derived from heterozygous littermates. Our results indicate that, in addition to its provision of structural support to ECM, type I collagen influences the spatial distribution of the matricellular protein SPARC. Since SPARC binds regulatory proteins such as platelet-derived growth factor B chain, plasminogen, and thrombospondin-1 (Clezardin et al., 1988; Sage et al., 1989; Raines et al., 1992), it is likely that type I collagen provides extracellular sites for a variety of protein complexes that alter cellular functions.

RESULTS

Extracellular Deposition of SPARC Protein In Vivo Is Impaired in *Mov*-13 Animals

We have examined the distribution of SPARC in 15 *Mov/Mov* embryos, and in an equal number of *Mov*/+ and +/+ littermates, between d10.5 and d14.5 of development. Figure 1A illustrates a typical Southern blot of genomic DNA: *Mov/Mov* embryonic DNA after digestion with *Eco*RI exhibited a unique band of 23 kb, whereas +/+ DNA exhibited a single band of 14 kb. *Mov*/+ embryos contained both 14 kb and 23 kb restriction fragments. *Mov/Mov* animals also showed profuse hemorrhagia that typified the homozygous phenotype (Fig. 1B).

Immunohistochemical localization of SPARC protein in d14.5 *Mov*/+ embryos showed a distribution identical to that in +/+ embryos. SPARC was identified at sites of type I collagen deposition, including perichondrium (Fig. 2A,C) as previously described (Holland et al., 1987; Lane and Sage, 1994). In contrast, *Mov/Mov* embryos displayed no evidence of type I collagen (Fig. 2B) or extracellular SPARC (Fig. 2D), although intracellular SPARC protein was evident in chondrocytes. The lack of extracellular SPARC was observed in all (15/15) *Mov/Mov* embryos examined and was most evident in comparisons between older *Mov/Mov* and +/+ embryos, due to the progressive accumulation of SPARC in +/+ embryos as development proceeded.

The lack of extracellular SPARC prompted us to question whether the level of SPARC synthesis was affected in *Mov*-13 animals. We examined steady-state levels of SPARC mRNA in *Mov/Mov, Mov*/+, and in +/+ littermates. Figure 3 shows a representative Northern blot hybridized with both SPARC and α1(I) collagen cDNA probes. The results indicated that transcription of SPARC in vivo was not altered in tissues of *Mov/Mov* mice lacking type I collagen mRNA.

SPARC mRNA and Protein Are Synthesized by *Mov*-13 Fibroblasts In Vitro

We characterized the effect of type I collagen on the synthesis and extracellular deposition of SPARC protein by fibroblasts cultured from d13 *Mov/Mov, Mov*/+, and +/+ littermate embryos. Immunocytochemical analysis with anti-type I collagen antibodies confirmed that +/+ fibroblasts synthesized type I collagen in vitro, whereas *Mov/Mov* fibroblasts did not (Fig. 4A,B). Similar cultures were assayed for expression of SPARC by immunofluorescence: no differences were detected

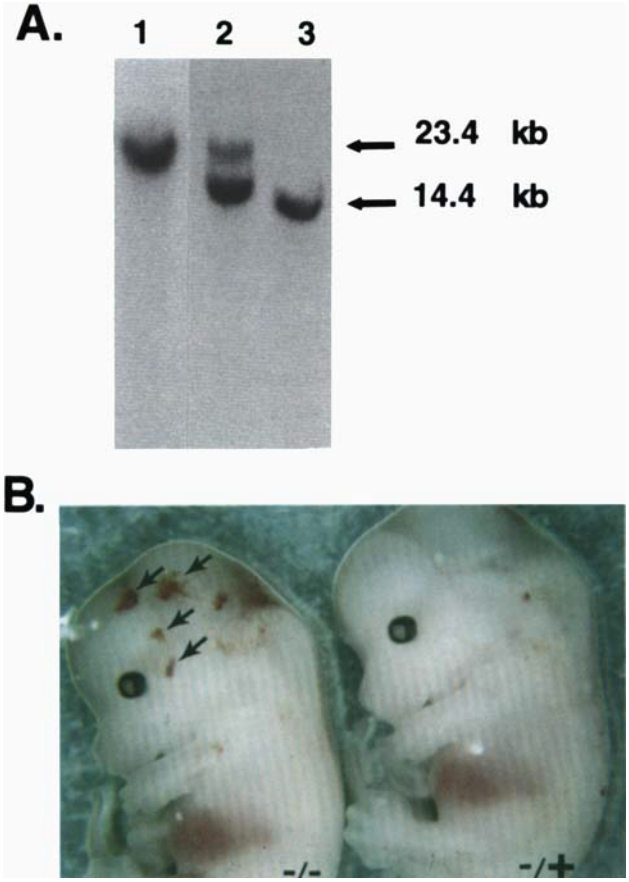

Fig. 1. Genotype and appearance of *Mov*-13 embryos. **A:** Southern blot of genomic DNA from three representative littermates. **Lane 1,** *Mov/Mov;* **lane 2,** *Mov/+*; **lane 3,** *+/+*. The blot was hybridized with an α1(I)-collagen cDNA probe that overlaps the MMLV insertion site in *Mov*-13 animals. Insertion of the proviral DNA results in a 23.4 kb fragment, compared to a 14.4 kb fragment in the DNA from wild-type embryos, after digestion with *Eco*RI. **B:** *Mov/Mov* (−/−) and *Mov/+* (−/+) littermates (day 13.5 embryos) were photographed to show the extensive hemorrhagia (arrows) present in older homozygotes. Genotype was confirmed by Southern blot analysis (A).

in the levels of intracellular SPARC between *Mov/Mov* and *+/+* fibroblasts (Fig. 4C,D). Moreover, differences in expression of cellular fibronectin between *+/+* (Fig. 4E) and *Mov/Mov* fibroblasts (Fig. 4F) were not apparent.

Northern blot analysis of RNA from the cultured fibroblasts indicated that steady-state levels of SPARC mRNA were similarly unaffected. Quantification of Northern blots, after normalization to the 28S rRNA signal, showed no significant differences among 7 independent isolates of *Mov/Mov* fibroblasts, in comparison to their *+/+* littermates (Fig. 5). The results were consistent with the findings obtained from RNA extracted from embryonic tissues (Fig. 3).

Cultures of *Mov*-13 homozygous, heterozygous, and wild-type fibroblasts were also labeled metabolically with [^{3}H]-proline, and the secreted proteins were analyzed by SDS-PAGE (Fig. 6A). All forms of type I collagen, including pro α1(I) and pro α2(I) collagen chains and the pC- and pN-forms (Sage and Bornstein, 1982), were absent from the conditioned medium of *Mov/Mov* fibroblasts (Fig. 6A, lane 1, arrows). Moreover, a number of other differences were seen in the secretory profile of fibroblasts from *Mov/Mov* vs. wild-type embryos: some proteins were decreased in *Mov/Mov* fibroblasts (Fig. 6A, arrowheads), whereas one protein appeared to be increased (Fig. 6A, asterisk). Although we did not pursue the characterization of these proteins, these data are consistent with the contention that an absence of type I collagen synthesis influences the expression of some secreted proteins.

The absence of type I collagen from cultured *Mov/Mov* fibroblasts was verified immunologically by Western blot assays of conditioned media (Fig. 6B). The secretion of SPARC was not impaired by the absence of type I collagen synthesis, as shown in Figure 6C: levels of SPARC were indistinguishable among fibroblasts from *Mov/Mov, Mov/+*, and *+/+* littermates, as summarized in Table 1.

Type I Collagen Promotes the Accumulation of SPARC In Vitro

Our data indicated that the synthesis and secretion of SPARC in vivo and in vitro were not impaired by the absence of type I collagen synthesis. Nonetheless, extracellular SPARC was absent from the tissues of *Mov/Mov* mice. We therefore postulated that type I collagen might promote the accumulation of SPARC in interstitial ECM in vivo and we performed experiments to determine whether type I collagen incorporated into ECM in vitro was associated with SPARC. Fibroblasts were cultured for 20 days to allow accumulation of insoluble ECM on the glass substrate. Cells were removed by incubation with Triton X-100, and the ECM that remained on the glass was analyzed for the presence of type I collagen, SPARC, fibronectin, and type III collagen. Figure 7 shows the immunolocalization of these proteins in *Mov/+* (Fig. 7A, C, E, and G) and *Mov/Mov* (Fig. 7B, D, F, and H). Type I collagen was abundant in the ECM of *Mov/+* cultures (Fig. 7A), but it was absent from ECM of *Mov/Mov* cultures (Fig. 7B). SPARC was detected at very low levels in *Mov/Mov* cultures (Fig. 7D), in comparison to cultures from *Mov/+* littermates (Fig. 7C). Insoluble fibronectin and type III collagen were deposited similarly by both *Mov/+* and *Mov/Mov* cultures (Fig. 7E–H).

A quantitative comparison of the abundance of type I collagen and SPARC deposited into the ECM by cultured wild-type and *Mov*-13 fibroblasts is shown in Figure 8. Triton X-100-insoluble ECM proteins from 20 day cultures of *Mov/Mov, Mov/+*, and *+/+* fibroblasts were extracted in hot SDS-PAGE buffer, resolved by electrophoresis, and subjected to Western blot analysis. Levels of fibronectin in the ECM from the different cultures were equivalent and therefore independent of type I collagen. In contrast, levels of SPARC were high in ECM that contained type I collagen, but were low in

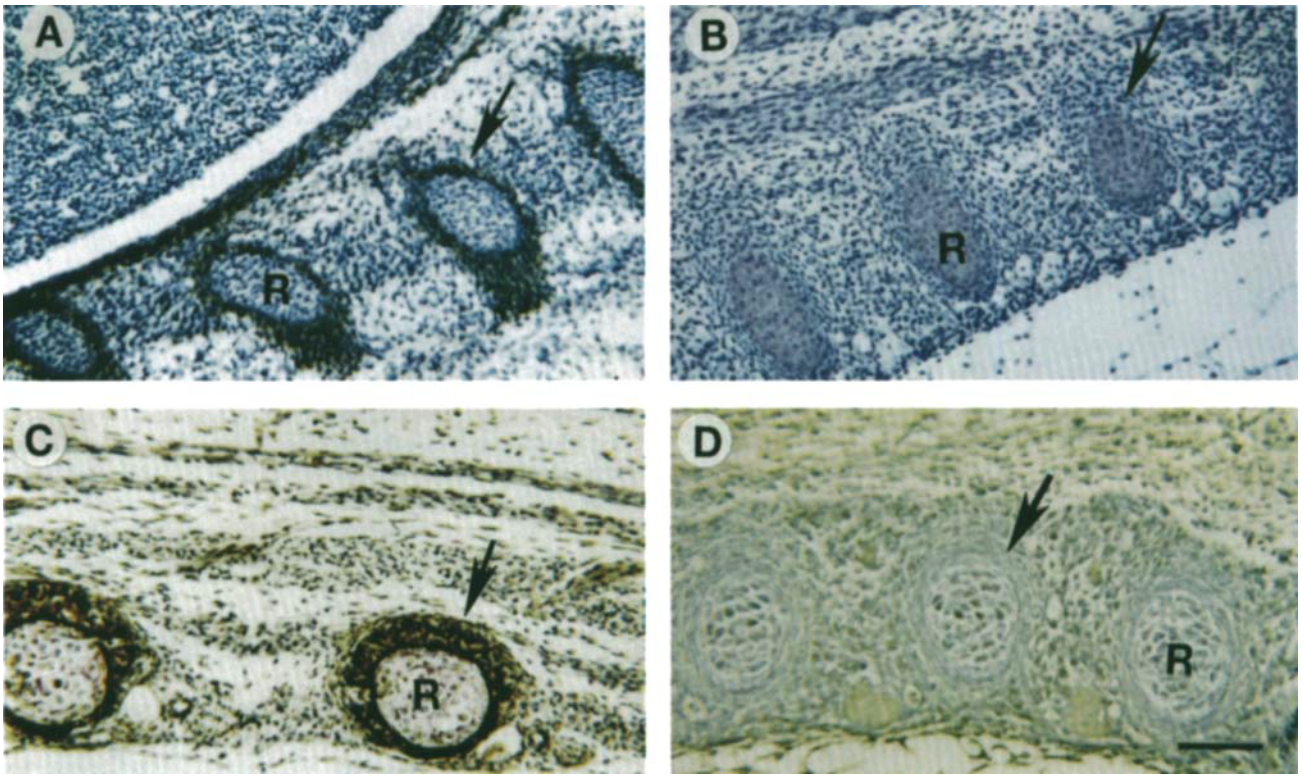

Fig. 2. SPARC protein is absent from the ECM of *Mov*-13 embryos. Antibodies specific for type I collagen (**A, B**) and SPARC (**C, D**) were used for immunohistochemical analysis of +/+ (A, C) and *Mov/Mov* (B, D) littermates (day 13.5 embryos). Bound antibodies were detected by an avidin-biotin-peroxidase system which generated a brown reaction product. Sections were counterstained with toluidine blue to identify nuclei. Perichondria (arrows) of the rib primordia (R) of +/+ embryos were reactive with both anti-type I collagen and anti-SPARC antibodies. In contrast, the rib primordia of *Mov/Mov* embryos were unlabeled. Bar = 80 μm.

ECM that lacked type I collagen (Fig. 8, lanes 1 and 3 vs. lanes 2 and 4). Therefore, it appeared that type I collagen was integral to the accumulation of SPARC in extracellular deposits of ECM.

Lack of Extracellular SPARC Has Functional Consequences for *Mov*-13 Fibroblasts

Fibroblasts cultured within malleable gels of native type I collagen in vitro pull strongly on the collagen fibrils by a process referred to as traction (Harris et al., 1981). Traction-mediated reorganization of collagen is considered to be an important aspect of fibroblast function that contributes to a variety of morphogenetic processes in vivo, e.g., the contraction of ECM in dermal wounds (Montesano and Orci, 1988), the eruption of teeth (Bellows et al., 1981), and the compaction and alignment of collagen fibers within developing tendons, ligaments, periosteum, and capsules of organs (Stopak and Harris, 1982). Therefore, it was of interest to determine whether the reorganization of collagen by fibroblasts could be influenced by the lack of ECM-associated SPARC. Traction applied to collagen by a population of cells can be studied quantitatively by means of a collagen gel contraction assay in vitro (Reed et al., 1994; Vernon et al., 1995; Vernon and Sage,

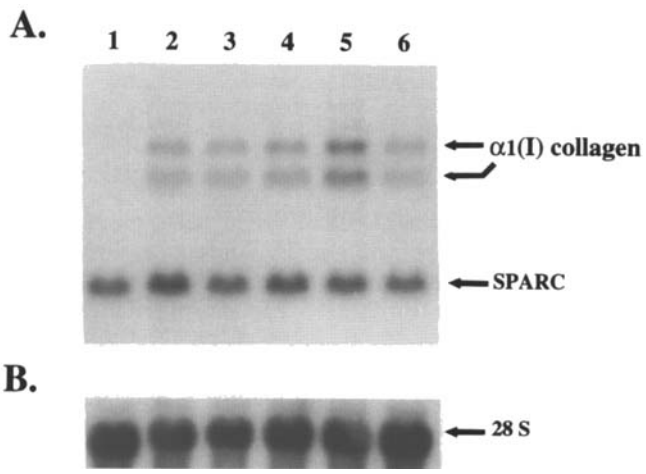

Fig. 3. SPARC mRNA is present in homozygous *Mov*-13 embryos. Blots of total RNA were prepared from *Mov/Mov* (**lane 1**), *Mov*/+ (**lanes 2–4**), and +/+ (**lanes 5–6**) embryos. Genotypes of the individual embryos (littermates derived from a *Mov*/+ × *Mov*/+ mating) were determined by Southern blot analyses of genomic DNA. RNA blots were probed with α1(I) collagen and SPARC cDNAs (**A**), and were subsequently reprobed with a cDNA corresponding to 28S rRNA (**B**) as a control for RNA loading. Although the *Mov/Mov* embryonic tissue lacked α1(I) collagen RNA, SPARC mRNA was expressed at levels comparable to those of *Mov*/+ and +/+ embryos.

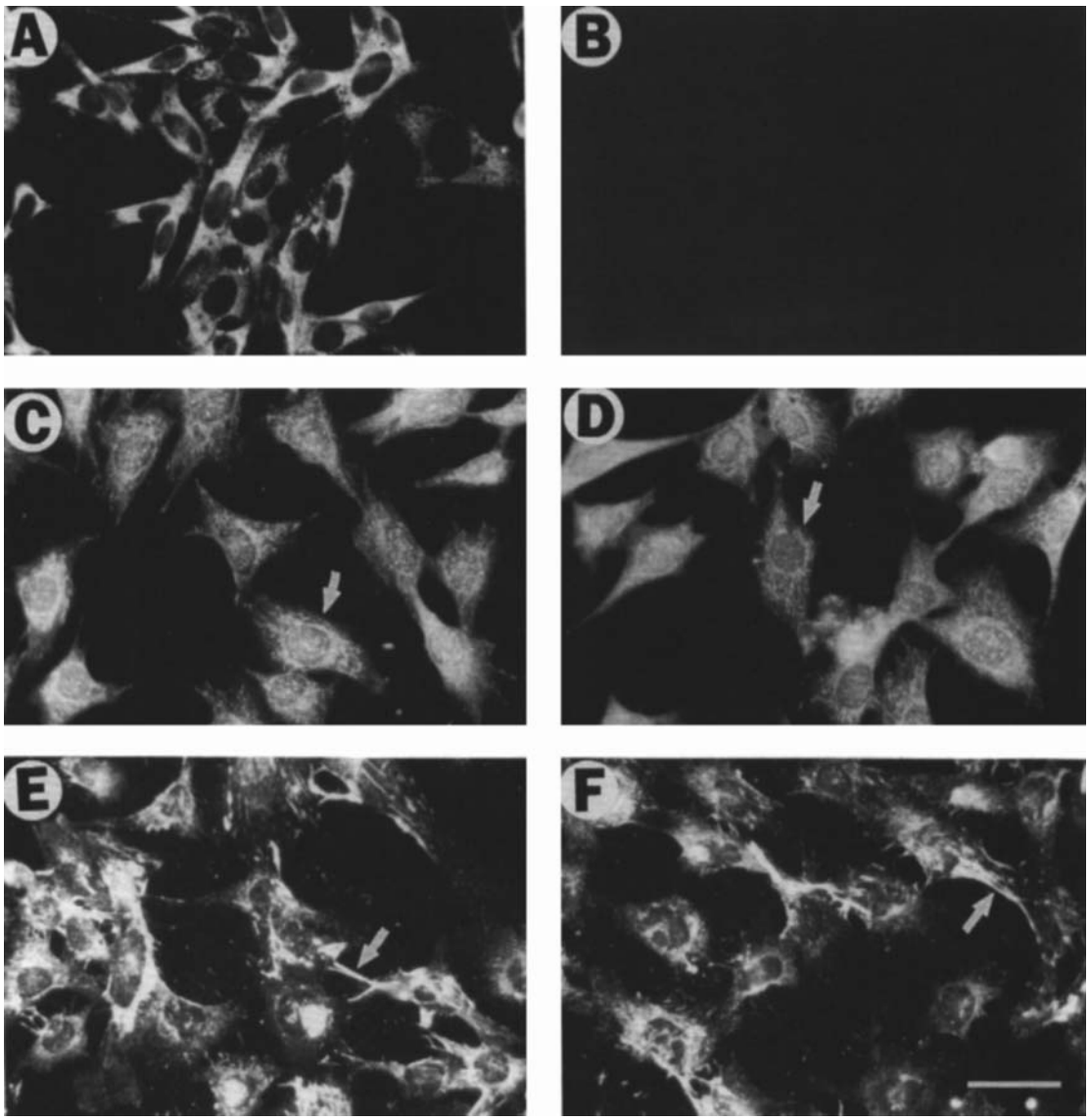

Fig. 4. *Mov*-13 fibroblasts have similar levels of intracellular SPARC and extracellular fibronectin in comparison to wild-type cells. Fibroblasts were isolated from +/+ (**A, C, E**) and *Mov/Mov* (**B, D, F**) embryos. Cultured cells were fixed and were stained by indirect immunofluorescence with antibodies specific for: α1(I)procollagen (A, B), SPARC (C, D), or fibronectin (E, F). Arrows identify intracellular SPARC (C, D) or extracellular fibronectin (E, F) in +/+ and *Mov/Mov* fibroblasts. Bar = 20 μm.

1996). Cells are embedded in disk-shaped collagen gels. As the cells are cultured, the gathering and compression of collagen fibers around individual cells causes a progressive reduction in diameter of the disks. The ability of *Mov/Mov* and *Mov/+* fibroblasts to reorganize collagen was compared by use of such assays.

Fibroblasts from *Mov/Mov* embryos contracted type I collagen gels less efficiently, in comparison to *Mov/+* fibroblasts (Fig. 9A). Differences in contraction were reproducible and were seen across the range of malleabilities of collagen gels tested. Interestingly, addition of purified SPARC protein to the collagen matrix prior to gelation significantly enhanced the capacity of *Mov/Mov* fibroblasts to contract collagen gels (Fig. 9B): a 70% increase in gel contraction was observed in cultures treated with 1.3 μM SPARC. This effect was specific for SPARC, since addition of other proteins such as ovalbumin or purified bovine fibronectin had no effect on contraction of collagen gels. The stimulatory effect of SPARC was neutralized with antibodies against the amino terminus of the protein (anti-1.1 IgG), but not by antibodies against the carboxyl terminus that bind only to denatured SPARC (anti-4.2 IgG) (Fig. 9C).

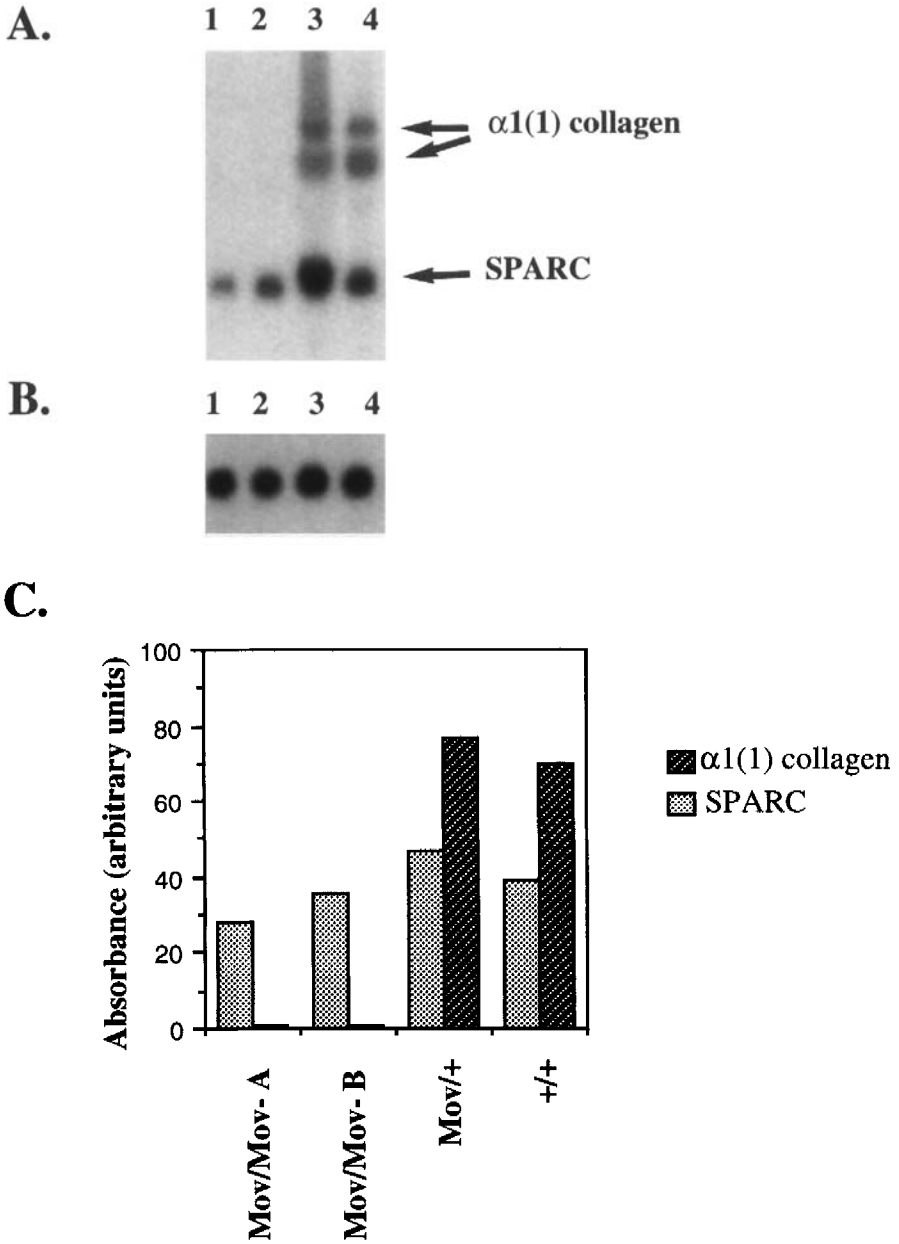

Fig. 5. SPARC mRNA is transcribed at equivalent levels in wild-type, homozygous, and heterozygous cultures. RNA was isolated from cultures of *Mov/Mov* (**lanes 1, 2**), *Mov*/+ (**lane 3**), and +/+ fibroblasts (**lane 4**). RNA blots were probed sequentially with α1(I) collagen and SPARC cDNA (**A**) and with G3PDH (**B**). Northern blots were quantified by a Molecular Dynamics phosphorimager and signals were normalized to the G3PDH hybridization signal (**C**).

DISCUSSION

In this study we have utilized *Mov*-13 animals to determine whether the ablation of collagen synthesis influences the synthesis and secretion of SPARC and its subsequent binding to the ECM. Our results demonstrate that the synthesis and secretion of SPARC is not affected by the absence of type I collagen; however, the lack of extracellular type I collagen significantly impairs the accumulation of SPARC in the ECM. Moreover, we find a functional relationship between SPARC and type I collagen: SPARC stimulates the remodeling of type I collagen by fibroblasts. These results demonstrate the interdependence among components of the ECM and promote the concept that the phenotype of ECM-knock-out mice reflects changes in the composition and/or organization of other ECM proteins that interact reciprocally (Flaumenhaft and Rifkin, 1991, 1992).

That type I collagen could influence the organization of other ECM proteins was demonstrated by Dzamba et al. (1993). In their study, *Mov*-13 homozygous fibroblasts were transfected with α1(I) collagen cDNAs con-

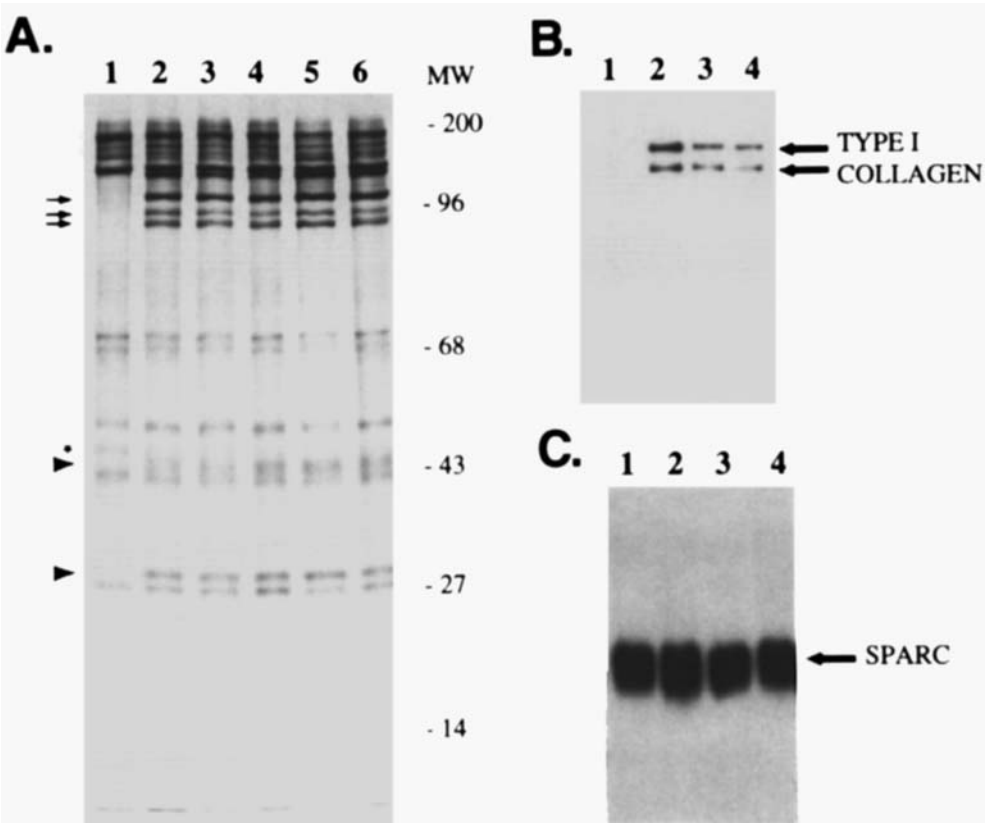

Fig. 6. The *Mov*-13 mutation does not affect synthesis or secretion of SPARC by embryonic fibroblasts in vitro. **A:** Cultures of *Mov/Mov* (**lane 1**), *Mov*/+ (**lanes 2–4**), and +/+ (**lanes 5, 6**) fibroblasts were incubated for 16 hr in the presence of [^{3}H]-proline; radiolabeled proteins in culture media were resolved on an 8% polyacrylamide gel in the presence of 10 mM DTT and were visualized by autoradiography. Arrows indicate the position of type I procollagen and its processed forms. *Mov*/+ and +/+ fibroblasts secreted type I procollagen, whereas *Mov/Mov* fibroblasts did not. Arrowheads indicate other proteins that were synthesized by *Mov*/+ and +/+ fibroblasts, but not by *Mov/Mov* fibroblasts. The asterisk indicates a protein that was synthesized by *Mov/Mov* fibroblasts only. **B:** Conditioned media from cultures of *Mov/Mov* (**lane 1**), +/+ (**lane 2**), and *Mov*/+ (**lanes 3, 4**) fibroblasts were subjected to Western blot analysis with an anti-type I collagen antibody. Immunoreactive bands corresponding to α1(I) and α2(I) collagen chains were present in the media of *Mov*/+ and +/+ cultures, but were absent from *Mov/Mov* culture medium. **C:** A Western blot of proteins from *Mov/Mov, Mov*/+, and +/+ fibroblast culture media similar to that shown in B was incubated with anti-SPARC antibodies. All samples showed similar levels of secreted SPARC protein.

TABLE 1. Quantification of SPARC Secreted by *Mov/Mov, Mov*/+, and +/+ Fibroblasts[a]

Genotype of fibroblasts	Absorbance (relative units) ± SD
Mov/Mov	69 ± 12
Mov/+	75 ± 7
+/+	72 ± 10

[a]Levels of SPARC in conditioned media were determined by densitometry of autoradiograms of five Western blots. Blots were probed with anti-SPARC antibodies and [^{125}I]-protein A as described in Experimental Procedures.

taining mutations in the fibronectin binding site of type I collagen that prevented the binding of fibronectin to collagen. The fibrillogenesis of fibronectin was consequently abnormal: fibrils were shown to be shorter and similar to those produced by cultures of untransfected *Mov/Mov* fibroblasts.

In addition to fibronectin, other components of ECM that include SPARC, decorin, biglycan, heparin, osteopontin, von Willebrand factor, vitronectin, and TGF-β have been shown to bind to type I collagen (Gebb et al., 1986; Ingham et al., 1988; Sage et al., 1989; Brown and Vogel, 1989; Pringle and Dodd, 1990; Chen et al., 1992; Takagi et al., 1992; San Antonio et al., 1994; Schonherr et al., 1995a,b). Our interest has focused on SPARC, a collagen-binding protein that regulates the cell cycle and angiogenesis (Lane and Sage, 1994; Iruela-Arispe et al., 1995). Assays that quantified the interaction of SPARC with collagens adsorbed to plastic substrates showed that the binding of SPARC to collagen types II, III, IV, and V was greater on a molar basis than its binding to type I collagen (Sage et al., 1989). Our findings in *Mov*-13 mice, however, indicate that type I collagen is perhaps the most relevant collagen for binding and accumulation of SPARC in vivo. *Mov/Mov* mice had apparently normal levels of SPARC transcripts and intracellular SPARC protein, but SPARC did not accumulate extracellularly. Moreover, the maintenance of SPARC in the perichondrial ECM was depen-

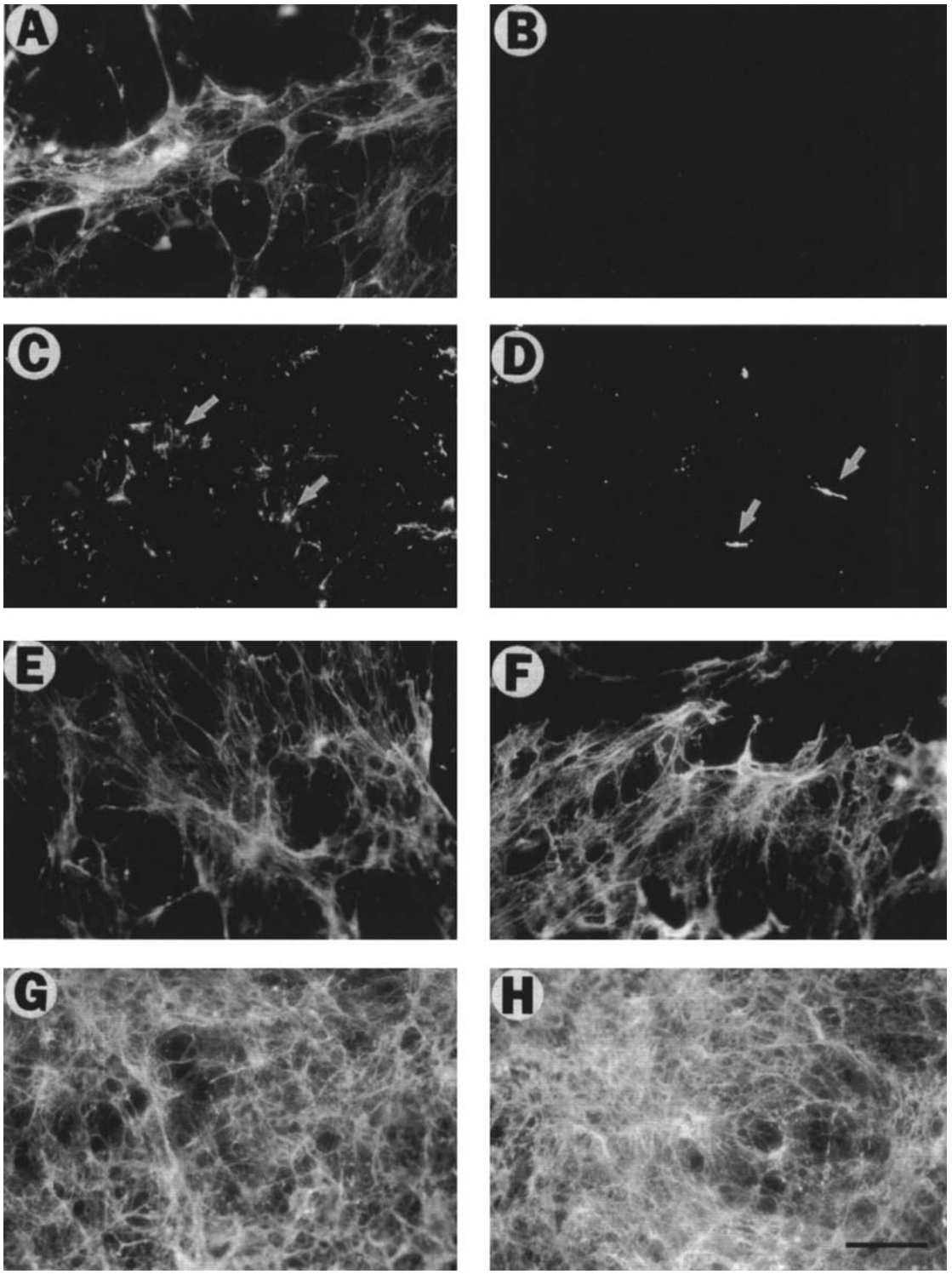

Fig. 7. Extracellular accumulation of SPARC in vitro is impaired in the absence of type I collagen. Cultures of *Mov/+* (**A, C, E, G**) and *Mov/Mov* (**B, D, F, H**) fibroblasts were grown for 20 days to promote accumulation of insoluble ECM on the glass substrate. Cells were removed, and the residual ECM was stained by indirect immunofluorescence. Antibodies used were: anti-type I collagen (A, B); anti-SPARC (C, D); anti-type III collagen (E, F); anti-fibronectin (G, H). *Mov/+* fibroblasts deposited significant quantities of type I collagen (A) and SPARC (C, arrows). In contrast, the deposition of SPARC by *Mov/Mov* fibroblasts (D, arrows) was minimal and was correlated with the absence of type I collagen (B). Deposition of type III collagen and fibronectin by *Mov/Mov* cultures (F, H) was similar to that seen in *Mov/+* cultures (E, G). Bar = 20 μm.

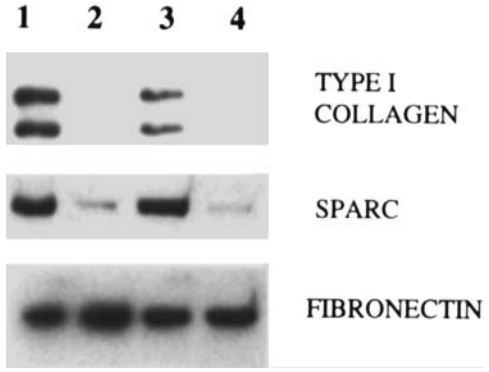

Fig. 8. Composition of ECM in cultures of +/+ and *Mov/Mov* fibroblasts. Cells were cultured for 20 days and were subsequently removed from the substrate. The residual ECM was subjected to Western blot analysis. Loading of ECM proteins was normalized to total cell number. Lanes were loaded with ECM deposited by +/+ fibroblasts (**1**); *Mov/Mov* fibroblasts (**2,4**); and *Mov*/+ fibroblasts (**3**). Absence of type I collagen from the ECM is correlated with a significant reduction in the levels of SPARC. In contrast, the deposition of fibronectin in the ECM was independent of the presence of type I collagen.

dent on the presence of type I collagen, regardless of the presence of other collagens (e.g., types III, IV, and V, as well as fibril-associated collagens) in the perichondrium at this time in development.

We observe that *Mov/Mov* fibroblasts are less effective contractors of collagen in vitro than are *Mov*/+ fibroblasts. This finding suggests that the absence of newly synthesized pericellular collagen compromises fibroblast-mediated restructuring of extant collagen. We found a similar relationship between synthesis of type I collagen and collagen gel contraction by bovine aortic endothelial cells: clones of endothelial cells that synthesized type I collagen in vitro were better contractors of collagen gels than were strains of endothelial cells that did not synthesize this protein (Vernon et al., 1995; Vernon and Sage, unpublished observations). The mechanism by which endogenous synthesis of type I collagen affects the contraction of surrounding collagen matrix is unclear. It has been reported that the contraction of collagen gels by human skin fibroblasts is facilitated by the endogenous synthesis of the cellular form of fibronectin (Asaga et al., 1991). In this circumstance, cellular fibronectin might serve as a mechanical link between cells and collagen fibrils via its cell-binding and collagen-binding domains. Similarly, newly synthesized pericellular type I collagen might transmit forces of cellular traction to distal, extant collagen via noncovalent/covalent cross-links between fibrils. It is noteworthy that addition of SPARC to the collagen matrix significantly enhanced contraction of collagen by *Mov/Mov* fibroblasts. We do not currently understand why SPARC stimulates the reorganization of collagen; however, the effect might relate to the capability of SPARC to modify the adhesion of cells to ECM (Sage and Bornstein, 1991). The physical interactions between cells and ECM involve a continuous

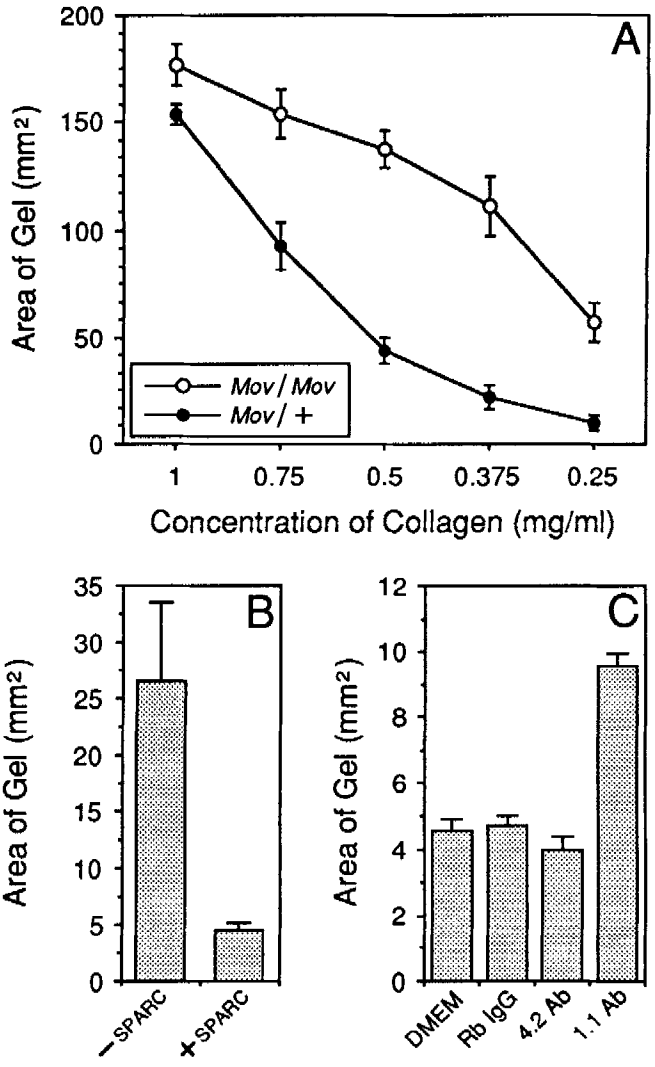

Fig. 9. Contraction of collagen gels by *Mov/Mov* fibroblasts is impaired, but is stimulated by exogenous SPARC. **A:** Equal numbers of *Mov/Mov* and *Mov*/+ fibroblasts were tested for their capacity to contract 15 mm disks of type I collagen that varied in malleability (0.25–1 mg/ml collagen). After 18 hr of culture, *Mov*/+ fibroblasts had contracted all gels to a greater degree than was seen with *Mov/Mov* fibroblasts. Measurements are the average of quadruplicate samples. **B:** Addition of 1.3 μM SPARC significantly enhanced the contraction of 0.375 mg/ml collagen gels by *Mov/Mov* fibroblasts. Uncontracted gels were 11 mm in diameter. Measurements are the average of quadruplicate samples. **C:** The stimulation of contraction of 0.375 mg/ml collagen gels (11 mm diameter) by SPARC was abrogated by antibodies that bind to domain I of the protein (1.1 Ab), but not by DMEM alone, normal rabbit IgG (Rb IgG), or antibodies that bind only to denatured SPARC (4.2 Ab). Measurements are the average of quadruplicate samples.

disassembly and reestablishment of focal contacts (Ruoslahti and Pierschbacher, 1987). SPARC has been shown to affect a number of cellular parameters related to a decrease in the number and strength of focal adhesions or ECM attachments (Murphy-Ullrich et al., 1995). It is possible that specific interactions between SPARC and nascent type I collagen fibrils potentiate the effects of SPARC on cells. The lack of pericellular

type I collagen in *Mov* homozygotes would therefore compromise not only the retention of SPARC in the vicinity of cells, but also the capacity of SPARC to stimulate cells.

Another aspect of the *Mov*-13 phenotype for which SPARC could potentially be a contributor is the increase in vascular permeability and fragility typical of homozygous embryos. Studies with vascular endothelial cells in vitro have shown that SPARC inhibits cellular attachment to the ECM (Sage et al., 1989, Lane and Sage, 1990), diminishes focal contacts (Murphy-Ullrich et al., 1995), and inhibits proliferation (Funk and Sage, 1991, 1993). In addition, SPARC increases the permeability of confluent endothelial cell monolayers in vitro (Goldblum et al., 1994). Since SPARC is not bound to type I collagen in *Mov/Mov* mice, there is a potential for free SPARC to enter the circulation; indeed, SPARC is present at low levels in the plasma of normal mice. Since SPARC is secreted by endothelial cells, it is not clear whether its presence in blood results from luminal secretion by endothelial cells or via diffusion from the interstitium. It has been proposed that hemorrhagia in *Mov/Mov* embryos results from the increased fragility of the vascular wall due to the lack of supportive type I collagen. However, abundance of free SPARC could also compromise the mechanical integrity of the endothelial layer.

The structure of the extracellular environment is complex. Collagens, glycoproteins, and proteoglycans are known to interact in extracellular spaces to create a physically integrated matrix that is, in turn, a substrate for growth factors, proteases, and other molecules (Fukamizu and Grinnell, 1990; Flaumenhaft and Rifkin, 1992). The complexity of ECM is reflected in the substantial diversity of ECM gene products. For example, more than 18 different collagen types have been identified, along with an array of fibronectin isoforms, proteoglycans, and laminins, many of which appear to be expressed in a tissue-specific manner. The importance of this diversity to biological function is confirmed by observations that the organization and composition of the ECM can cause growth factors to exert different effects on cellular behavior (Madri et al., 1988; Nathan and Sporn, 1991; Flaumenhaft and Rifkin, 1991, 1992; Raines et al., 1992; Iruela-Arispe and Sage, 1993). The ECM thus appears to consist of a series of modular elements that provide mechanical support and that facilitate interactions between cells and molecules that mediate cell behavior and growth. Loss of any one component of ECM, or mutations that render ECM molecules biologically inactive, have the potential to compromise the function of other components of ECM. Through the use of mice deficient in particular ECM proteins or proteoglycans, it might be possible to define the molecular hierarchies that control the spatial distribution and turnover of ECM and that mediate the effects of ECM on cells. The biological effects of a purified protein on cell cultures in vitro might be significantly different from the function of the protein in vivo; therefore, it becomes important to study the role of ECM proteins in a context as similar to the natural state as possible.

Our present results in *Mov*-13 mice constitute a clear demonstration of the interaction between two ECM proteins in vivo: SPARC and type I collagen. It is likely that similar types of interaction between proteins of the ECM will contribute to the phenotype of mice in which synthesis of other forms of ECM is ablated.

EXPERIMENTAL PROCEDURES

Animals

Mice heterozygous for the *Mov*-13 locus were mated, and the females were checked daily for a vaginal plug (day 0) (Jaenisch et al., 1983; Schnieke et al., 1983; Löhler et al., 1984; Harbers et al., 1984). Embryos were removed from the uteri of pregnant females at days 11, 12, and 13 post-coitus. For analysis of embryonic genotypes, genomic DNA was extracted from the posterior limbs and tail of each embryo, digested with *Eco*RI, subjected to electrophoresis, blotted onto nylon membranes, and hybridized with a 14 kb probe that spanned the viral insertion site of the α1(I) collagen gene. This probe identifies 14 kb or 23 kb *Eco*RI fragments in wild-type and mutant alleles, respectively (Harbers et al., 1984). The ages of the embryos were confirmed by morphological characteristics according to Rafferty (1970). Embryos were embedded in paraffin, and sagittal sections of 5 μm were used for immunohistochemical analysis.

Isolation of Embryonic Fibroblasts

The dermis was dissected from day 13 embryonic skin, minced in small pieces, and incubated, with agitation, in a solution of versene containing 0.5% trypsin for 30 min at room temperature. Trypsin was inactivated with an equal volume of Dulbecco's Modified Eagle's Medium (DMEM) that contained 10% fetal calf serum (FCS). Free cells and tissue fragments were centrifuged and were washed several times with DMEM/10% FCS supplemented with penicillin G, streptomycin SO_4, and fungizone. Isolated cells were plated on tissue culture dishes coated previously with 50 μg/ml fibronectin (Telios, San Diego, CA). Cells were allowed to attach for 1–2 hr, and the adherent cells were grown for 2–3 days in DMEM/10% FCS/antibiotics prior to use.

Immunohistochemistry and Immunocytochemistry

The upper half of each embryo was fixed by immersion in methyl-Carnoy's solution for 1–3 hr. A sagittal incision was made in 13 day embryos to ensure adequate penetration of fixative. Immunolocalization was performed following procedures previously described for cells and embryos (Sage et al., 1989; Iruela-Arispe et al., 1991b). The primary antibodies included: (1) a rabbit polyclonal antibody against a peptide sequence from the carboxyl terminus of murine SPARC (NH_2-

TCDLDNDKYIALEEWAGCFG) (Lane and Sage, 1990), (2) a rabbit polyclonal affinity-purified antibody against intact murine SPARC (Sage et al., 1989), (3) a guinea pig polyclonal antibody against rat tail type I collagen (Iruela-Arispe et al., 1991b), (4) a rabbit polyclonal antibody against cellular fibronectin (Iruela-Arispe et al., 1991b), and (5) a rabbit polyclonal antibody against type III collagen (Iruela-Arispe et al., 1991b).

For immunohistochemistry, immune complexes were localized with an avidin-biotin-peroxidase amplification system (Vector Laboratories, Burlingame, CA), and tissues were counterstained with 1% toluidine blue. Immune complexes in cultured cells were localized with fluorescein isothiocyanate-conjugated second antibodies (Vector). Photomicrographs were taken on an Axiophot microscope equipped for epifluorescence.

Northern Blot Analysis

Total RNA was purified from homozygous (*Mov/Mov*), heterozygous (*Mov/+*), and wild-type (+/+) embryonic fibroblasts as described by Chomczynski and Sacchi (1987). RNA was fractionated by electrophoresis on 1.2% denaturing agarose gels and was transferred to Nytran membranes by vacuum blotting (Schleicher & Schuell, Keene, NH). Blots were prehybridized for 3–5 hr and were hybridized consecutively with cDNA probes specific for (1) murine SPARC (Iruela-Arispe et al., 1991b), (2) human α1(I) collagen (Iruela-Arispe et al., 1991b), (3) human glyceraldehyde 3-phosphate dehydrogenase (G3PDH), and (4) the 28S ribosomal subunit (Iruela-Arispe et al., 1991a). cDNA inserts were purified from vector sequences, radiolabeled, and hybridized to blots as described previously (Iruela-Arispe et al., 1991a). Filters were washed in a solution containing 15 mM sodium chloride and 1.5 mM of sodium citrate at 60°C and were placed in contact with X-ray film. Autoradiographic images were quantified with the ImageQuanta program on a computerized densitometer (Molecular Dynamics, Sunnyvale, CA). All values were normalized to the 28S rRNA signal.

Metabolic Labeling and Western Blot Analysis of Secreted Proteins

Fibroblasts from *Mov/Mov, Mov/+*, or +/+ animals were grown to 80% confluence and were preincubated for 30 min in serum-free DMEM containing 50 μg/ml sodium ascorbate and 64 μg/ml β-aminopropionitrile (GIBCO/BRL, Grand Island, NY). Cultures were subsequently incubated in fresh medium containing 50 μCi/ml L-[2, 3, 4, 5, ^{3}H]-proline (100 Ci/mol, New England Nuclear, Boston, MA). After 18 to 20 hr, media containing radiolabeled proteins were removed and were subsequently mixed with proteinase inhibitors (1 μg/ml pepstatin A, 10 mM N-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride). Conditioned media were centrifuged to remove cell debris, dialyzed against 0.1N acetic acid, and lyophilized. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), radiolabeled proteins were solubilized in sample buffer, heated at 95°C for 3 min in the presence of 5 mM dithiothreitol, and separated on an 8% acrylamide gel (Laemmli, 1970). Proteins resolved by SDS-PAGE were stained with Coomassie-brilliant blue and the gels were incubated in Enhance™ (New England Nuclear) prior to drying and autoradiography.

For Western blot analysis, proteins from conditioned medium, prepared in the absence of radiolabel, were resolved on SDS-PAGE gels and were transferred to nitrocellulose. Blots were incubated for 2 hr at room temperature in MT-buffer (PBS, pH 7.7, containing 1% nonfat dry milk and 0.05% Tween-20) prior to the addition of primary antibodies. Immune complexes were identified with 0.5 μCi/ml [^{125}I]-protein A (New England Nuclear) in MT-buffer. Radiolabeled complexes were detected as autoradiographic images on X-ray film exposed at −70°C with two intensifying screens (Kodak,Rochester, NY).

Isolation of Insoluble ECM

Mov-13 fibroblasts from homozygous and heterozygous littermates were plated onto coverslips for 2 weeks. Cells were removed after a 5 min incubation with 0.5% Triton X-100 in DMEM, and the underlying matrix was either fixed in methyl-Carnoy's fixative for immunocytochemistry, or was extracted with an SDS buffer (Laemmli, 1970) for protein analysis by SDS-PAGE. ECM that was resistant to Triton X-100 was stained routinely with anti-β tubulin antiserum to assess the efficiency of the removal of cell membranes. β-tubulin-positive material was never observed in these preparations.

Cell Contraction Assays

We used a collagen gel contraction assay to compare the remodeling of type I collagen by *Mov/Mov* and *Mov/+* murine embryonic fibroblasts (Reed et al., 1994; Vernon et al., 1995; Vernon and Sage, 1996). Twenty-four well tissue culture plates (wells were 15 mm^2 in diameter) were made non-adhesive with a coating of 1% agarose (Sea-Kem LE; FMC BioProducts, Rockland, ME). One volume of a solution of 3 mg/ml bovine type I collagen (Vitrogen™, Celtrix Corp., Palo Alto, CA) was combined with 1/6 volume of 7 × DMEM and was adjusted with 1 × DMEM to yield gels with final collagen concentrations of 1, 0.75, 0.5, 0.375, or 0.25 mg/ml. Suspensions of fibroblasts at 2 × 10^6/ml in DMEM were combined with 9 volumes of the type I collagen solutions, made 2% with FCS, dispensed into the agarose-coated wells (500 μg/well), and gelled for 2 hr at 37°C. DMEM (500 μl) with 2% FCS was subsequently added to float the collagen disks. Areas of gels were calculated from the average of two measurements made at 90° angles.

To test the effect of SPARC on collagen gel contraction, we suspended fibroblasts in 11 mm diameter gels of 0.375 mg/ml collagen with 2% FCS, with or without 1.3 μM SPARC (human platelet osteonectin, Hemato-

logic Technologies, Essex Junction, VT). Gels were floated in DMEM + 2% FCS with or without added SPARC, maintained in vitro for 18–24 hr at 37°C, and measured for area determination. In selected experiments, collagen gels and floatation solutions were supplemented with antibodies against oligopeptides that represented regions within SPARC domains I or IV (Lane and Sage, 1989). As controls, either DMEM or the IgG fraction of normal rabbit serum was substituted for the anti-SPARC antibodies.

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