REGULATION OF SPARC EXPRESSION AND FUNCTION DURING EARLY EMBRYONIC DEVELOPMENT

by

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ABSTRACT

Migratory and signalling pathways require the interplay of extracellular and cell-surface molecules which govern many aspects of cell differentiation and tissue morphogenesis. The extracellular matrix glycoprotein SPARC is expressed by many tissues as they undergo rapid proliferation, differentiation, and morphogenesis. In vitro studies have also demonstrated that SPARC interferes with cell-substratum interactions and modulates cell-cycle progression. The present study examines the role that SPARC plays during the embryonic development of *Xenopus laevis*.

*In situ* hybridization and immunohistochemistry were used to examine the relationship between SPARC localization and important developmental events. SPARC expression was abundant, though transient, within the early axial mesoderm, particularly the notochord and the somites. The spatial-temporal localization of SPARC within the somites paralleled the presence of gap junctions which are believed to play a role in the electrical coupling of pre-innervated *Xenopus* somites. A lack of correlation between SPARC transcript expression and protein localization was observed within the neural tube.

The effects of ectopic SPARC expression on early developmental events was examined. Ectopic introduction of SPARC RNA, protein and peptides resulted in a number of shared developmental phenotypes, most prevalent of which was a reduction on embryonic axis. A
peptide representing the EF-hand related calcium-binding domain of SPARC produced the most dramatic and dose-dependent effects. Though exterior morphological criteria suggested that gastrulation was not affected, resultant headless or axis-reduced embryos indicated that important events, such as cell-cell signalling, may have been affected. Other SPARC peptides which displayed potent effects on cellular activities \textit{in vitro} had no effects on early development.

The regulation of SPARC was also examined. SPARC expression by ectodermal animal cap cells required cell-cell interaction. Mesoderm inducers, such as activin, elevated SPARC expression within intact animal cap explants. \textit{Xenopus} genomic sequences were identified and analysis revealed that the intron-exon organization of the 5' regulatory region of \textit{Xenopus} SPARC was similar to that of mammalian SPARC. Though overall similarity between \textit{Xenopus} and mammalian regulatory sequences was very low, several individual regulatory elements were conserved. The overall importance of proper SPARC expression and function during early development is discussed.
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**General Introduction**

The development, stability and functioning of tissues depend on dynamic interactions and communication between cells and their extracellular matrix (ECM) environment. Nowhere is this more dramatically illustrated than during early embryonic development. Not only do ECM macromolecules form elaborate three-dimensional frameworks to establish tissue patterns and boundaries, but they also play critical roles in regulating cell proliferation, adhesion, migration, and differentiation. Consequently, a dynamic reciprocal dialogue is established whereby secretions and the organization of the extracellular matrix in turn influence the activity of secreting cells, promoting a permissive environment for further growth and development. ECM macromolecules are divided into four classes based on shared structural motifs; collagens, elastins, proteoglycans and glycoproteins. The glycoproteins, of which SPARC (Secreted Protein, Acidic, Rich in Cysteine) is a member and the focus of my thesis, represent a complex class of structurally and functionally diverse macromolecules. This general introduction will begin by examining some of the important roles that ECM glycoproteins play during early embryonic development. This will be followed by an overview of key aspects of *Xenopus laevis* development and its advantages for use as an embryological model. Specific aspects of *Xenopus* development, such as somitogenesis, will be highlighted as they are important to the discussion of SPARC function. Lastly, a detailed examination of the structure, physicochemical binding properties, and putative functions of SPARC will be presented.

**Extracellular matrix glycoproteins during early embryonic development**

At the turn of the century, cell membranes and the surrounding ECM were believed to be inert structures unimportant for cellular activities. If anything, the ECM was viewed by some
as an unfortunate nuisance that complicated analysis of intracellular molecules. However as investigators began to more closely examine events such as axonal (Weiss, 1934) and cell migration (Townes and Holtfreter, 1955), it became apparent that matrix macromolecules played dynamic structural and informational roles. With the advent of more sophisticated techniques, matrix glycoproteins have been demonstrated to have important functions in a number of morphogenetic processes. For example, laminin promotes the differentiation of polar endothelial cells into tubular structures (Grant et al., 1989), neural differentiation of the central nervous system (David et al., 1995) and neural crest cell migration (Lallier and Bronner-Fraser, 1991; Bronner-Fraser 1993). Addition of thrombospondin and tenascin to neurons promotes axonal differentiation and migration (O’Shea and Dixit, 1988; Chiquet et al., 1990; Wehrle and Chiquet, 1990). Interference with fibronectin binding to integrin receptors leads to arrest of gastrulation in axolotls (Boucaut et al., 1984)

Matrix glycoproteins also have the ability to: (1) sequester and/or release ECM macromolecules, growth factors and cytokines (Rifkin et al., 1991; Klagsburn, 1992; Miao et al., 1996), (2) modulate cell cycle progression (Templeton, 1992), and (3) via binding to cell surface receptors (such as integrins, Tuckwell et al., 1993), modulate intracellular signalling events, changing the phenotypic character and function of the target cells (Lin and Bissell, 1993). The latter provides a means whereby ECM macromolecules can not only regulate their expression, but also the synthesis and release of enzymes which participate in the turnover and remodelling of the matrix (Canfield et al., 1990; Streuli and Bissell, 1990). Remodelling of the ECM is not only essential for growth, but also for tissue repair (Reed et al., 1993) and wound healing (Mackie et al., 1988; Dostal and Gamelli, 1993).
An Overview of Embryonic Development

The development of multicellular organisms begins with a fertilized egg dividing into embryonic cells which in many animals are initially morphologically indistinguishable. As a result of maternal and/or embryonic signals, the embryonic cells progress towards specific fates, eventually leading to the formation of highly differentiated cell types and tissues. Our understanding of the molecular and cellular events underlying the early embryological development of multicellular organisms is a result of the use and study of a broad spectrum of organisms, each of which afford certain biological and technical advantages for embryological research. Several advantages of using *Xenopus laevis* are (1) the large size of the eggs, (2) the abundance of eggs, and (3) synchronous development following *in vitro* fertilization. In addition many of the developmental stages of the embryos can be readily identified and experimentally manipulated. For example, the morphogenetic contributions of different macromolecules are often assayed by the microinjection of RNA, gene-plasmid constructs, antibodies, cytokines, etc. into oocytes and embryos. Other micromanipulatory approaches include the use of (1) animal cap assays to examine inductive interactions, (2) cell and tissue transplantations within and between embryos to study inductive and other cell-cell interactions, (3) oocyte extracts to examine cell cycle progression, and (4) embryonic extracts to examine transcription regulation (see Kay and Peng, 1991). As a result many inductive agents and developmental pathways have been identified and characterized using *Xenopus* (reviewed in Dawid and Taira, 1994). Moreover, recent advances in frog transgenics has expanded the technical repertoire of *Xenopus* as a developmental model (Kroll and Amaya, 1996).
Inductive centres and events during early Xenopus development

Maternal molecules, which will play important roles in inductive signalling, begin to be localized during oogenesis in *Xenopus*, so that a prepattern exists before fertilization (Thomson and Melton, 1993). Following fertilization formation of dorsal structures is established, in part, through pathways that involve the participation of the wnt, glycogen synthase kinase and β-catenin pathway (Cornell et al., 1995; Dominguez et al., 1995, Yost et al., 1996). The TGF-β and bFGF family of growth factors then work in concert to pattern the differentiation of embryonic cells that give rise to mesodermal tissues, such as notochord and somites (Green et al., 1992), and may also be involved in the preliminary induction and patterning of early neural tissues (Hemmati-Brivanlou and Melton, 1994). The region within the embryo where this dorsal and mesodermal patterning occurs is termed the "Nieuwkoop centre" (reviewed by Slack, 1994, Jones et al., 1996). The critical cell-cell interactions and movements of gastrulation are centered around this region. Subsequent patterning have been shown to involve other macromolecules, such as products of the goosecoid, brachyury and hedgehog families of genes. (Steinbeisser at el., 1993; Johnson et al., 1994: Parr and McMahon, 1995). These inducing signals/patterns also compete with ventralizing signals provided by a subset of the BMP (Bone Morphogenic Protein) family of growth factors (Dale et al., 1992). Thus through the localization, interplay and overlap of several factors, gradients are created within the embryo which guide the patterning of both dorsal-ventral and anterior-posterior axes.
Xenopus somitogenesis

Shortly after gastrulation, embryonic cells which give rise to somites can be distinguished morphologically from the remainder of the mesoderm (Hamilton, 1969). Segmentation of individual somites proceeds as a wave from the head to the tail. In most vertebrates, mesodermal cells form condensations which give rise to somites composed of three tissue types, dermatome, sclerotome and myotome (Carlson et al., 1981). In Xenopus, the process of somitogenesis has several distinguishing features. The cells which give rise to the dermatome do not segment, but instead form an epithelial-like sheet which lies lateral to the segmented myotomes. During early embryonic stages, there are very few sclerotome cells associated with the segmented somites. Myotomes therefore, make up the majority of the somites. The mechanism of Xenopus myotome segmentation itself also has unique feature (Radice et al., 1989). The large, rectangular myotomes are initially perpendicular to the dorsal axis of the embryo. In a head to tail wave, groups of myotomes rotate 90° so that they now lie parallel to the body axis. As a result, stacks of segmented myotomes, which can initiate contractions, now lie end to end along the axis. In most organisms, myotome segmentation and the first embryonic muscle contractions are preceded by a complex series of events, that include cell divisions, cell migrations and rearrangements, fusion, and the formation of neuromuscular junctions. Relative to other organisms, Xenopus myotome segmentation and function occur very early. As a result, within 48 hours after fertilization, Xenopus embryos can swim (Nieuwkoop and Faber 1956) and escape predators. Many other species, including frog, take several days to weeks before synchronized muscle contractions occur.
At the time when the embryos first begin to swim, innervation of trunk myotomes has not yet been completed, raising the question as to how myotome contractions are controlled. An explanation lies in data generated from the elegant work of Blackshaw and Warner (1976) who demonstrated that as the wave of somite segmentation progressed, the myotomes became electrically coupled to each other via gap junctions. They hypothesised that contractions in the early embryo are initiated by neural signalling of the unsegmented head somites. The depolarization signals were then propagated down the embryonic axis through the inter- and intra-somitic gap junctions which couple the myotome. When innervation of the myotomes was completed and neural impulses controlled the contractions, gap junctions decreased dramatically. The use of gap junctions to propagate muscle contractions is not unique to *Xenopus* myotomes. Heart muscle contractions, which are initiated at a node, are propagated principally through gap junctions which couple the cardiac muscle cells (Green and Severs, 1993; Sun et al., 1995).
**SPARC Overview**

SPARC was first isolated in 1981 from bovine bone. As a result of its enrichment in bone, ability to bind calcium and inhibit hydroxyapatite growth *in vitro*, it was first called osteonectin (Termine et al., 1981). However, it soon became evident that matrix glycoprotein termed BM-40, isolated from Engelbreth-Holm-Swarm (EHS) mouse tumour cells (Dziadek et al., 1986), a glycoprotein isolated from bovine aortic endothelial cells (Sage et al., 1986), and SPARC isolated from mouse parietal endoderm (Mason et al., 1986) were the same protein.

SPARC is a 43 kDa secreted matrix glycoprotein with a core molecular weight of ~30 kDa (~300 amino acids). SPARC sequences have been isolated from an evolutionarily diverse group of organisms, ranging from human, mouse, pig, cow, frog, chicken and nematode (Termine et al., 1981; Mason et al., 1986; Domenicucci, et al., 1988; Swaroop et al., 1988; Damjanovski et al., 1992; Schwarzbauer and Spencer, 1993). Mammalian SPARCs share between 95 and 98% amino acid similarity, while identities to other SPARCs (compared to mouse SPARC) range between 78% (frog) and 85% (chicken) and 37% (nematode). The primary amino acid sequences of SPARC can be divided into three structural domains; (1) an N-terminal glutamic acid-rich domain, (2) a cysteine-rich domain, in which 11 cysteine residues are perfectly conserved in all vertebrates; and (3) a C-terminal EF-hand related calcium binding domain (Lane and Sage, 1994).

*In situ* hybridization and immunocytochemical studies have shown that SPARC is expressed at high levels by many embryonic tissues undergoing rapid morphological development, differentiation and remodelling, e.g. during neurogenesis, somitogenesis,
chondrogenesis, osteogenesis and angiogenesis, but is not detectable in development of the intestine, liver and kidneys (Sage et al., 1989a; Ringuette et al., 1992, Damjanovski et al., 1994). Injection of SPARC antibodies into embryos, or ectopic overexpression of SPARC, leads to severe defects and/or developmental arrest, reflecting the fact that SPARC mis-expression can be deleterious to embryogenesis (Purcell et al., 1993; Schwarzbauer and Spencer, 1993). Though the precise function(s) of SPARC during embryonic development remains to be established, numerous in vitro binding and tissue culture studies indicate that like most matrix glycoproteins, it has multiple activities. SPARC is a potent inhibitor of calcium-phosphate mineral growth in vitro (Romberg et al., 1986; Maurer et al., 1992), raising the possibility that SPARC may inhibit tissue mineralization, a role which is likely to be carried out by the glutamic acid-rich N-terminal domain which can bind 5-8 Ca^{2+} ions. SPARC also binds to and inhibits the action of platelet derived growth factor (PDGF) (Raines et al., 1992). Even though SPARC has no affinity for transforming growth factor beta (TGF\(\beta\)), it blocks binding of TGF\(\beta\) to its receptors (Hasselaar et al., 1992). Therefore, SPARC may have important functions in regulating cell proliferation by buffering the effects of these cytokines. In vitro studies have demonstrated that SPARC inhibits the G1 to S transition during the cell cycle by an unknown mechanism (Funk and Sage, 1991). Data also indicate that SPARC has the potential to regulate the expression of matrix-degrading proteases and their cognate inhibitors (Tremble et al., 1993). SPARC inhibits cell spreading of endothelial, fibroblast and smooth muscle cells in culture by decreasing the number of focal contacts. These observations indicate that SPARC is a counter-adhesive glycoprotein like tenascin and thrombospondin (Hay 1991). Through the use of synthetic peptides, the
counter-adhesive ability of SPARC has been attributed to regions within the N-terminal and C-terminal domains (Lane and Sage, 1994). Thus, SPARC can be envisioned to regulate cell shape and migration \textit{in vivo} by modulating cell-matrix contacts. The diversity of functions exhibited by SPARC \textit{in vitro} may allude to equally diverse functions during development.
**Approach**

My M.Sc. research data (Damjanovski 1991) demonstrate that SPARC is highly conserved between amphibians and mammals (Ringuette et al., 1991), indicating that its functions are also likely to be conserved in evolution. My doctoral research has been focused on examining the functions of SPARC by exploring its contribution to early embryonic development. Using *Xenopus* as a model, three approaches were used: (1) whole mount *in situ* hybridization and immunohistochemistry, (2) ectopic expression and (3) analysis of factors controlling SPARC activation and expression. The logic and details of these approaches are detailed in the following three chapters. Since the chapters represent data presented in multi-authors publications, my contribution is outlined in appendix A.
Chapter 1

Transient Expression of SPARC in the Dorsal Axis of Early Xenopus Embryos: Correlation with Calcium-Dependent Adhesion and Electrical Coupling
Abstract

Our comprehension of embryonic development has been greatly enhanced by the identification and characterization of associated extracellular matrix macromolecules. Using *Xenopus laevis* as a model, the expression and distribution of SPARC (Secreted Protein, Acidic, Rich in Cysteine; also called osteonectin and BM-40) during early embryonic development was investigated. SPARC has been found to be enriched in tissues undergoing rapid morphological development, differentiation, and remodelling. In *Xenopus*, SPARC transcripts were first expressed by embryonic cells which give rise to the first embryonic tissues, the notochord and somites. SPARC RNA levels remained high throughout the rapid morphological development and differentiation phase of these tissues, and then rapidly decreased. Of particular interest, SPARC protein began to accumulate within the intersomitic clefts at the onset of trunk myotome contraction. The intersomitic enrichment of SPARC remained high as long as the myotomes remained electrically coupled, principally by gap junctions. As myotomes became innervated and the number of gap junctions decreased, SPARC expression decreased dramatically within the somites. SPARC was also found to be enriched within other gap junction-rich tissues, such as the neural tube, epidermis and heart primordia. In addition to gap junction communication, several of the morphogenetic events underlying early embryonic development have been shown to be modulated by calcium. The selective enrichment of SPARC during these events suggests it makes an important calcium-dependent contribution to early morphological development.
Introduction

Several calcium-binding transmembrane and extracellular matrix (ECM) glycoproteins have been identified which have important structural and morphoregulatory functions during embryonic development (Hay, 1991; Adams and Watt, 1993). For example, selective cell-cell aggregation and sorting is in part promoted by cadherins, a family of calcium-dependent transmembrane cell adhesion receptors (Takeichi, 1991). When tissue culture cells expressing various cadherins are mixed together, those expressing the same cadherins preferentially aggregate (homophilic binding). Since an embryonic tissue is usually enriched in one type of cadherins, homophilic binding is likely to be an important in vivo mechanism for tissue assembly and stability. In addition to cadherins, laminin, (LM), a major glycoprotein constituent of basement membranes, may also make use of calcium to promote cell-substratum adhesion. At low laminin concentrations, binding to neural crest cells via a subset of integrin receptors is divalent cation independent. However at high LM concentrations, not only does the LM aggregate appear to bind to a different subset of integrin receptors, but binding requires divalent cations such as Ca$^{2+}$ or Mn$^{2+}$ (Lallier and Bronner-Fraser, 1991). Fibulin (FB), a newly identified calcium-binding glycoprotein which binds to fibronectin [FN (Argraves et al., 1989; Pan et al., 1993)], is expressed within the neural tube, cardiac primordia and somites. Although the function of FB in these tissues is unknown, it is of particular interest that it is enriched in basement membranes where epithelial-mesenchymal transformations are occurring. Another calcium-binding ECM glycoprotein expressed at high levels during early embryonic development, is SPARC [also called osteonectin, BM (Basement Membrane Protein)-40 (Termine et al., 1981; Dziadek et al., 1986; Mason et al.,
SPARC is a 43 kDa calcium-binding glycoprotein which is evolutionarily conserved among mammals, amphibians, and nematodes (Termine et al., 1981; Mason et al., 1986; Swaroop et al., 1988; Damjanovski et al., 1992; Schwarzbauer and Spencer, 1993). Analysis of amino acid sequences derived from cDNA sequences demonstrates that amphibian and nematode SPARC share 80% and 38% sequence identity, respectively, to mammalian SPARC. In situ hybridization and immunocytochemical studies have shown that SPARC is expressed at high levels by many embryonic tissues undergoing rapid morphological development, differentiation and remodelling, e.g. neurogenesis, somitogenesis, chondrogenesis, osteogenesis and angiogenesis, but is not detectable in development of the intestine, liver and kidneys (Sage et al., 1989a; Ringuette et al., 1992; Damjanovski et al., 1994). Injection of anti-SPARC antibodies into developing embryos, or ectopic overexpression of SPARC, leads to severe developmental defects and/or developmental arrests, reflecting a critical requirement for SPARC during embryonic development (Purcell et al., 1993; Schwarzbauer and Spencer, 1993).

As a rule, most ECM macromolecules have multiple morphogenetic roles during early embryonic development. Although the precise function(s) of SPARC during early embryonic development remains to be established, numerous in vitro binding and tissue culture studies indicate that it is also a multifunctional protein. SPARC has an affinity for collagens and hydroxyapatite and is a potent inhibitor of calcium-phosphate growth in vitro (Romberg et al., 1986) raising the possibility that SPARC may inhibit tissue mineralization. SPARC may have important functions in regulating cell proliferation by binding to and buffering the effects
of cytokines (Hasselaar et al., 1992; Raines et al., 1992). SPARC can regulate the expression of matrix-degrading proteases and cognate inhibitors (Tremble et al., 1993) and inhibits the spreading and focal contacts of cells in culture, indicating that SPARC may play a role in facilitating migration of cells through the ECM. Synthetic peptides indicate that the anti-adhesive ability of SPARC can be attributed to its N-terminal and C-terminal domains (Sage et al., 1989b). Thus, SPARC can be envisioned to regulate cell shape and migration *in vivo* by modulating cell-matrix contacts. In order to assess if any of the above functions observed *in vitro* reflect *in vivo* SPARC functions during embryonic development, we have examined its precise spatiotemporal distribution in early *Xenopus* embryos. Previous studies in our laboratory had demonstrated that SPARC transcripts are first expressed by mid-gastrulation/early neurula embryos (Ringuette et al., 1992). Both transcripts and protein were found to be particularly enriched in the dorsal axis. By whole mount *in situ* hybridization and immunohistochemical analysis we now report that SPARC undergoes dramatic changes in expression during axial development and decreases significantly once axial structures are formed. SPARC levels are also found to be significantly high during the initial phases of the morphogenetic development and differentiation of other tissues.
Materials and Methods

Embryo rearing

Albino *Xenopus* embryos were fertilized and grown as follows. Three days prior to ovulation, mature *Xenopus* females were primed with a subcutaneous injection of 50 units of pregnant mares serum (Sigma). About sixteen hours prior to ovulation, the females were injected with 500-700 units of human chorionic gonadotropin and were placed at 15°C overnight. The following morning, egg-laying was facilitated by squeezing the female. For fertilization, a fragment of fresh *Xenopus* testis was macerated in a small volume (1 ml) of 80% Steinberg’s (100% Steinberg’s=60 mM NaCl, 0.67 mM KCl, 0.34 mM Ca(NO₃)₂, 0.83 mM MgSO₄, 10 mM Hepes pH 7.4) in a Petri plate. Eggs were squeezed directly into the macerated testis, gently mixed, and left undisturbed for 5 minutes. The egg/sperm mix was then flooded with an excess of 20% Steinberg’s (50 ml) and left undisturbed for a further 20 minutes. Fertilized eggs were then dejellied. Eggs were transferred into a solution of 3% L-cysteine at pH 8 in which they were gently agitated until no signs of a jelly coat remained between them. The fertilized eggs were then rinsed with several (5-6) washes of 20% Steinberg’s and transferred into a Petri plate in fresh 20% Steinberg’s in which they were reared. Following the first cell cleavage, abnormal or dead embryos were removed.

Albino *Xenopus* embryos staged according to Nieuwkoop and Faber (1956) were used. Following removal of the vitelline membrane with fine forceps, embryos were fixed in 10 ml MEMPFA (0.1 M MOPS, 2 mM EGTA, 1 mM MgSO₄, 3.7% paraformaldehyde) for 1-2 hours. The fixative was then replaced with two washes of methanol, in which the embryos were stored at -20°C.
Whole mount *in situ* hybridization

Whole mount *in situ* hybridization was performed as described in Harland (1991).

RNA probe synthesis

A 460 bp EcoRI fragment of the 5' region of *Xenopus* SPARC in the EcoRI site of pGEM4Z (Damjanovski et al., 1992) was used to generate an RNA probe. Digoxigenin-labelled sense and anti-sense transcripts were generated using SP6 and T7 viral promoters as follows. The *Xenopus* SPARC-plasmid construct was linearized on either side of the SPARC insert using either PstI or HindIII for use with SP6 and T7 RNA polymerase, respectively. Subsequent to the restriction enzyme linearization, the DNA was phenol/chloroform extracted and precipitated with 1/10 volume 3 M sodium acetate and 2.5 volumes of ethanol. Complete digestion of the DNA was confirmed by agarose gel electrophoresis of a small sample of the restricted DNA. Following optical density quantitation of the DNA, the appropriate volume was used to generate labelled transcript. The following reagents were combined: 3.5 µl linearized DNA template (1 µg/µl), 4 µl dNTP mix [2 mM each ATP CTP GTP, 1.25 mM UTP, and 3.5 µl DIG UTP (25 nmol-Boehringer 1209 256)], 6 µl dH₂O, 2 µl dithiothreitol (250 mM), 0.5 µl RNase inhibitor (20 u/µl), 2 µl 10X transcription buffer (Boehringer), and 2 µl SP6 or T7 RNA polymerase (=90 units). The mixture was incubated at 37°C for 2 hrs. To remove the DNA template, 1 µl DNase 1 (1 mg/ml) was added and the mixture incubated for a further 15 minutes at 37°C. The reaction was terminated with the addition of 80 µl of 1% SDS, 20 mM Tris pH 7.5, 20 mM EDTA, 100 mM NaCl. To confirm transcription, 3-4 µl of sample were examined after agarose gel fractionation. The remaining transcript was then precipitated with 10 µl 5 M ammonium acetate and 220 µl cold ethanol. The transcript
was hydrolysed with a 35 minute incubation in 40 mM sodium bicarbonate, 60 mM sodium carbonate at 60°C. The size of the hydrolysed fragment was confirmed using agarose gel electrophoresis of an aliquot of the sample. The hydrolysed probe was then precipitated with 200 µl H₂O, 25 µl 3 M NaOAc and 600 µl cold ethanol. The pellet was then resuspended in 500 µl of hybridization buffer (see later) and stored at -20°C.

Type 8 cytoskeletal actin anti-sense cRNA probe (Mohun and Garrett, 1987) was used as a control.

Hybridization.

Embryos stored in methanol were rehydrated (in small glass vials) 5 minutes each in 75%, 50%, and 25% methanol, and four final washes with PBS (pH 7.4) 0.1% Tween-20. This was followed with 4 washes in PBS (pH 7.4) 0.1% Tween-20 (PTw). Samples were then treated with proteinase K (5 µg/ml) for 15-20 minutes at room temperature. Embryos were then rinsed 2 times in 5 ml 0.1 M triethanolamine for 5 minutes each rise. To the final 5 ml of triethanolamine, 12.5 µl of acetic anhydride was added, and incubated for a further 5 minutes. Subsequently a further 12.5 µl of acetic anhydride was added for 5 minutes. Samples were then washed 2 times in PTw for 5 mins each and refixed for 20 minutes in 4% paraformaldehyde in PTw (pH 7.4). Samples were then washed 5 times 5 minutes each with PTw. Samples were left in 1 ml of the PTw to which 250 µl of hybridization buffer was added [50% formamide, 5X SSC, 1 mg/ml tRNA, 0.1X Denhart's, 0.5 mM EDTA, 100 µg/ml heparin, 0.1% Tween 20, and 0.1% CHAPS] and incubated at room temperature for 5 minutes. The buffer was removed and a fresh 0.5 ml of hybridization buffer was added and incubated at 60°C for 10 minutes. Fresh hybridization buffer (0.5 ml) was added and
incubated for a further 6 hours at 60°C. This mix was replaced with 0.5 ml of hybridization buffer containing 25 μl of probe (1:20 dilution) and left incubating overnight at 60°C.

Washes

Probe was removed and replaced with 1 ml fresh (preheated) hybridization buffer 10 mins at 60°C. Samples were then washed in 1 ml 50% hybridization buffer: 50% 2XSSC, 0.3% CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate), 60°C, 10 minutes, followed by 1 ml 25% hybridization buffer: 75% 2XSSC (1XSSC=75 mM NaCl, 50 mM sodium citrate), 0.3% CHAPS for 10 minutes, at 60°C. Two 20-minute washes in 2XSSC, 0.3% CHAPS then followed during which time the temperature was slowly lowered to 37°C. 1 ml of 2XSSC, 0.3% CHAPS, RNAse A (20 μg/ml), RNAse T1 (10 U/ml) was then added for 30 mins at 37°C. A 10 minute room temperature 2XSSC 0.3% CHAPS wash was then followed by two 0.2XSSC, 0.3% CHAPS washes for 30 minutes at 60°C. Samples were then washed twice in PTw 0.3% CHAPS for 10 minutes at 60°C followed by a room temperature wash in PTw for 10 minutes.

RNA probe detection

Embryos in glass vials were then incubated with 1 ml PBT (PBS, 2 mg/ml BSA, 0.1%triton X-100) for 15 minutes at room temperature. Fresh PBT (1 ml) with 20% heated (55°C, 30 mins) lamb serum was then added for 1 hour at room temperature. This blocking mix was replaced with 0.5 ml of fresh PBT, 20% serum containing 2.5 μl sheep anti-DIG antibody coupled to alkaline phosphatase (Boehringer 1093 274). Samples were left gently rocking overnight at 4°C. Samples were then washed at least 5 times at room temperature, in PBT followed by one overnight wash at 4°C. Samples were then transferred into 1 ml
alkaline phosphatase buffer (100 mM Tris pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween 20, 5 mM levamisol) for 5 minutes. Following a fresh 5-minute wash in alkaline phosphatase buffer, 4.5 µl NBT (4-nitro blue tetrazolium) and 3.5 µl BCIP (5-bromo-4-chloro-3-iodolyl-phosphate) per ml was added (NBT 75 mg/ml in 70% dimethyl formamide, BCIP 50 mg/ml in 100% dimethyl formamide). The mix was incubated at room temperature and examined periodically. Following the colourmetric reaction embryos were fixed in Bouin's fixative overnight (Bouin's = 1 g picric acid in 75 ml H₂O, 25 ml 37% formaldehyde, 5 ml glacial acetic acid). Refixed embryos were dehydrated in methanol and cleared in a 2:1 mix of benzyl benzoate and benzyl alcohol.

For sectioning, in situ stained, cleared embryos were placed back into methanol. These dehydrated embryos were embedded in molten (55°C) paraplast overnight before they were hardened, and 30 µm sections were taken. These sections were placed on slides, dewaxed using xylene and mounted with Permount (Fisher Scientific) for examination.

**Whole mount immunohistochemistry.**

Embryos were fixed at room temperature for 2 hours using 25% DMSO in methanol. Embryos were washed and blocked as follows: 2 times 30 minutes in PBS, 0.1% triton-X100, 2 times 1 hour PBS, 0.1% Triton-X100, 0.1% BSA, 0.1% dry milk powder at room temperature. All subsequent washes and incubations were at 4°C. Anti-human SPARC monoclonal anti-body ON3 (Breton-Gorius et al., 1992), which cross-reacts with *Xenopus* SPARC (Ringuette et al., 1992) was diluted 1:50 in PBS, 0.1% Triton-X100, 0.1% BSA, 0.1% dry milk powder. The embryos were incubated with the diluted antibody, rocking gently overnight. Embryos were then washed 5 times for 1 hour each in PBS, 0.1% Triton-
X100, and once overnight in PBS, 0.1% Triton-X100, 0.1% BSA, 0.1% dry milk powder. An alkaline phosphatase tagged goat anti-mouse secondary antibody (Boehringer-Mannheim) was diluted 1:200 in PBS, 0.1% Triton-X100, 0.1% BSA, 0.1% dry milk powder and incubated overnight with the embryos. Embryos were subsequently washed 5 times for 1 hour in PBS, 0.1% Triton-X100, and once overnight with rocking. Embryos were incubated twice, 10 minutes each in alkaline phosphatase buffer (100 mM Tris pH 9.5, 50 mM \(\text{MgCl}_2\), 100 mM NaCl, 0.1% Tween 20, 5 mM levamisol). The colourmetric reaction was achieved using NBT and BCIP at room temperature (as above). Embryos were post-fixed in Bouin's fixative for 2 hours (as above). Embryos were then dehydrated in methanol and cleared with benzyl alcohol, benzyl benzoate (BBBA) for photography. For sectioning embryos were taken from methanol, embedded in molten Paraplast, and 30 \(\mu\)m sections were taken as described above. As a control, a neural specific antibody (2G9, Jones and Woodland, 1989) was used.
Results

Expression of SPARC transcripts during early notochord and somitic development

It was demonstrated by Northern analysis that SPARC RNA was first detectable at stage 13, early neurula (Ringuette et al., 1992). Transcripts accumulated rapidly, reaching a plateau by tailbud stage 30, when 24-25 paired somites had formed. To obtain a three dimensional view of the spatiotemporal expression of SPARC transcripts, digoxigenin-labelled cRNA probes, corresponding to N-terminal glutamic acid and cysteine-rich domains of SPARC, were used for whole mount in situ hybridization. Staining was observed shortly after a notochord rod became morphologically distinct (Fig 1A). A staining gradient, decreasing in a cranial to caudal direction, was observed within the longitudinal ridges of the paraxial mesoderm by stage 18 (Fig 1A). The staining intensity was greatest within the anterior trunk region where 2-3 paired somites had formed. As the wave of somitogenesis progressed towards the posterior end, a matching shift in the gradient was also observed. By stage 25 (pre-tailbud), when somite segmentation had reached the caudal region, the anterior to posterior SPARC stain gradient was no longer visible (Fig 1B). Similar levels of staining were observed at the centre of each segmented myotome, overlapping the nuclei (Fig 1E). High levels of staining were visible within notochord and overlying neural floor plate. To confirm the distribution of SPARC transcripts, mid-trunk transverse sections of the stained embryos were performed. Analysis of sections confirmed that mRNAs were abundant within the notochord, somites and the floor plate of the neural tube (Fig 3A). However, low level staining was also observed overlapping the endodermal region fated to form the
Figure 1. Whole mount in situ localization of SPARC RNA during early Xenopus development.

A 460 bp EcoRI fragment of the 5' region of Xenopus SPARC was subcloned into pGEM-4Z, and digoxigenin-labelled transcripts were generated using the flanking viral promoters and cognate RNA polymerases. (A) Shortly after neurulation (stage 18) SPARC RNA was expressed by the notochord (n), and in the segmented (s) and unsegmented somitic mesoderm (um). (B) At stage 25, SPARC message was abundant in the notochord (n), the neural floor plate (fp), and in the somites (s). (C) By stage 33 SPARC messages were no longer evident in the notochord (n), but were abundant in the floor plate (fp) of the neural tube and subnotochordal rod (sr) of the endoderm. (D) Towards the end of somitogenesis (stage 43) the presence of SPARC message in the dorsal axis was greatly diminished. Expression was now upregulated in the anterior region of the embryo where organogenesis was progressing. (E) Closer examination of the distribution of SPARC RNA within maturing somites revealed that SPARC messages at stage 25 were localized primarily over the centrally located nuclei within each myotome (sn). Very little SPARC RNA was found near the anterior and posterior poles of the myotome adjacent to the intersomitic cleft (i). (F) As somites matured (stage 33), SPARC RNA was no longer centrally localized above the nuclei (sn), but was now abundant at the anterior and posterior poles of the somite, adjacent to the intersomitic cleft (i). As controls, Xenopus cytoskeletal actin cRNA (G) and SPARC sense cRNA probes (H) were used.
sub-notochordal rod; a staining masked by the notochord staining when viewed in whole mounts. The above data indicated that by the early tailbud stage, expression of SPARC transcripts by ectodermal and endodermal tissues was mainly restricted to tissues adjacent to the notochord.

**Transient patterns of SPARC RNA expression in the tailbud embryo**

SPARC transcripts continued to be expressed by notochordal cells until tailbud stage 27. However by stage 33, a period corresponding to the beginning of notochord cell vacuolation, mRNA expression by notochordal cells was significantly lower (Fig 1C). In contrast, increased expression of SPARC transcripts by the neural floor plate was evident. The decreased staining within the notochord made the staining of the sub-notochordal rod much more visible. Indeed, by stage 33 staining levels appeared very similar for both the neural floor plate and sub-notochordal rod, giving a double track appearance when the embryos were viewed laterally (Fig 1C). Transverse sections through the trunk of stained embryos confirmed high levels of SPARC mRNA expression by the above tissues and its virtual absence within the notochord (Fig 3B).

At higher magnifications, the non-uniform staining of somitic myotome cells of pre-tailbud stage 25 embryos was more apparent. The most intense staining within the myotomes forming the 15-16 paired somites was observed overlapping the centralized nuclei (Fig 1E). As somitogenesis progressed and spontaneous contraction of primary myotomes began, the staining intensity was gradually shifted towards the rostral and caudal poles of the somites. Staining adjacent to the intersomitic cleft reached a maximum by stage 33, when 30-32 paired somites had formed (Fig 1F) and the hatched embryos were swimming due to rhythmic
contractions of segmented myotomes.

At later stages, staining in the trunk of the embryo gradually diminished. Shortly after stage 37/38 (when migration of secondary myoblasts had begun to invade the intermyotomal septa of paraxial myotomes) staining was still visible within the lateral region of anterior somites but could not be detected along the remainder of the dorsal axis (Fig 1D). In contrast, staining began to increase within developing ventral anterior structures, such as the pharyngeal folds, gills and region of the heart primordia. Staining of the surface ectoderm was also more apparent than at earlier stages.

Control anti-sense Xenopus cytoskeletal actin cRNA probe revealed a distribution pattern distinct from that of SPARC (Fig 1G), whereas no significant staining was observed with SPARC sense cRNA (Fig 1H).

**SPARC protein expression and distribution**

Since SPARC is a secreted glycoprotein, its tissue distribution could not be predicted based solely on its pattern of mRNA expression. Therefore I made use of anti-SPARC monoclonal antibody (MAb-ON3), which cross-reacted with Xenopus SPARC, to localize by whole mount immunohistochemistry the distribution of SPARC relative to its mRNA distribution. Because of the auto-fluorescence of yolk protein enriched cells, alkaline phosphatase-tagged secondary antibodies were used to visualize ON-3 binding.

**SPARC localization during early notochord and somite morphogenesis and differentiation**

SPARC immunoreactivity was visible on the dorsal aspect of neurula embryos at stage 18 (Fig 2A) in a pattern similar to its mRNA distribution (Fig 1A). The medial dorsal notochord, which was still undergoing antero-posterior elongation and secreting an ECM sheath, showed
pronounced staining. Similarly, significant levels of staining were evident within the presomitic and somitic mesoderm. Higher levels of staining were observed in the central regions of the segmented myotomes overlapping the nuclei, with no significant accumulation at somite boundaries. The staining highlighted segmented somites as the wave of segmentation progressed towards the rostral end of the embryos. Anterior staining of the region corresponding to the eye anlage was more evident than observed by whole mount \textit{in situ} hybridization (Fig 2A).

Relative to neurula embryos (stages 14-20), significantly higher dorsal axis staining was observed during tailbud stages 20-30. Notochord staining remained high throughout its elongation phase. When stage 25 embryos were viewed dorsally (Fig 2B), no significant staining of the neural tube was evident, making the underlying stained notochord clearly visible. The 16 paired somites at this stage also expressed high levels of SPARC protein. As observed with its cognate mRNA distribution, high levels of SPARC protein were observed along the entire dorsal axis, highlighting individual somites. The protein also appeared evenly distributed along the entire length of the elongated myotomes (Fig 2E). Anterior mediolateral staining overlapping the developing eye was also pronounced, and punctate staining of the surface ectoderm was now evident (Fig 2B). When a neural specific antibody was used (Fig 2G) a distribution pattern distinct from that of SPARC was observed.

\textbf{SPARC protein distribution in tailbud and tailed embryos}

Notochord staining, which began to decrease at stage 30, was no longer visible by stage 33/34 (Fig 2C). However at stage 33, significant staining was observed throughout the neural tube, with the exception of the floor plate (Fig 3C). The absence of SPARC protein from the
Figure 2. Whole mount immuno-localization of SPARC during early *Xenopus* embryogenesis.

An anti-SPARC monoclonal anti-body (MAb-ON3), which cross-reacted with *Xenopus* SPARC, was used to localize SPARC. (A) During early somitogenesis (stage 18) SPARC was detectable within the notochord (n), the unsegmented dorsal mesoderm (um), segmented somites (s), and eye anlage (ea). (B) By stage 25 the notochord tube (n), eyes (e) and somites (s) all express high levels of SPARC. At higher magnification (E) SPARC was found within the somites, and was becoming detectable within the intersomitic cleft (i). (C) By stage 33 SPARC levels decreased in the notochord (n), but remain elevated within the neural tube (nt). The protein exhibits a striated chevron distribution along the somites. At higher magnification (F) this striation can be seen as a result of SPARC accumulation within the intersomitic clefts (i). (D) The stage 45 embryo showed decreased levels of SPARC in the dorsal axis, but increased anterior accumulation. As a control, a neural specific antibody (G) was used.
floor plate was surprising, in light of the fact that its mRNA expression in the neural tube was restricted to this region. By this stage, staining within the intersomitic cleft was higher than within myotomes, accentuating the chevron topography of the segmented somites (Fig 2F). The accumulation of SPARC within the intersomitic cleft coincided with the translocation of its cognate mRNA to the cranial and caudal poles of the elongated myotomes. The intersomitic enrichment of SPARC confirmed previous immunofluorescent studies (Ringuette et al., 1992). Staining of the surface ectoderm observed in stage 25 embryos was now much more intense and could be localized to the sensorial layer of the surface ectoderm (Fig 3C).

Immunostaining within the intersomitic clefts was still visible at stage 40/41, though SPARC RNA transcripts were absent from the myotomes approximately 24 hours earlier (Fig 2D). Within the rostral region, the pattern of SPARC expression coincided with tissues undergoing rapid development, e.g., laryngeal visceral pouches, gills, cardiac primordia, and tracheal cavity. Even though albino embryos were used, periodic albinism resulted in the pigmentation of the retinal pigmented epithelial, masking, to a degree, immunostaining in the eye. Significant immunostaining continued to be visible within the surface ectoderm, which by this stage of development was now undergoing rapid morphogenetic development and differentiation.
Figure 3. Mid-trunk cross-sectional analysis of SPARC RNA and protein localization.

To more closely examine the distribution of SPARC RNA and protein within the embryo, mid-trunk cross section were performed. (A) At stage 18 SPARC message was found to be abundant in the notochord (n), flanking somites (s), floor plate (fp) of the neural tube (nt), and the endodermal cells fated to become the sub-notochordal rod (sr). (B) By the early tailbud stage (33), the highly vacuolated notochord (n) contained few SPARC messages. The somites (s), neural tube (nt), particularly its floor plate (fp) and the sub-notochordal rod (sr) all express high levels of SPARC message. SPARC RNA was also detected in the surface ectodermal (se). To compare the distribution of SPARC protein to its cognate mRNA, immunohistochemistry was performed using ON3. (C) At stage 33 SPARC protein was no longer present in the notochord (n) and sub-notochordal rod (sr). SPARC staining was still visible in the neural tube (nt), and the sensorial layer of the surface ectoderm (se).
Discussion

Previous Northern blot studies demonstrated that embryonic expression of SPARC begins at neural stage 13 (Ringuette et al., 1992). SPARC transcript levels increased rapidly until early organogenesis. Whole mount immunofluorescence indicated that SPARC protein was particularly enriched within dorsal axial structures and surface ectoderm. However, autofluorescence signals from the yolk enriched cells prevented a precise mapping of the staining pattern. To circumvent this limitation advantage was taken of recent advances in whole mount in situ hybridization and immunohistochemical techniques specifically adapted for Xenopus embryos. Both techniques make use of enzyme-linked colourmetric detection, eliminating the problems associated with autofluorescence.

Whole mount analyses demonstrated that the first significant levels of SPARC transcript and protein were found associated with the notochord shortly after it became morphologically distinct. Shortly thereafter, SPARC was also observed within the longitudinal ridges of the paraxial mesoderm, just prior to the appearance of the first somites. SPARC levels increased as notochord elongation and somitogenesis progressed. The data therefore indicated that SPARC was involved in mesoderm development only after mesoderm induction had occurred.

Extensive studies have demonstrated that notochord and somites have similar developmental strategies (Keller, 1991). However, they form radically different structures with different developmental fates, which also reflect mechanistic differences. Since SPARC has been implicated to have multiple functions, it may have common and distinct functions in these tissues. In light of this potential complexity, the expression and potential role(s) of SPARC in the formation of notochord, somites and other tissues will be discussed separately.
Notochord morphogenesis

Very shortly after the involution of axial mesoderm in the blastoporal region (stage 11), prospective notochordal cells begin to undergo convergent extension movements to form an axial epithelial-like rod (Keller, 1991). By scanning electron microscopy (SEM), the notochordal cells can be distinguished from adjacent presumptive somitic cells by their flatter appearance and tighter associations. Their homotypic associations and rearrangements are in part dependent on cadherin and integrin receptors, which promote cell-cell and cell-matrix adhesion respectively. Adhesion by cadherins, and a subset of the integrin family, is calcium-dependent (Takeichi, 1991; Bronner-Fraser, 1993), and several integrins are expressed by presumptive notochordal cells by mid-gastrulation (Wittaker and DeSimone, 1993). Increases in EP-cadherins expression are also detectable in the notochord at this stage (Levi et al., 1991). In addition to these receptors, SEM and immunohistochemical studies have shown that notochord cells secrete large amounts of ECM macromolecules. A basement membrane (BM)-like structure composed of fibronectin, laminin and glycosaminoglycans surrounds the extending notochord by stage 14/15 (Hay, 1991; Perris et al., 1991; Welsch et al., 1991; Ueda et al., 1992). By the time the notochord induces the overlying ectoderm at the beginning of neurulation, collagen types IV and VI are also detectable in the BM. An ECM sheath composed of collagen types II, X, and proteoglycans is then laid down around the BM (Perris et al., 1991; Welsch et al., 1991). Both the BM and the perinotochordal sheath alter their molecular composition slightly as development progresses.

Our data now demonstrates that SPARC is also a major ECM glycoprotein secreted by the developing notochord. Its widespread distribution may reflect a role in modulating the
adhesive ability of calcium-dependent cadherin and integrin receptors. The N-terminal domain of SPARC has been demonstrated to bind up to 8 calcium ions with a dissociation constant of $10^{-3} - 10^{-5}$ M (Maurer et al., 1992), approximately equal to ECM calcium levels (Romberg et al., 1986). Hence, one of SPARC's functions may be to modulate the binding affinities of these receptors by acting as a local calcium-reservoir and chelator.

**Somitogenesis**

Similar levels of SPARC were detected within the axial and paraxial mesoderm during the early stage of neurulation. It is therefore conceivable that SPARC carries out similar functions during the early phases of notochord and somitic formation. However, when 20-22 paired somites had formed (stage 28), dramatic changes in the spatial distribution of SPARC mRNA began to occur. The biological significance of these changes may be linked to the unique features of segmentation in *Xenopus*. Relative to most vertebrates, *Xenopus* somite segmentation and differentiation occurs rapidly (Hamilton, 1969; Radice et al., 1989). The first segmented somites are visible in stage 16 embryos and segmentation progresses in a posterior direction at a rate of one pair of somites per 20 minutes. By stage 26, the segmented myotomes are sufficiently differentiated to undergo contractions (Radice et al., 1989). Consequently only 40 hours after fertilization, a swimming tadpole is hatched and the tadpole can escape from predators and search for food.

Rhythmic contraction of the early embryos is triggered by neural impulses to unsegmented head somites. The depolarization signals are then propagated, in part, through the segment trunk somites via gap junctions (Blackshaw and Warner, 1975; Kordylewski, 1978; Radice et al., 1989). In other model systems, the formation and stability of gap junctions is
promoted by calcium dependent cadherins (Jongen et al., 1991), and gating of gap junctions is controlled by intracellular calcium ions (Bennet and Verselis, 1992). It was therefore of special interest that the commencement of trunk myotome contractions coincided with the appearance of SPARC within the intersomitic clefts. This accumulation was preceded by the translocation of SPARC mRNA to the apices of the myotomes, indicating that SPARC protein which accumulated within the clefts was synthesized adjacent to the cleft. Since SPARC can bind calcium ions with dissociation constants similar to the ECM calcium levels (Maurere et al., 1992), it is conceivable that it acts as an extracellular calcium reservoir to affect: (1) the stability of the gap junctions; and (2) the gating required for the depolarization wave to be propagated down the embryonic axis. Consistent with this hypothesis is the observation that once trunk somites became innervated later in development (beginning at stage 36, Kordylewski, 1978), both gap junctions (Radice et al., 1989) and SPARC levels (Figs. 1 and 2) decrease dramatically within the segmented mesoderm.

The widespread distribution of SPARC within the unsegmented mesoderm suggests that it also has roles independent of modulating myotome contractions. Previous work in our laboratory has demonstrated that when anti-SPARC antibodies were microinjected into blastula embryos, somite segmentation was severely disrupted (Purcell et al., 1993). Myotomes were poorly stacked and embryos lacked normal swimming motion. Thus SPARC may also have important functions in somite segmentation and differentiation.

**Ectoderm tissues**

Unlike the co-localization of SPARC message and protein in notochord and somites, the pattern of SPARC protein distribution in the neural tube did not match the spatial expression
of its mRNA. Shortly after neural induction, SPARC transcript began to accumulate within the floor plate of the neural tube; however little protein could be detected. In contrast, little SPARC mRNA could be detected in the remainder of the neural tube, but it became enriched in protein. It is therefore conceivable that protein synthesized by the floor plate is recruited by the neural tube. Since the notochord and somites are both enclosed within ECM sheaths by this stage, it is not likely that they contribute to the accumulation of SPARC within the neural tube. Since during early neural tube development cells are extensively interconnected by gap junctions (Minkoff et al., 1991; Ruangvoravat and Lo, 1992), SPARC may have similar functions in the neural tube as in the somites in tailbud embryos. However caution must be applied since SPARC has been demonstrated to modulate cell migration in vitro. Therefore like other multifunctional ECM proteins, SPARC is likely to have other roles.

Punctate staining of the surface ectoderm was consistently observed by whole mount in situ hybridization. Sectional analysis revealed that punctate staining was generated by surface ciliated cells and a subset of cells associated with the sensorial layer. However, the staining pattern observed by whole mount immunohistochemistry was in part dependent on the fixative used. The DMSO/methanol fixative used in this study gave the best view of internal tissues. However, while comparable staining of internal tissues was obtained with paraformaldehyde fixation, a much stronger staining of the surface ectoderm was observed, in particular the ciliated cells (data not shown). These ciliated cells, are a subset of ectodermal cells which migrate from the deep ectoderm to the surface of the embryo at about the hatching stage (Drysdale and Elinson, 1992). SPARC could potentially play a role in this migration, as it is hypothesized to have anti-adhesive properties. It is also recognized that co-ordinated beating
of ciliated epithelial cells relies, in part, on the gap junctions which connect them within the epithelial sheet (Willecke et al., 1991; Hennemann et al., 1992). Thus electrical coupling could play a role in cilia action, and SPARC's presence could modulate this action. It is also important to note that Rohon-Beard sensorial neurons, which are extending within the sensorial layer, are also connected via gap junctions at this stage (Roberts, 1969; Roberts and Taylor, 1982).

**Cardiac expression**

Another intriguing localization of SPARC is its association with early cardiac development. The heart is one of the first organs formed. Cardiac muscle contractions are, in part, modulated by gap junctions (Green and Severs, 1993). SPARC may act as an important calcium reservoir, particularly during early development when the heart's calcium regulatory machinery is not yet functioning. Here too SPARC could be impinging on electrical coupling between gap junction connected cardiac cells.

Several tissue culture studies have demonstrated that SPARC inhibited cell migration, leading investigators to compare SPARC to other glycoproteins with anti-adhesive abilities, such as thrombospondin and tenascin (Sage and Bornstein, 1991). However, it is also important to note that the structural organization of SPARC bears strong similarities to other calcium-binding ECM glycoproteins that make use of acidic amino acid residue sequence motifs to bind high levels of calcium. For example, bone sialoproteins [BSP and OPN, (Butler, 1991)] share a number of characteristics with SPARC. They are small (32 - 34 kDa core peptide); each represent about 10% of the non-collagenous protein in bone; and have glu or asp-rich sequences which bind to calcium with dissociation constants similar to that of
ECM calcium levels. However they also differ in a number of important ways. Principal among them is that BSP is to promote ECM mineralization (Chen et al., 1992), while SPARC and OPN inhibit it (Romberg et al., 1986). Furthermore, SPARC expression is not restricted to mineralized tissues like BSP. Therefore although BSP, OPN and SPARC use similar sequence motifs to bind to ECM calcium, they appear to have discrete functions.

It was to some degree surprising how rapidly SPARC levels dropped once a tissue became morphologically distinct. While some overlap was apparent, the distribution pattern of SPARC was unique when compared to that of other calcium-dependent cell receptors and ECM macromolecules. This analysis is, however, likely to be too simple as several in vitro studies have demonstrated that the non-calcium binding regions of SPARC have biological activity, such as inhibiting cell migration and cell-cycle arrest (Lane and Sage, 1990; Funk and Sage, 1991). Consequently, it is likely that SPARC has both calcium-dependent and calcium-independent functions.

Deciphering the precise morphological contributions of any ECM macromolecule within a complex ECM network is very difficult due to functional redundancies and changes in activity due to subtle changes in ECM and cell microenvironments. The high levels and changes in SPARC expression during somitogenesis may offer the best opportunity to explore some of the functions of SPARC during early embryogenesis. Not only do somites represent the most abundant embryonic tissue, but they can be easily cultured in vitro and micromanipulated. Moreover the cellular and molecular events underlying somite formation, differentiation and function have been extensively studied in Xenopus. Hence the role of SPARC during somitogenesis represents a logical starting point for future studies.
Chapter 2

Ectopic expression of SPARC in Xenopus embryos interferes with tissue morphogenesis:
Identifiation of a bioactive region encompassing the EF-hand related motif
Abstract

SPARC is an extracellular matrix Ca\textsuperscript{2+}-binding glycoprotein that exhibits both counteradhesive and antiproliferative effects on cultured cells. It is secreted by cells of various tissues as a consequence of morphogenesis, response to injury, and cyclic renewal and/or repair. In an earlier study with *Xenopus* embryos, we had shown a highly specific and regulated pattern of SPARC expression. These studies now show that ectopic expression of SPARC prior to its normal embryonic activation produces severe anomalies, some of which are consistent with the functions of SPARC proposed from studies *in vitro*.

Microinjection of SPARC RNA, protein, and peptides into *Xenopus* embryos, prior to endogenous embryonic expression, generated different but overlapping phenotypes: a) Injection of SPARC RNA into one cell of a two-cell embryo resulted in a range of unilateral defects; b) precocious exposure of embryos to SPARC by microinjection of protein into the blastocoel cavity was associated with certain axial defects comparable to those obtained with SPARC RNA; c) SPARC peptides containing follistatin-like and copper-binding sequences were without obvious effect, whereas SPARC peptide 4.2, corresponding to a disulphide-bonded, Ca\textsuperscript{2+}-binding domain, was associated with a dose-dependent reduction in axial structures, eventually leading to the complete ventralization of the embryos. The histological analysis of ventralized embryos indicated that the morphogenetic movements associated with gastrulation may have been inhibited. Microinjections of other Ca\textsuperscript{2+}-binding glycoproteins, such as osteopontin and bone sialoprotein, demonstrated that the phenotypes generated were unique to SPARC. We probed further the structural correlates of this region of SPARC in the context of tissue development. Co-injection of peptide 4.2 with Ca\textsuperscript{2+} or EGTA, and
injection of peptide 4.2K (containing a mutated consensus Ca\(^{2+}\)-binding sequence), demonstrated that the developmental defects associated with peptide 4.2 were independent of Ca\(^{2+}\). However, the disulphide bridge in this region of SPARC was found to be critical, as injection of peptide 4.2AA, a mutant lacking the cystine generated no axial defects. We have therefore shown, for the first time \textit{in vivo}, that the temporally inappropriate presence of SPARC is associated with perturbations in tissue morphogenesis. Moreover we have identified at least one bioactive region of SPARC as the C-terminus disulphide-bonded, Ca\(^{2+}\)-binding loop that was previously shown to be both counteradhesive and growth-inhibitory.
Introduction

Extracellular matrix glycoproteins make major morphoregulatory contributions to early embryonic development. Their complex, transient expression patterns, structural heterogeneity and functional diversity indicate important roles in the modulation of a broad spectrum of cellular activities, such as adhesion, migration, communication, and proliferation. For example, the blastocoel roof of amphibian embryos is coated with an elaborate network of fibronectin fibres prior to the involution of mesodermal cells during gastrulation (Adams and Watt, 1993; Boucaut et al., 1984). Microinjection of anti-fibronectin antibodies into the blastocoel cavity of newt embryos inhibited gastrulation, and the use of *Xenopus* cells and RGD peptides *in vitro* confirmed that fibronectin was an adhesive substrate that promoted cell migration (Boucaut et al., 1984, Smith et al., 1990). Tenascin has also been found to be associated with mesodermal cell migration during amphibian gastrulation *in vivo* (Riou et al., 1990) and promotes neural crest cell migration in avian embryos (Akitaya and Bronner-Fraser, 1992). Since both tenascin and thrombospondin inhibit cell adhesion to fibronectin substrates *in vitro*, these two large ECM glycoproteins might have anti-adhesive potential *in vivo* (Chiquet-Ehrismann et al., 1988, Bornstein, 1995).

It has become apparent that proteins such as tenascin, thrombospondin and SPARC (secreted protein, acidic, and rich in cysteine) primarily modulate cell-matrix interactions and do not serve structural roles in the ECM *per se*. This group of proteins has been termed "matricellular" and is typified by the Ca$^{2+}$ and Cu$^{2+}$-binding glycoprotein SPARC, a prominent component of remodelling tissues (Bornstein, 1995; Lane and Sage, 1994; Sage and Bornstein, 1991).
In the previous chapter I demonstrated that SPARC expression begins at early neurula stage 13 in *Xenopus* embryos. Whole mount in situ hybridization and immunohistochemical analysis demonstrated staining within the notochord and somites from the onset of their development (Damjanovski et al., 1994). As development progressed, SPARC RNA and protein underwent marked and rapid changes in expression during neurulation and early organogenesis. In particular, SPARC transcripts and protein were found to be enriched in cells known to undergo Ca\(^{2+}\)-dependent adhesion and that were electrically coupled via gap junctions. The highly regulated pattern of expression indicates that SPARC has important functions in early embryonic development. This hypothesis is based in part on experiments in which microinjection of anti-SPARC antibodies into the blastocoel cavity led to major trunk defects (Purcell et al., 1993). Ectopic expression of SPARC produced comparable muscle defects in *C. elegans* (Schwarzbauer and Spencer, 1993). Although the precise functions of SPARC during early embryonic development are not known, a variety of potential functions have been described for SPARC in vitro, e.g., inhibition of cell-substratum adhesion and cell spreading (Sage et al., 1989b), binding to and modulating growth factors (Raines et al., 1992; Hasselaar and Sage 1992), regulation of cell-cycle progression (Funk and Sage, 1991, 1993), and augmentation of inter-endothelial cell permeability (Goldblum et al., 1994; reviewed in Lane and Sage 1994). Furthermore, SPARC interacts with several ECM macromolecules in vitro (Kelm and Mann, 1991; Tremble et al., 1993).

In chapter 1 examined the developmental consequences of the ectopic expression of SPARC prior to its normal embryonic activation. Here, I report that microinjection of
SPARC RNA, native protein, and synthetic peptides have distinct, but non-exclusive developmental consequences. These observations from a developmental system \textit{in vivo} are consistent with some of the functions previously identified for SPARC \textit{in vitro}. 
Materials and Methods

Embryo rearing

Embryos were reared as described in chapter 1. Staging of embryos was according to Nieuwkoop and Faber (1956).

SPARC RNA and protein

Full length mouse SPARC cDNA (Mason et al., 1986) was subcloned, in both orientations, into the PstI site of pGEM4Z. Orientation was determined using restriction enzyme analysis. Both orientations were linearized with HindIII such that T7 RNA polymerase would produce sense RNA with one orientation and anti-sense RNA with the other. Both capped and poly(A)-tailed full-length sense, and (control) anti-sense mouse RNAs were synthesized with Boehringer Mannheim's cap-scribe kit as follows: 0.5 μg of linear DNA, 5 μl of cap scribe buffer (Boehringer Mannheim); and 2 μl of T7 RNA polymerase (in a 20 μl reaction) were incubated for 1 hour at 37°C. EDTA (0.5 μl of 0.5M) was added to terminate the reaction following which 2 μl of sample were removed for analysis by agarose gel electrophoresis. The remaining sample was precipitated with 10 μl 10 M NH₄OAc and 100 μl ethanol. The RNA pellet was resuspended in 12 μl of water and was polyadenylated with the addition of 4 μl 5X polyadenylation buffer, 4 μl 2.5 mM ATP, and 2 μl polyA polymerase. The reaction was carried out at 37°C for 30 minutes and terminated with the addition of 1 μl 0.5 M EDTA and 180 μl water. Samples were then extracted two times with phenol/chloroform and precipitated with 75 μl 10 M NH₄OAc and 1000 μl ethanol. The RNA pellet was resuspended in water and quantitated using optical density measurements. An aliquot was analysed by agarose gel electrophoresis to examine the quality
of the RNA and to compare the RNA samples before and after polyadenylation.

Rat bone sialoprotein cDNA (a gift of Dr. Jaro Sodek, University of Toronto) was similarly subcloned and transcribed. Intact mouse SPARC protein was isolated from the conditioned medium of PYS-2 cells and was purified as previously described (Sage et al., 1989b). This protein preparation was shown to disrupt barrier function in cultured endothelial cells, as described by Goldblum et al., (1994) and to inhibit endothelial and smooth muscle cell proliferation \textit{in vitro} according to Funk and Sage (1993). Porcine SPARC (Domenicucci et al., 1988) and porcine osteopontin (Zhang et al., 1990) obtained from Dr. Jaro Sodek, University of Toronto, porcine bone sialoprotein (Goldberg et al., 1988) obtained from Dr. Harvey Goldberg, University of Western Ontario), bovine brain calmodulin (Calbiochem), and bovine serum albumin (Pharmacia, fraction V) were used as controls.

**Immunoprecipitation**

To confirm that SPARC RNA, injected 2 hours post-fertilization (pf) into two-cell embryos, was translated, we injected $^{35}$S methionine with the RNA. Ten embryos were lysed, at 2 and 6 hours after injection, in 200 $\mu$l 150mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% SDS, 0.5% BSA. This is prior to SPARC's embryonic induction at stage 13, 16 hours pf (Ringuette et al., 1992). For immunoprecipitation, the lysed embryos were centrifuged at 14000 g for 10 minutes at room temperature. To the supernatant, 200 $\mu$l of immunoprecipitin (BRL) was added and incubated at room temperature for 15 minutes. The tubes were centrifuged at 14000 g for 10 minutes at room temperature to remove non-specific binding of immunoprecipitin. The supernatant was incubated with the appropriate antibody. Supernatant (50 $\mu$l) was incubated for 2 hours with rocking at room temperature,
with 5 µg (2-10 µl) of antibody (monoclonal mouse SPARC antibody ON3, and control mouse fibronectin antibody (Fisher Scientific), and goat anti-rabbit IgG antibodies. BRL immunoprecipitin reagent (protein A, 75 µl) was then added and the sample rocked for 30 minutes at room temperature. The sample was pelleted by centrifugation for 5 minutes at 14000 g. The pellet was washed and repelleted 3 times with 500 µl of BSA free lysis buffer. The pellet was then resuspended in 50 µl protein gel loading buffer. An aliquot was heated for 2 minutes at 85°C, and fractionated on a 8% SDS PAGE gel. Following electrophoresis, the gel was dried and exposed to x-ray film.

**Synthetic peptides**

Peptides representing various regions of mouse SPARC (Fig 4), synthesized by the Howard Hughes Institute (University of Washington) or by Zymogenetics (Seattle, WA), were purified by high-performance liquid chromatography (Lane and Sage, 1990). All peptides were assayed *in vitro* according to Lane and Sage (1990) and Funk and Sage (1993). In this study we injected peptide 2.1 (amino acids 54-73 [CQNHHCKHGKVCELDESNTP]) corresponding to the N-terminal end of the cysteine-rich domain II; peptide 2.3 (amino acids 113-130 [TLEGTKKGHKLHLDYIGP]) representing the C-terminal end of domain II; and peptide 4.2 (amino acids 254-273 [TCDLDNDKYLAEWAGCFG]) representing the C-terminal EF-hand related motif of domain III of SPARC (Sage et al., 1995). Mutant peptide 4.2K (identical to peptide 4.2 except for an aspartic acid-to-lysine substitution at amino acid 258) and peptide 4.2AA (also identical to peptide 4.2 except for two cysteine-to-alanine substitutions at amino acids 255 and 271) were also microinjected into the embryo.
Figure 4. Diagrammatic representation of SPARC domains and position of synthetic peptides used for microinjection.

SPARC can be subdivided into three distinct structural domains. The N-terminal glutamic acid-rich domain I can bind with low affinity up to eight Ca^{2+} ions (Kd 10^{-3}-10^{-2} M), making SPARC sensitive to changes in ECM Ca^{2+} ion levels. Domain II, contains a follistatin-like module with 10 conserved cysteine residues and two Gly-His-Lys copper-binding sites. Domain III representing the C-terminal half contains a series of alpha-helical segments. In addition, domain III also has a unique disulphide-bridge calcium-binding EF-hand like motif which can bind one Ca^{2+} ion with a K_d^{7}10 M. Peptide 2.1 (amino acids 54-73 [CQNHHCKHGKVCHELDESNTP]) corresponds to the N-terminal end of domain II. Peptide 2.3 (amino acids 113-130 [TLEGTKKGHLHLDYIGP]) is adjacent to peptide 2.1. Peptide 4.2 (amino acids 254-273 [TCDLDNDKYIALEEWAGCFG]) represents the EF-hand of domain III. Mutant peptides 4.2K and 4.2AA are identical to peptide 4.2 except for an aspartic acid-to-lysine substitution at amino acid 258 and two cysteine-to-alanine substitutions at amino acids 255 and 271 respectively.
Leader Peptide

Domain I  Domain II  Domain III
GLU-rich  CYS-rich  EF-hand like motif

NH$_2$ COOH

2.1  2.3  4.2

4.2K
4.2AA
**Microinjection**

Embryos were injected with glass needles prepared with a Narishige glass needle puller and a Narishige forced-gas system. Stage 2 (RNA injections, two hours p.f) and stage 8 (protein and peptide injections, eight hours p.f) *Xenopus* embryos in 100% Steinberg's solution supplemented with 3% ficoll were injected with 5 to 200 pg of RNA (in 5 to 50 nl water) into 1 cell of a 2-cell embryo. Between 10 ng to 1000 ng (in 10 to 300 nl PBS) of protein was injected into the blastocoel cavity of stage 8 embryos. Embryos were allowed to heal for several minutes in 100% Steinberg's solution before transfer into 20% Steinberg's solution.

**Whole mount immunohistochemistry**

Embryos were fixed for 2 hours in Dent's fixative (25% dimethylsulphoxide in methanol), washed several times in PBS, and incubated with a neural-specific 2G9 antibody (1:250 dilution) overnight at 4°C (Jones and Woodland, 1989). Following several washes with PBS, the neural tissue distribution was visualized with an anti-mouse alkaline-phosphatase-conjugated secondary antibody reacted with NBT and BCIP. Embryos were immersed in Bouin's fixative, dehydrated in methanol, and cleared in benzyl benzoate and benzoic acid prior to photography.

**Histology**

Embryos were fixed for 2 hours in 4% formalin, washed several times in PBS, and dehydrated in ethanol. Dehydrated embryos were embedded in paraplast, sectioned (10 μm), and placed on collagen-coated slides. Sections were stained with haematoxylin and eosin according to standard protocols (Kelly et al., 1991) and were mounted with permount for photography.
Results

Generation of unilateral defects by microinjection of SPARC RNA into early blastomeres

Microinjection of 5-25 pg of RNA had no effects on development, whereas microinjection of 200 pg of RNA resulted in death in greater than 75% of the embryos. Microinjection of 100 pg of capped and poly(A)-tailed mouse SPARC sense RNA into one cell of two-cell embryos (Table 1) resulted in defects restricted to one side of some embryos (Fig. 5, A to D). Co-injection of rhodamine dextran sulfate revealed that the affected region corresponded to the progeny of the injected blastomere (data not shown). Eyes were either small and deformed (arrow, Fig. 5, A) or absent, on the left or right side (Fig. 5, B vs C), coincident with the blastomere injected. Defects were also observed within the trunk. The absence of somites on one side resulted in embryos with severely curved axes (triangles, Fig. 5, A and D). Histological analysis revealed somite malformations that corresponded with the kinked axis (Fig. 5, E). More severe axial defects (Fig. 5, D) were associated with malformed axial features, such as notochords, identified histologically (Fig. 5, E). Control SPARC anti-sense RNA-injected embryos (Table 1) were normal, both morphologically (Fig. 5, F) and histologically (Fig. 5, G). Similarly, control BSP sense or anti-sense RNA-injected embryos were normal (Table 1).

To confirm that injected SPARC sense RNA (100 pg) was translated into SPARC protein I immunoprecipitated labelled embryonic protein with SPARC antibody 2 and 6 hours after RNA injections and 4-8 hours before embryonic activation of SPARC RNA occurred. The Mr of SPARC on SDS-polyacrylamide gels, is 43,000. $^{35}$S-methionine injected alone, or with
antisense RNA was not associated with a band of Mr 43,000 in embryos up to 6 hours after injection (Fig. 6, A and B). After injection of ³⁵S-methionine with sense RNA, two bands were immunoprecipitated at both 2 and 6 hours post-injection (Fig. 6, C and D). The upper bands with an estimated molecular weight of 43 kDa matched the molecular weight expected for SPARC. The lower molecular weight band (middle band, lanes C and D) likely represents a degradation product of SPARC. An unidentified band present in all lanes,
Figure 5. Injection of SPARC RNA into early blastomeres causes head and axis defects. Microinjection of 100 pg capped and poly(A)-tailed mouse SPARC sense RNA into one cell of a two cell embryo resulted in the reduction of axial structures on the injected side. These defects included: a reduced eye on one side of the embryo (A, arrow), an eye present on one side (B) but missing from the other side (C) of the embryo, and various axial kinks (A and D, triangles). Histological sections of sense RNA injected embryos revealed asymmetric somites and poorly formed notochords (E). In contrast microinjection of anti-sense RNA had no effects on development as seen both externally (F) and histologically (G). [n, notochord; nt, neural tube; s, somites]. Bar represents 300 μm.
Table 1. Summary of macromolecules injected and their phenotypic effects.

<table>
<thead>
<tr>
<th>Macromolecule</th>
<th>Amount injected</th>
<th>Number of embryos</th>
<th>Axis defects</th>
<th>Head defects</th>
<th>Other observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse sense RNA</td>
<td>100 pg</td>
<td>65*</td>
<td>20 (30%)</td>
<td>15 (25%)</td>
<td>27 (40%) normal</td>
</tr>
<tr>
<td>Mouse anti-sense RNA</td>
<td>100 pg</td>
<td>44</td>
<td>3 (7%)</td>
<td>2 (4%)</td>
<td>39 (90%) normal</td>
</tr>
<tr>
<td>Rat BSP sense RNA</td>
<td>100 pg</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>23 (90%) normal</td>
</tr>
<tr>
<td>Rat BSP anti-sense RNA</td>
<td>100 pg</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>18 (90%) normal</td>
</tr>
<tr>
<td>Mouse SPARC</td>
<td>750 ng</td>
<td>78</td>
<td>40 (50%)</td>
<td>20 (25%)</td>
<td>18 (25%) normal</td>
</tr>
<tr>
<td>Mouse SPARC plus Ca²⁺</td>
<td>500 ng</td>
<td>20</td>
<td>8 (40%)</td>
<td>7 (35%)</td>
<td>5 (25%) normal</td>
</tr>
<tr>
<td>Porcine SPARC</td>
<td>500 ng</td>
<td>30</td>
<td>12 (40%)</td>
<td>9 (30%)</td>
<td>9 (30%) normal</td>
</tr>
<tr>
<td>Porcine OPN</td>
<td>400 ng</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>100% death prior to gastrulation</td>
</tr>
<tr>
<td>Porcine BSP</td>
<td>400 ng</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>100% normal</td>
</tr>
<tr>
<td>Peptide 2.1</td>
<td>10-1000 ng</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>100% normal</td>
</tr>
<tr>
<td>Peptide 2.3</td>
<td>10-1000 ng</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>100% normal</td>
</tr>
<tr>
<td>BSA</td>
<td>1000 ng</td>
<td>85</td>
<td>0</td>
<td>0</td>
<td>100% normal</td>
</tr>
<tr>
<td>Peptide 4.2</td>
<td>60-250 ng</td>
<td>193</td>
<td>0</td>
<td>191 (99%)</td>
<td>0</td>
</tr>
<tr>
<td>Peptide 4.2</td>
<td>500-1000 ng</td>
<td>154</td>
<td>151 (98%)</td>
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<td>0</td>
</tr>
<tr>
<td>Peptide 4.2 plus Ca²⁺</td>
<td>60-250 ng</td>
<td>20</td>
<td>0</td>
<td>20 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Peptide 4.2 plus Ca²⁺</td>
<td>500-1000 ng</td>
<td>19</td>
<td>18 (95%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peptide 4.2K</td>
<td>60-1000 ng</td>
<td>58</td>
<td>57 (98%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peptide 4.2AA</td>
<td>1000 ng</td>
<td>45</td>
<td>0</td>
<td>0</td>
<td>50% normal, 50% ventral edema</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>500 ng</td>
<td>30</td>
<td>9 (30%)</td>
<td>0</td>
<td>21 (70%) death</td>
</tr>
</tbody>
</table>

*Where the number of embryos injected is not equal to the number of phenotypes reported, the difference is due to embryos that died early in development as a result of injection damage.

bone sialoprotein, osteopontin

calmodulin-injected embryos that displayed exogastrulation-like phenotypes.
corresponds to a non-specific reactivity of the polyclonal SPARC antibody.

Microinjection of mouse SPARC in the blastocoel cavity causes a broad spectrum of defects

Control uninjected embryos and those injected with protein initiated gastrulation at the same
time and appeared to progress through gastrulation and early neurulation in a similar manner,
as judged by the morphological criteria of blastopore and neural tube formation. However,
shortly after neurulation a range of defects was apparent for the embryos injected with
SPARC. Approximately 25% of the embryos (20 of 78) had minor abnormalities in head
structure and defective truncated axes by stage 28 (Fig. 7 A); 50% (40 of 78) appeared to
have a broad range of severe head and axial defects (Fig. 7 B); and 25% of injected embryos
appeared unaffected (see also Table 1). Head defects included a lack of eyes and reduced
cement glands (arrows, Fig. 7, A and C). To confirm that observed effects were not unique
to mouse SPARC, we also injected porcine SPARC. Amino acid analysis of SPARC, based
on cDNA sequences, has demonstrated a 90% amino acid identity among mammals and 80%
between *Xenopus* and mammals (Damjanovski et al., 1992). The effects of porcine SPARC
(Fig. 7, C) were very similar to those of mouse SPARC (Table 1, Fig. 6, A and B), indicating
that the high amino acid conservation of SPARC also reflects conservation of functions.
Sagittal sections of embryos with defective axes confirmed the absence of anterior structures
and a lack of tissue differentiation, with the exception of an unidentified group of densely
packed columnar cells (arrow, Fig. 7, D and E). Within the trunk region of some of these
embryos, a notochord-like structure could also be identified (Fig. 7, E).
Figure 6. Translation of microinjected SPARC sense RNA.

An autoradiograph of $^{35}$S-methionine-labelled protein extracts immunoprecipitated with anti-SPARC IgG and fractionated by SDS-PAGE. (A) control $^{35}$S-methionine injected, and (B) $^{35}$S-methionine, anti-sense SPARC RNA co-injected embryos did not express SPARC-like protein after 6 hours. In contrast, co-injection of $^{35}$S-methionine with sense SPARC RNA resulted in the detection of a SPARC-like protein at 2 hours (C), and 6 hours (D) post-injection. Molecular weight standards of 43kDa (ovalbumin) and 30 kDa (carbonic anhydrase) are shown.
Figure 7. Microinjection of SPARC protein into the blastocoel cavity leads to severe axial defects.

Injection of mouse SPARC into a stage 8-9 blastocoel cavity resulted in reduction of head structures, including reduced cement glands (arrow in A and C) and severe axis defects (A, B and C). Defects in axes were confirmed with histological sections which revealed densely packed columnar cells (arrow in D and E), notochord-like structures (n in E) but few other discernible tissues (D and E). Effects of mouse SPARC were confirmed through the similar injection of porcine SPARC (C). Bar represents 200 μm.
SPARC binds to a variety of macromolecules (Lane and Sage, 1994). Therefore, developmental defects generated by precocious overexpression of SPARC might have been due to non-specific binding of macromolecules that play critical roles in early embryonic development. To address this concern, we injected equivalent amounts of two structurally-related Ca$^{2+}$-binding glycoproteins into the blastocoel cavity. Embryos injected with porcine osteopontin were arrested in development prior to gastrulation, whereas no developmental defects were observed after injection of porcine bone sialoprotein (Table 1).

Since the N-terminal, glutamic acid-rich region of SPARC can bind up to 8 Ca$^{2+}$ with $K_d$ ranging from $10^{-1}$-10$^{-4}$ M, we were also concerned that the effects shown in Fig. 2 might have been due to the binding of extracellular Ca$^{2+}$ by SPARC; both inter- and intracellular Ca$^{2+}$-mediated pathways would thus be compromised. However, preincubation and co-injection of SPARC with 0.3-10 mM CaCl$_2$ did not alter the results (Table 1). Since the blastocoel cavity of Xenopus embryos is known to contain 1.5 mM free Ca$^{2+}$, we also tested Ca$^{2+}$-dependent effects of SPARC by the injection of SPARC with EGTA. Co-injection with 0.3-3 mM EGTA did not interfere with the phenotypic effects of SPARC. However, further increases in EGTA concentration (with or without SPARC) led to embryonic death as a result of the dissociation of blastomeres, which depend on cadherins for their cohesion (Angres et al., 1991).

Increasing doses of SPARC were required over time to obtain consistent results due to possible SPARC lability or contaminating protease degradation. Therefore, the data presented were generated by microinjection from 80-750 ng of SPARC. Similar results with respect to phenotypes were also obtained with 300 ng of porcine SPARC microinjected as described.
Microinjection of synthetic peptides

Synthetic peptides corresponding to domains II and III of SPARC (Fig. 4) were found to mimic some of the major biological effects of native SPARC on tissue culture cells (Lane and Sage, 1990; Sage et al., 1995). Therefore, we sought to determine whether these peptides could mimic the developmental changes associated with the microinjection of native SPARC.

Microinjection of peptides 2.1 and 2.3 does not interfere with embryonic development

Peptide 2.1, representing the N-terminal region of the cysteine-rich, follistatin-like domain II (Fig. 4), arrests endothelial cells and fibroblasts at the G1 stage of the cell cycle (Lane and Sage, 1994). Surprisingly, no effects on development were observed when 10-1000 ng of peptide 2.1 were injected into Xenopus embryos (Table 1, and Fig. 8, C). Similar results (Fig. 8, D) were also obtained with peptide 2.3, a cationic peptide C-terminal to peptide 2.1 (Fig. 4). Injection of up to 1000 ng of BSA (Fig. 8, A) or up to 300 nl of PBS (Fig. 8, B) had no apparent effects on development. Sections of control uninjected, PBS, BSA, peptide 2.1 or peptide 2.3-injected embryos were indistinguishable (data not shown).

Abnormalities associated with injection of peptide 4.2

Microinjecting 60 ng of peptide 4.2, representing the Ca\(^{2+}\)-binding EF-hand related motif of SPARC (Fig. 4), generated dorso-anterior defects. Virtually all embryos (63 out of 65) had small heads, with abnormal eyes and cement glands by stage 30 (Table 1, and Fig. 9, A). Also visible were large ventral swellings, which sometimes collapsed to form highly convoluted surface folds. The trunk of the embryos appeared to have developed normally. Increasing the level of peptide 4.2 100 ng led to a complete absence of head development in 100% (58 out of 58) of the embryos (Table 1, and Fig. 9, B). Greater than 90% of these
Figure 8. Microinjection of SPARC cysteine domain peptides did not affect development.

Control microinjections of 300 nl PBS (A) or 1000 ng BSA (B) had no effect on development. Embryos injected with 1000 ng SPARC peptide 2.1 (C), representing the N-terminal region of the cysteine-rich, follistatin-like domain, also appeared normal. Similar results were also obtained with 1000 ng peptide 2.3 (D), representing a cationic region adjacent to region 2.1.
embryos had relatively normal dorsal axes and continued to develop well into the late tailbud (stage 50) and died shortly thereafter (Fig. 9, C). Histological sections revealed that otic vesicles (Fig. 10, D) were the first anterior tissues formed and thus confirm that anterior development was truncated (Fig. 10, A - G). The neural tube and notochord were always present (Fig. 10, F), but differed in their anterior progression. Use of the neural 2G9 antibody, however, revealed that neural tissue did indeed reach the extreme anterior of most embryos (Fig. 12, C, middle embryo).

The above results indicated that there was a concentration-dependent decrease in anterior to posterior development associated with the microinjection of peptide 4.2. This observation was confirmed by microinjection of 250 ng peptide 4.2. All of the 70 injected embryos lacked heads, and now had significantly shorter dorsal axes (Table 1, and Fig. 9, D). Histological analysis revealed that the otic vesicles were also missing from the anterior region (Fig. 10, H to J). The somites, notochord, and neural tube were always present but often did not span the entire length of the dorsal axis as is characteristic of normal embryos. Frequently, transverse sections of the extreme anterior end of the axis revealed that the axial ridge was composed of only somite-like cells (Fig. 10, I). Posterior sections also revealed a loosely organized neural tube-like structure (Fig. 10, I and J). Similarly, the notochord frequently failed to extend into the anterior region (Fig. 10, H and I).

Increasing the concentration of microinjected peptide 4.2 from 250 ng to 500 ng or 1000 ng resulted in more severe axial defects (Fig. 9, E and F; and Table 1). The increased volume required to microinject larger amounts (due to constraints on solubility) caused significant swelling of the blastocoel cavity. For some embryos (29 of the 151 with injection defects)
Figure 9. Abnormalities associated with microinjection of peptide 4.2.

Microinjecting 60 ng of peptide 4.2 (A), representing the high-affinity, Ca\(^{2+}\)-binding, EF-hand related motif of SPARC, generated embryos with small heads, and abnormal eyes and cement glands (A, single arrows). Large ventral swellings were visible, which sometimes collapsed to form highly convoluted surface folds (double arrows). Injection of 100 ng peptide 4.2 (B) was associated with a complete absence of head development in 100% of the embryos. As in (A), swelling in the anterior ventral region or surface-convoluted folds (double arrows) was present. As a result of this swelling or excess tissue, the ventral endoderm, anterior regions of latter-stage 35-42 embryos (C) appeared malformed and swollen. Microinjection of 250 ng peptide 4.2 (D) was associated with embryos lacking heads, and with significantly shorter dorsal axes and a prominent collapsed ventral mass (double arrows). Microinjection of up to 1000 ng peptide 4.2 was associated with a broad range of axial defects: (E) were distorted or had non-existent axes, whereas (F) had formed prominent axial ridges (arrow), which spanned about one-fifth of the circumference of the embryos. Peptide 4.2 results were reproduced by peptide 4.2K, a non-Ca\(^{2+}\) binding mutant of peptide 4.2. Injection of 100 ng (G) or up to 1000 ng (H) peptide 4.2K produced results indistinguishable from 100 ng (B) or 1000 ng (E) peptide 4.2.
Figure 10. Histological analysis of embryos injected with SPARC peptide 4.2.

Transverse serial histological sections (anterior to posterior series, A to G) of eyeless embryos that were injected with 100 ng peptide 4.2 (shown in Fig 5, B) revealed otic vesicles (o in D) to be the first discernible anterior structure. The ventral swellings or convoluted folds were continuous with the surface epidermis (arrows, E to G) and were coincident with the region containing the remnants of the blastocoel cavity (b in F). In embryos (shown in Fig 5, D) injected with 250 ng peptide 4.2, transverse serial histological sections (anterior to posterior series, H to J) revealed the somites (s) were the most anterior structures identified, followed by the neural tube (nt) and notochord (n)-like structures. Dorsal is top. Bar represents 50 μm.
the swelling was so significant that external signs of gastrulation were not be seen. However, by the time sibling control embryos had reached stage 35 (tailbud), these embryos had formed an axial ridge (Fig. 9, F). The ridges spanned about one-fifth of the circumference of the embryos. Embryonic death occurred shortly thereafter. Transverse and sagittal sections of embryos with severely deficient axes revealed that many had a notochord (Fig. 11, A and B). In some cases notochords and somites formed a hook-like structure at the posterior end, indicative of interference with gastrulation and convergent extension of the notochord (arrow, Fig. 11, A and B). Antibody 2G9 revealed no neural tissues within these embryos (bottom embryo, Fig. 12, C). Histological sections of the most severely malformed, axis-deficient embryos, revealed no distinct morphological structures (Fig. 11, C). It appeared that cellular migration might have occurred without giving rise to distinctive morphological tissues. For embryos which formed ridges (Fig. 9, F) late in development relative to sibling embryos, histological sections revealed the ridges were not underlain by any discernable tissues (Fig. 11, D). As we had observed with native SPARC, preincubation of peptide 4.2 with 0.3-10 mM CaCl₂ or 0.3-3.0 mM EGTA did not alter these results (Table 1).

**Effect of peptide 4.2 mutants**

To determine whether binding of Ca²⁺ to the EF-hand related motif contributed to the effect of peptide 4.2 on development, I injected peptide 4.2K, in which an aspartic acid was substituted by a lysine (Sage et al., 1995). Peptide 4.2K displays a significantly reduced affinity for Ca²⁺ (Sage et al., 1995). No differences between the effects of peptides 4.2 and 4.2K were observed in intact or sectioned embryos. Injection of 100 ng to 1000 ng of
Figure 11. Histological analysis of severe effects caused by the injection of peptide 4.2. Sagittal sections of embryos injected with 1000 ng of peptide 4.2 (A and B, dorsal is top, anterior to the right) revealed that most embryos contained a notochord (n) and somites (s). The notochord and somites often formed a hook-like structure in the posterior end (arrow, A and B). Mid-embryo sections of severely axis-deficient embryos revealed no discernible tissues (C). Of the embryos which formed a late axial structure (as shown in Fig. 5, F), sections of these embryos at the mid-line showed cellular rearrangement, but no distinct morphologies (D). Bar represents 100 μm.
peptide 4.2K (Fig. 9, G and H) or wild-type peptide 4.2 (Fig. 9, B and E) generated near identical dose-dependent effects (see also Table 1).

An unusual feature of the EF-hand related motif of SPARC is that it is stabilized by a disulfide bridge. To determine whether this disulfide bond contributed to the biological activity of peptide 4.2, we microinjected peptide 4.2AA, in which the two cysteine residues of peptide 4.2 had been replaced by alanines. Microinjection of up to 500 ng peptide 4.2AA did not interfere with development. Embryos injected with between 500 and 1000 ng underwent normal development until the tailbud stage, at which time ventral swellings became prominent (Table 1, and Fig. 12, A). The effect (or cause) of this phenotype is unclear as these embryos were otherwise morphologically normal and had intact head and axial structures.

As an additional control, a protein with four Ca\(^{2+}\) binding EF-hands (bovine brain calmodulin) was also injected. Injection of up to 200 ng calmodulin did not interfere with development. Injection of 500-1000 ng lead to embryonic death in 20 of 30 injected embryos by the time sibling control embryos reached late gastrulation. The remaining 10 injected embryos underwent an exogastrulation-like process (Table 1, and Fig. 9, B), and appeared as endodermal masses which were capped by ectoderm (arrows, Fig. 12, B). Antibody 2G9 revealed that the ridge of the ectodermal cap was neural tissue (arrows, Fig. 12, D).
Figure 12. Injection of a mutant SPARC domain III peptide results in different phenotypes.

Microinjection of 1000 ng peptide 4.2AA resulted in embryos which underwent normal development until the tailbud stage, at which point ventral swelling became prominent (A, arrow). Head and axis structures in 4.2AA-injected embryos appeared normal (A). Microinjection of 500 ng calmodulin caused about 30% of the embryos to undergo an exogastrulation-like process resulting in protruding endoderm (B). The external pigmented ectoderm of these embryos seemed to be turned inside-out, as the un-pigmented internal cells protruded to form an elongated axis (B, arrows). Use of an anti-neural antibody (2G9) revealed that the ectoderm ridge of the calmodulin-injected embryos was neural (D, arrows). In normal, and peptide 4.2-injected headless embryos, 2G9 revealed a broad neural stripe (C, top 2 embryos), whereas embryos injected with peptide 4.2 (showing severely reduced axes) exhibited no neural tissues (C, bottom embryo).
Discussion

Whole mount in situ hybridization and immunohistochemical analysis demonstrated that SPARC was expressed by Xenopus embryos at stage 13, from the onset of the formation of the notochord and somites (Damjanovski et al., 1994). Rapid changes occurred in the level and spatial distribution of SPARC throughout the neurulation and tailbud stages of development. In this study I show that precocious ectopic expression of SPARC affects Xenopus development. Microinjection of capped and poly(A)-tailed full-length mouse SPARC RNA into one blastomere of two-cell embryos (stage 2, 14 hours before the expression of endogenous SPARC occurred) often generated unilateral defects. For example, eyes were usually absent or defective only on the injected side. In addition, some embryos had both dorso-anterior and trunk defects only on one side. Microinjection of SPARC peptides into the blastocoel resulted in a similar phenotype, though the effects were bilateral.

In Xenopus, eye development (the first anterior organ formed) is affected by a broad variety of treatments including injection of anti-gap junction antibodies into blastomeres of eight-cell embryos (Warner et al., 1984) and injection of reagents that affect dorsol-anterior development (e.g. retinoic acid, Xgsk, Xwnt-8, trypan blue, heparin, suramin, reviewed in Kao and Danilchik, 1991). Embryos with defective eyes or lacking eyes have also been generated by microinjection of anti-SPARC antibodies into the blastocoel cavity (Purcell et al., 1993). Moreover, in situ and immunohistochemical studies have demonstrated that SPARC is a major component of developing retina in chicken, bovine, and monkey embryos (M. Ringuette, Q. Yan and H. Sage, A. Henchickson, unpublished experiments).

Although eye development in Xenopus is sensitive to a variety of experimental
perturbations, several factors could account for the observations reported here. Lineage tracing, separation, and perturbation experiments indicate that the three body axes (anterior-posterior, dorsal-ventral, and left-right) are linked developmentally (reviewed in Almirantis, 1995); it is therefore likely that their formation is in part influenced by common axis determinants. SPARC has been shown to modulate the activity of growth factors (Lane and Sage, 1994; Raines et al., 1992). As such, binding of SPARC to inducing factors could interfere with the specification of embryonic tissues that would have occurred later in development. That the injection of other extracellular glycoproteins (osteopontin and bone sialoprotein) resulted in unique phenotypes, indicates that non-specific chelation of macromolecules by these acidic proteins was not a causal factor. However, it remains to be established if SPARC binds to and modulates the activity of inducing factors in early Xenopus embryos. We are currently investigating whether or not SPARC has an affinity for, or interferes with other known embryonic inducing factors.

Microinjecting SPARC into the blastocoel cavity (a region into which proteins can be injected within a large ECM cavity) prior to gastrulation enabled us to assess the impact of the precocious presence of SPARC (1) just prior to the start of major morphogenetic movements, (2) while major cell-cell inductive events were still occurring and, (3) 8-9 hours before endogenous expression of SPARC began. As with the microinjection of SPARC RNA, a variety of head and tail defects were observed, e.g., defective cement gland and eyes, somite misalignment, axis bending, and ventral swelling. However, in contrast to the microinjection of RNA, bilateral defects were observed. A plausible explanation is that the injected SPARC was distributed evenly within the blastocoel cavity. Since Xenopus embryos
do not begin to express SPARC until mid-gastrulation, and SPARC does not accumulate to any significant levels until neurulation (Damjanovski et al., 1994), the defects generated here were likely due to the precocious presence of SPARC prior to its endogenous activation. Of concern was that the effects could have been due to sequences and structures unique to murine SPARC, amphibian SPARC has not been purified nor characterized functionally. As SPARC is a highly conserved protein (90% amino acid identity among mammalian species and 80% between Xenopus and mammals, [Damjanovski et al., 1992]), it is likely that common functions would be shared in different organisms. Indeed, when we microinjected porcine SPARC, the defects generated were very similar to those obtained with the murine protein.

Studies in vitro on endothelial cells, smooth muscle cells and fibroblasts have demonstrated that SPARC delayed cell cycle progression and reduced cell-substratum interactions (reviewed in Lane and Sage, 1994). However, SPARC had no obvious morphological effect on early cell division and movement associated with gastrulation in Xenopus as judged by the timing of the closure of the blastopore. Nevertheless, the effects observed at the tailbud stage indicate that SPARC did interfere with events, and at high doses resulted in the ventralized embryos. However, I have recently observed that SPARC did not interfere with the induction of mesoderm in Xenopus animal cap explants by activin or bFGF (chapter 3), two factors which play important inductive roles in early development. One potential target of SPARC could have been PDGF (platelet derived growth factor), since SPARC binds to PDGF-AB and -BB with high affinity. Interference with PDGF signalling in Xenopus has also been shown to generate some axis and anterior defects (Ataliotis et al.,
1995) that are comparable to those observed with microinjected SPARC. However, PDGF is only one of several factors known to participate in early development. It is conceivable that the more severe defects observed with SPARC injection were due to its binding to multiple factors. Moreover, any potential effects of SPARC on cell division and adhesion might have been subtle and therefore not obvious until later in development. Interestingly, the microinjection of two other acidic, Ca$^{2+}$-binding glycoprotein, BSP and OPN, resulted in developmental effects that were distinct from each other and from SPARC; these results indicate that the phenotypes generated were protein-specific.

The effects of native SPARC on tissue culture cells have been recapitulated by synthetic peptides corresponding to discrete domains of SPARC. For example, peptides from the cysteine-rich, follistatin-like domain (2.1) and the C-terminal EF-hand related motif (4.2) inhibit cell cycle progression in mid-G$_1$ (Funk and Sage, 1993). In addition to inhibiting cell cycle progression, peptides 2.1 and 4.2 were found to compete for the binding of SPARC to collagen types I and III and to endothelial cells. No effects on Xenopus development were observed when 60-1000 ng of peptide 2.1 or 2.3 were microinjected into the blastocoel cavity. However, injection of 60 ng of peptide 4.2 generated dorso-anterior defects. When the concentration of peptide 4.2 was increased to 100 ng, 100% of the embryos were headless. Further increases were associated with concentration-dependent defects in an anterior to posterior direction. The substantial effects of peptide 4.2 were surprising since, as discussed above, peptides 2.1 and 4.2 have similar effects on cultured cells in vitro. A potential explanation is that peptide 4.2 is a more potent inhibitor of cell-cycle progression than is peptide 2.1 (4 to 100 fold) (Sage et al., 1995). It also inhibits cell spreading in vitro.
The combination of cell-cycle modulation, cell surface receptor and collagen-binding, and counteradhesion may account for the interference of peptide 4.2 with *Xenopus* development. Whereas gastrulation did not appear to be affected by peptide 4.2, as judged by closure of the blastopore, the absence of dorso-anterior axis and structures indicate it is very likely that interference with cell movement and/or inductive events occurred. This gastrulation defect is similar to that observed when gastrulation proceeds in the absence of a blastocoel roof (Keller et al., 1992). In this scenario gastrulation is driven by convergent extension of the axial mesoderm and not by migration of cells. A similar situation may be taking place with SPARC peptide 4.2, which may inhibit mesoderm cell migration, but not convergent extension. The phenotype generated by peptide 4.2, however, differs in that at high doses peptide 4.2 forms no detectable axis. The high dose phenotype does lack all morphological signs of a blastopore, which are present at lower doses, thus a different mechanism may operate between the two doses.

The inhibition of endothelial cell proliferation by peptide 4.2 was abrogated by two mutant peptides, 4.2AA and 4.2K (Sage et al., 1995). Peptide 4.2 K was associated with apparently the same developmental defects as were seen with peptide 4.2. In contrast, embryos injected with peptide 4.2AA embryos appeared normal until the tailbud stage, with the exception of a subset which had ventral swelling. The effects *in vivo* of peptide 4.2 are therefore in part dependent on the formation of the disulphide bridge but are tolerant of a disruption of the Ca$^{2+}$-binding consensus sequence. The potentially Ca$^{2+}$-independent effect was confirmed by the observation that the co-injection of peptides 4.2, 4.2AA, or 4.2K with Ca$^{2+}$ or EGTA did not alter the results seen with the peptide alone. Although direct comparisons between the
effects observed in tissue culture vs. whole embryos are difficult, the Ca\(^{2+}\)-independent effects of peptide 4.2 \textit{in vivo} might be consistent with data that SPARC does not act as a chelator of Ca\(^{2+}\) in the extracellular space, despite its ability to modulate endothelial cell shape (Sage, 1992). The requirements of a disulphide bridge, an unusual feature of the EF-hand related motif of SPARC, indicates that the secondary structure of this region is critical. That microinjection of calmodulin, a protein containing four prototypic non-disulphide-bonded EF-hands, generated substantially different results indicated that the phenotypic effects of peptide 4.2 were particular to SPARC.

It was demonstrated that precocious expression of SPARC led to a variety of developmental defects in \textit{Xenopus} embryos. As a matricellular multifunctional protein, SPARC has been shown to affect a variety of cell behaviours, including counteradhesion and regulation of the cell cycle. While the molecular basis of the phenotypes generated by precocious expression of SPARC, in particular with peptide 4.2, remains to be determined, the data indicate that cell motion and cell proliferation were likely affected during \textit{Xenopus} gastrulation. Therefore the counteradhesion and inhibition of cell cycle activities observed \textit{in vitro} appear to be magnified \textit{in vivo} by precocious overexpression of SPARC while rapid cellular migration and proliferation are occurring.
Chapter 3

Regulation of SPARC expression during early Xenopus development: Similarities and differences between amphibian and mammalian regulation
Abstract

SPARC [(Secreted Protein, Acidic, Rich in Cysteine)/osteonectin/BM-40] is a highly conserved metal-binding extracellular matrix (ECM) glycoprotein which is first expressed by Xenopus embryos during early neurulation (stage 13) by presumptive notochord and somitic cells. Therefore I examined whether potent mesoderm inducing factors could directly regulate SPARC expression. When animal cap explants of stage 9 embryos were cultured in vitro, SPARC expression was not detected until sibling embryos reached late neurula (stage 19), indicating that expression of SPARC by stage 13 embryos is in part dependent on inductive signals that are absent in animal caps. Indeed, addition of activin, a potent mesoderm inducer, to animal cap explants resulted in SPARC being expressed when sibling embryos had reached stage 16. While bFGF, a ventral mesoderm inducer, had modest effects on SPARC mRNA expression, the combination of both activin and bFGF was synergistic. The appearance of SPARC transcripts 11 hours after the addition of activin and bFGF, indicates that unknown intermediates were likely to be involved in activating SPARC expression. Dissociated animal cap cells failed to express SPARC, indicating that activation of SPARC expression depends on cell-cell interactions. In order to identify the potential intermediate regulatory factors which may activate and control SPARC expression, we examined the genomic organization of the 5' end of the Xenopus SPARC gene. No significant homology to the equivalent region that is highly conserved in the mouse, bovine and human SPARC genes was observed. Thus while mammalian SPARC promoters lack TATA or CAAT-boxes, the Xenopus gene contains a consensus TATA-box. Moreover, promoter-proximal GGA-box repeats necessary for high level expression of mammalian SPARC are absent in Xenopus. When reporter constructs containing 5' flanking DNA linked to a β-
galactosidase reporter gene were microinjected into two-cell embryos, 868 bp of 5′ flanking DNA was sufficient to mimic the temporal and tissue-specific pattern of SPARC expression observed in whole embryos. The data indicate that (1) zygotic activation of SPARC mRNA transcription is likely to be mediated by regulatory factors acting downstream of major mesoderm induction events and that (2) the high sequence conservation at the 5′ end of mammalian SPARC genes is not evolutionarily conserved in *Xenopus*. 
Introduction

Early embryonic development in *Xenopus* is dependent on a complex series of rapid inductive interactions, mediated principally by the binding of secreted peptide growth factors to cognate receptors on adjacent cells (Kimelman et al, 1992). As a consequence, within ten to twelve hours after fertilization, the three primary germ layers are established. These layers in turn express a broad spectrum of macromolecules that play major roles in directing development. An important subset of these morphoregulatory molecules are those which constitute the extracellular matrix (ECM): collagens, proteoglycans and glycoproteins (Hay, 1991). Because of their ability to form elaborate architectural frameworks, ECM macromolecules were first perceived to function mainly as inert structural elements, giving tissues their unique shapes, strengths and resiliency. However it is now clear from extensive studies that they also play critical roles in regulating cell proliferation, adhesion, migration and differentiation throughout development. For example changes in the levels of laminin (LN), a major adhesive glycoprotein component of these basal lamina sheaths, promote the transformation of epithelium to mesenchyme and mesenchyme to epithelium, as exemplified by somitogenesis and neural crest cell emigration, respectively (Lallier and Bronner-Fraser, 1991). Recent evidence indicates that tenascin not only promotes the emigration of neural crest cells (Chiquet et al., 1991), but also regulates their progression through their differentiation cascades (Wehrle and Chiquet, 1990). Moreover, proteoglycans have been shown to chelate growth factors/inducing factors, hence modulating their biological activity during development (Klagsburn, 1992; Rifkin et al., 1991; Miao et al., 1996).

Numerous studies indicate that SPARC, a 43 kDa calcium-binding ECM glycoprotein,
also has complex multiple functions during development. SPARC is expressed in mammalian and amphibian embryos by all three primary germ layers, undergoing rapid turning “on” and “off” as development progresses (Damjanovski et al., 1994). However, its functions are not restricted to embryonic development as SPARC has been shown in several mammalian species to remain associated with adult tissues undergoing turnover, remodelling, secretion and repair (reviewed by Lane and Sage, 1994). Interference with its regulated pattern of expression leads to severe developmental defects during early embryonic development.

Precocious expression of SPARC cRNA in one cell of two cell *Xenopus* embryos, generates lateral defects in head and axis development, e.g. missing eyes, disorganized and missing somites (Chapter 2). Similarly, microinjection of anti-SPARC antibodies into the blastocoel cavity causes a variety of head and axis defects (Purcell et al., 1993). In *C. elegans*, misexpression of SPARC, or SPARC gene “knock-out” leads to impaired muscle development (Schwarzbauer and Spencer 1993).

Several potential activities of SPARC suggested from studies performed on tissue culture cells provide an indication of possible SPARC functions. (reviewed by Sage and Lane, 1994).

Thus, native SPARC and synthetic peptides representing distinct domains of SPARC, decrease cell-matrix interactions (Murphy-Ullrich et al, 1995), bind and potentially buffer the effects of cytokines such as PDGF AB and BB (Raines et al, 1992), regulate the expression of matrix-degrading proteases and inhibitors (Sage et al, 1989b), and modulate cell barrier permeability (Goldblum et al, 1994).

The complex patterns of SPARC expression during early mammalian development have led investigators to examine the regulatory mechanisms controlling SPARC expression.
DNA sequence analysis of the 5' flanking region of the mouse, bovine and human SPARC genes revealed repeated GCA-boxes and an absence of both a “TATA” box and “CAAT” box (Hafner, et al., 1994; Nomura et al., 1989; Young et al., 1989). Using promoter-reporter gene constructs of bovine and human SPARC it was demonstrated that approximately 500 bp of 5' flanking regulatory sequence is sufficient to generate levels of expression similar to those observed in vivo (Young et al., 1989). However, regulation of SPARC expression at the murine locus was found to be more complex. Reporter constructs indicated that accurate tissue-specific expression required up to 3.0 kb of the 5' flanking region (Nomura et al., 1989). Consistent with other regulatory domains the 5' flanking regions are punctuated with numerous potential transcription factor target sites, some of which are conserved among mammalian SPARC genes, e.g. the cAMP response, heat shock, and glucocorticoid response elements.

The above studies demonstrated a high degree of interspecies conservation of promoter-proximal regulatory elements in mammals which can direct cell-specific expression of SPARC in cultured cells. Since Xenopus SPARC transcripts are expressed by both neural and mesodermal tissues, we examined if activin could activate SPARC expression. Addition of activin to Xenopus ectodermal animal caps results in the expression of mesodermal and neural genes from some of the animal cap cells, and their differentiation into mesodermal and neural tissues. Using a combination of animal cap explants and whole embryos, we report that in Xenopus zygotic activation of SPARC mRNA transcription is likely to be mediated by regulatory factors acting downstream of major mesoderm induction events, and that the repeated sequence elements and motifs conserved at the 5' end of mammalian SPARC genes
was not conserved in *Xenopus* during evolution. *Xenopus* SPARC genomic sequences did, however, contain many of the cis-elements found in mammalian SPARC sequences.
Materials and Methods

Animal cap assays

Animal caps were excised from stage 8 *Xenopus* embryos which were bathed in 1X MMR (100 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM N-2-hydroxyethylpiperazine-N’-2-eteane sulfonic acid pH 7.4, Peng, 1991) and immediately transferred into 1 ml 0.5X MMR, containing 1 mg/ml BSA, 50 µg/ml gentamicin, with or without factors. After 2 hours, the animal caps were transferred into 100% Steinberg's, containing 50 µg/ml gentamicin. All steps were carried out at 20°C.

A) Growth factors

Animal caps were treated with human activin A (Genentech, San Francisco) at concentrations ranging from 1 to 100 ng/ml (Smith et al., 1990). Mouse bFGF and EGF (Boehringer Manheim) were used at concentration of 100 ng/ml and 10 ng/ml respectively (Green et al., 1992).

B) SPARC and synthetic peptides

Mouse SPARC (Sage et al., 1989a) and synthetic peptides (Lane and Sage, 1990) were generous gifts from Dr. Helene Sage, University of Washington, Seattle. Animal caps were cultured with SPARC, peptide 2.1 (amino acids 54-73 [CQNHCKHGKVCEDSNTP]) or peptide 4.2 (amino acids 254-273 [TCDLDNDKYLAEWAGCFG]), at concentrations of 1, 10 and 100 µg/ml, with or without a 1 hour preincubation with activin A (10 ng/ml) or bFGF (100 ng/ml).

C) Dissociation of animal caps cells

Animal caps (10) were placed in 2 ml of calcium-chelating buffer (60 mM NaCl, 0.67 mM
KCl, 0.4 mM EDTA, 5 mM Na$_2$HPO$_4$, 5 mM NaH$_2$PO$_4$, 10 mM Hepes pH 7.4, Sato and Sargent, 1989), gently dissociated with a wide bore pipet every 30 minutes. After 2 hours, the dissociated cells were allowed to settle to the bottom of the well and incubated in a Ca$^{2+}$, Mg$^{2+}$-free buffer containing (60 mM NaCl, 0.67 mM KCl, 0.4 mM EDTA, 10 mM Hepes pH 7.4) and incubated for 16 hrs before RNA extractions were performed.

D) SPARC antibody

As animal caps express SPARC message (this chapter), presence of SPARC protein may be required for induced animal cap differentiation. Animal caps, with or without activin A (10 ng/ml) or bFGF (100 ng/ml) treatment were cultured with (100 μg/ml) polyclonal anti-porcine SPARC antibody (previously demonstrated to bind to *Xenopus* SPARC [Ringuette et al., 1992]) in 100% Steinberg's, with 50 μg/ml gentamycin.

RNA isolation and Northern analyses

Embryos, animal caps, and dissociated cells were lysed in 500 μl of Chaos buffer (4M guanidium thiocynate, 0.1% Sarkosyl, 10 μg/ml β-mercaptoethanol) and extracted with a combination of 500 μl phenol, 100 μl chloroform and 50 μl 2 M NaOAc pH 4.5. The supernatants were precipitated with isopropanol and equal amounts of RNA (10 μg/lane, Fig 15, G) were fractionated by formaldehyde-agarose gel electrophoresis and transferred onto Duralose (Stratagene) (Sambrook et al., 1989). $^{32}$P-labelled SPARC cDNA probes were prepared using a Pharmacia random primer labelling kit. Hybridization and wash conditions were performed at high stringency, in 0.2X SSC at 60°C, were as described in chapter 1 (Damjanovski et al., 1992).
PCR analysis of animal cap explants

First strand cDNAs (using RNA from 10 animal caps) were prepared with BRL superscript II reverse transcriptase according to manufacturers' recommended conditions. Essentially 10 μg total RNA was hybridized to 10 ng oligo-dT in a 10 μl volume at 70°C for 5 minutes before being placed on ice. For reverse transcription 4 μl 5X reverse transcription buffer (7.5 mM MgCl₂, 1 mM each dATP, dCTP, dGTP and dTTP, 50 mM Tris pH 8.8, 250 mM KCl) was added along with 2 μl (10 units) Superscript II reverse transcriptase (BRL) in a 20 μl reaction, and incubated at 42°C for one hour. Two microliters of the reverse transcription mix was used in a 10 μl PCR reaction with 1 μl 10X PCR buffer (250 mM Tris pH 9.3, 20 mM MgCl₂, 500 mM KCl, 10 mM DTT), 2 μl dNTP mix (1.25 mM each dATP, dCTP, dGTP and dTTP), and 100 ng primer. Primers for detecting SPARC transcripts (Damjanovski et al., 1992, accession no. X62483) were 5'-CCTTGATTCAAAGCA-3' (nt 858) and 5'-AGGGCAGCTAGTGCTTT-3' (nt 39), and for detecting NCAM transcripts (Krieg et al., 1989, accession no. M25696) were 5'-CACAGTTCCACCAAATGC-3' (nt 2717) and 5'-GGAATCAAGCGGTACAGA-3' (nt 3142). PCR amplifications were carried out according to manufacturers' recommended conditions (Perkin-Elmer). The annealing temperatures were 60°C (25 cycles) and 55°C (30 cycles) for SPARC and NCAM respectively.

Isolation and analysis of Xenopus SPARC Genomic sequences

A Xenopus lambda-EMBL4 red blood cell genomic library (a gift from Dr. Thomas Sargent, NIH, Bethesda MD) was screened with a full length 1.6 kb Xenopus SPARC cDNA probe (Damjanovski et al., 1992). A 4800 bp genomic insert containing 1400 bp of 5'
flanking region, the first exon and intron, and the second exon and a portion of the second intron was subcloned in the HindIII site of the β-galactosidase reporter plasmid pSDK (Kothary et al., 1989). Two clones, XG6 (sense orientation) and XG10 (anti-sense orientation) were used to assay for β-galactosidase activity. A 561 base pairs deletion from the 5' end of XG6 was generated (clone XG6-K) by digesting with Ksp II. The Ksp II fragment was subcloned and sequenced.

Double stranded DNA sequencing was performed according to USB's Sequenase II sequencing kit. Due to the absence of convenient restriction endonuclease sites, sequencing was carried out by walking along the genomic DNA using 5 forward primers 5'-GGGATCCAGGCACAAGACGAAG-3' (nt +1880), 5'-GGCACCCCACACAGGCC-3' (nt +1341), 5'-ATGGCTACTAGCATC-3' (nt +1099) 5'-CTTCAGGCTCCTAGA-3' (nt +20) 5'-CATGTATGATCTGCAC-3' (nt -436), in addition to two reverse primers, 5'-CTAGGAGCCTGAAGT-3' (nt +15) and the universal T7 promoter primer. Sequences were analyzed using Intelligenetic's PCGENE software and the sequence was submitted to GenBank (Accession number U77424).

Transcription start site determination

The transcription start site was determined by 5' RACE analysis and S1 mapping. For 5'RACE analysis, first strand cDNA was generated using reverse primer 5'-GCAGCTAGTGCTTTGC-3' (nt +1900) according to Calzone et al., 1987. First strand cDNA was then synthesized in a 50 µl reaction with 10 µg RNA, 7.5 mM MgCl₂, 50 mM Tris pH 8.8, 250 mM KCl, 0.25 mM each dATP, dCTP, dGTP and dTTP, 5 µl RNase inhibitor (5 units) and 3 µl reverse transcriptase (Superscript II, 30 units, BRL) for 2 hours.
at 42°C. This first strand cDNA was then purified by phenol-chloroform extraction, ethanol precipitation. The first strand cDNA was polyadenylated with Pharmacia polyA polymerase according to manufacturer's conditions in a 20 µl reaction containing 4 µl (5X) polyA-polymerase buffer, 2 µl 1 mM dATP, and 2 µl polyA-polymerase incubated at 37°C for 20 minutes. The polyA-tailed construct was PCR amplified using oligo dT-XbaI forward primer and a *Xenopus* SPARC reverse primer 5'-CGGATCCAGGCACAAGACGAAGA-3' (nt +1879). The PCR product was cloned into the XbaI/Bam H1 site of pBluescript KS.

S1 mapping was carried out according to Sambrook et al., 1989, using the following substrates. A 445 bp single-stranded Taq I fragment labeled at its 5' end (overlapping the transcription start site, nts -407, +38) was hybridized to 10 µg poly(A)+ RNA isolated from *Xenopus* heart. Briefly 1 µg of the Taq I DNA fragment was end labelled in a 5 µl reaction with 1 µl 5X kinase buffer (250 mM Tris pH 7.5, 50 mM MgCl₂, 25 mM DTT, 0.5 mM spermidine), 1 µl γ-³²P ATP, 1 µl T4 kinase, incubated at 37°C for 30 minutes. Following NlaIII digestion a 140 bp fragment (which was now end labelled only at one end) was isolated. This fragment was denatured and hybridized with 10 µg of *Xenopus* heart poly(A)+ RNA in a 50 µl hybridization reaction with 400 mM NaCl, 40 mM PIPES (pH 6.5) and 1 mM EDTA at 60°C overnight. *Xenopus* heart RNA was used as it has been shown to be enriched in SPARC transcript (Damjanovski et al, 1992). The reaction was digested with 300 units of S1 nuclease with 280 mM NaCl, 50 mM sodium acetate (pH 4.5), and 5 mM ZnSO₄ at 30°C for 1 hour. The size of the protected fragment was determined by Urea-PAGE gel electrophoresis (Sambrook et al., 1989).
Reporter construct microinjection and enzyme assay

Genomic $\beta$-galactosidase reporter constructs ([50-300 pg] XG6 - 3400 bp sense insert, XG10 - 3400 bp anti-sense insert, XG6-K - 2550 bp sense insert) were co-injected with 10 pg of rhodamine dextran sulphate tracer into 1 cell of 2 cell embryos bathed in 1X MMR. After healing for 5 minutes, the embryos were transferred into 0.2X MMR and allowed to develop for times indicated in the figure legends. Embryos were fixed in 4% paraformaldehyde in PBS and colour reactions developed with 50 $\mu$g/ml X-gal according to Vize et al. (1991). Embryos were then post-fixed in 5% formaldehyde, dehydrated in methanol and cleared with (2:1) benzyl benzoate and benzoic acid for photography.
Results

Animal cap assays

SPARC transcripts are first expressed by the zygotic genome by early neurula (stage 13) embryos (Ringuette et al., 1992, Fig. 13 A). By mid-neurula (stage 18), transcripts were found by whole mount \textit{in situ} hybridization to be expressed by presumptive notochord and somitic cells (Fig. 1 A). Rapid increases in SPARC levels were then observed within the trunk mesoderm as development progressed (stage 25, Fig. 1 B). By stage 33, intense staining was found within the subnotochordal rod, floor plate of the neural tube and somites, relative to the anterior region (Fig. 1 C). To investigate the molecular events controlling the complex patterns of SPARC expression, I first sought to examine the effect of mesoderm inducers on SPARC expression in animal cap explants.

When animal cap explants excised from stage 9 embryos are cultured in the absence of exogenous factors, they give rise to an epidermal sphere (Fig. 14 A) which contains no mesoderm. Addition of activin to ectodermal animal caps alters animal cap morphology (Fig. 14 D) and results in the expression of mesodermal and neural genes from some of the animal cap cells, and their differentiation into mesodermal and neural tissues. Since SPARC transcripts are expressed by both neural and mesodermal tissues, we examined whether activin could activate SPARC expression in the explants. Untreated animal cap explants begin to express SPARC transcripts after 14 hours of culturing, at which time sibling embryos had reached stage 19 (Fig. 13 A, 13 B, and Table 2). This is 6 hours later than normal SPARC expression in stage 13 embryos. In the presence of activin, SPARC transcripts were
Figure 13. Delayed SPARC expression in animal caps is advanced by activin.

Expression of SPARC transcripts from 10 animal caps was analysed by Northern blot analysis using a $^{32}$P-labelled full length *Xenopus* SPARC cDNA. (A) SPARC transcripts were first detected in developing *Xenopus* embryos at stage 13. (B) Animal caps explants did not begin to express SPARC transcripts until sibling embryos had reached stage 19. (C) When animal caps were treated with activin (10 to 100 ng/ml), SPARC transcripts were now detectable by the time sibling embryos had reached stage 16, four hours earlier. Ethidium bromide staining of the gel was used to confirm the quality and quantity of the RNA (not shown).
Table 2: Growth factor induction of SPARC transcripts in animal caps.

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>SPARC transcript first detected (relative to sibling controls)</th>
<th>Time difference between expression in whole embryo and treated animal cap</th>
<th>Treatment length of stage 9 animal caps before SPARC transcript detected</th>
<th>Relative SPARC levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole embryos</td>
<td>stage 13</td>
<td>Stage 13 - Time 0</td>
<td>-</td>
<td>++++ (control)</td>
</tr>
<tr>
<td>Untreated animal caps</td>
<td>stage 19</td>
<td>6 hours</td>
<td>14 hours</td>
<td>+</td>
</tr>
<tr>
<td>Activin treated animal caps</td>
<td>stage 16</td>
<td>3 hours</td>
<td>11 hours</td>
<td>+++</td>
</tr>
<tr>
<td>bFGF treated animal caps</td>
<td>stage 19</td>
<td>3 hours</td>
<td>14 hours</td>
<td>++</td>
</tr>
<tr>
<td>EGF treated animal caps</td>
<td>stage 19</td>
<td>6 hours</td>
<td>14 hours</td>
<td>+</td>
</tr>
<tr>
<td>Activin+EGF treated animal caps</td>
<td>stage 16</td>
<td>3 hours</td>
<td>11 hours</td>
<td>+++</td>
</tr>
<tr>
<td>Activin+bFGF treated animal caps</td>
<td>stage 16</td>
<td>3 hours</td>
<td>11 hours</td>
<td>++++</td>
</tr>
</tbody>
</table>
expressed by animal cap explants 3 hours earlier, when sibling embryos had reached stage 16 (Fig. 13 C, and Table 2). However, this was still 11 hours from the start of incubation, supporting the proposal that the effect of activin on SPARC expression is indirect.

Since complete dorsal axis development is dependent on a series of inductive events orchestrated by a variety of growth factors, I also examined whether the addition of other inducers activated SPARC expression. Epidermal growth factor (EGF), which does not alter the epidermal fate or morphology of animal cap explants (Fig. 14 B), had no effect on SPARC expression (Fig. 15, lanes 1, 2, and Table 2). Basic fibroblast growth factor (bFGF), a ventral mesoderm inducer, which does not cause in animal caps to elongate (Fig 14 C) also did affect the temporal expression of SPARC in animal cap explants (Table 2). Slightly higher levels of SPARC transcripts were also consistently found in animal cap explants incubated with bFGF, compared to EGF (Fig. 15, lanes 2, 3, and Table 2). No significant differences were observed when animal cap explants were co-incubated with activin and EGF (Fig. 14 E), when compared to activin alone (Fig. 15, lanes 4,5 and Table 2). However, an additive or perhaps synergistic effect was observed when activin and bFGF (Fig. 14, F) were added together (Fig. 15, lanes 3, 4 and 6, and Table 2). These results were consistent with the observation that activin-mediated mesoderm induction is promoted by bFGF (Cornell and Kimelman, 1994).

Follistatin has been demonstrated to bind and inhibit the action of activin (Hemmati-Brivanlou, and Melton, 1994). Since domain II of SPARC has homology to the cysteine rich repeats of follistatin, the possibility exists that the inductive activity of activin might be affected by SPARC. However, pre-incubation of activin with SPARC protein or SPARC
Figure 14. Treatment of animal cap explants with activin, but not other growth factors, results in changes in morphology.

Animal caps, of equal size, removed at stage 8-9 and left untreated adopted a spherical morphology which was maintained for several days (A). Animal caps attained a similar morphology if they were treated with 10 ng/ml epidermal growth factor (B). Treatment with 100 ng/ml basic fibroblast growth factor did not alter the spherical shape of the animal caps (C), though swelling was evident, possibly due to the induction of vesicles. However if 10 ng/ml activin was added to the culture media the animal caps adopted an elongated morphology (D) after one day. This elongated morphology was similar if the animal caps were co-treated with activin and epidermal growth factor (E) or activin and basic fibroblast growth factor (F). (Magnification A-E 40X). RNA isolated from these animal caps was quantitated using optical density absorbance. (G) To confirm equal quantities, 10 µg of RNA from each animal cap treatment (first six lanes from left) was gel fractionated and stained with ethidium bromide. As a further control 10 µg of RNA from stage 18 embryos was used (G, lane 18).
Figure 15. Synergistic effect of activin and basic fibroblast growth factor on SPARC expression in animal caps.

Animal cap explants were incubated until sibling embryos reached stage 25. SPARC RNA expression was then measured by Northern blot analysis (10 µg of total RNA per lane, ethidium bromide staining of the gel was used to confirm equal loading see Fig. 14 G) using $^{32}$P-labelled full length *Xenopus* SPARC cDNA. Addition of EGF had no effect on SPARC expression as seen by comparing the hybridization signals obtained from untreated (lane 1) and EGF (lane 2) treated animal cap explants. While bFGF (lane 3) had a moderate effect on the level of SPARC mRNA expression, activin (lane 4) generated significantly higher levels of expression. Co-incubation of activin with EGF (lane 5) did not augment the effects of activin. However, a synergistic effect on SPARC expression was observed by co-incubating activin with bFGF (lane 6).
synthetic peptide (corresponding to the follistatin-like domain) did not affect the inductive properties of activin on animal cap explants, nor the timing of SPARC expression (data not shown). Moreover, incubation of activin-treated animal cap explants with anti-SPARC polyclonal antibodies also had no effect (data not shown).

Expression of embryonic muscle-specific gene products is dependent on both the action of inducing factors, and on the presence of cell-cell contact and extracellular calcium (Gurdon, 1988). Dissociated animal cap cells adopt a neural fate, as measured by NCAM expression, a gene not expressed by untreated intact animal caps (Sato and Sargent, 1989). As expected, when animal cap explants were dissociated in a calcium-free medium, NCAM expression was detected by RT-PCR (Fig. 16, A). SPARC transcripts were not expressed by dissociated cells (Fig. 16, B), indicating that SPARC expression in animal cap explants depends on cell-cell contact. Moreover addition of activin to the dissociated cells did not result in the expression of detectable levels of SPARC transcript (data not shown).

Characterization of the 5' end of the Xenopus SPARC gene

Tertiary screening of a lambda-EMBL4 Xenopus red blood cell genomic library with a full-length Xenopus SPARC cDNA led to the isolation of three unique clones with 15-20 kb inserts. A 4800 bp Hind III restriction fragment from one clone was found by Southern analysis to hybridize with a cDNA probe representing the 5' end of SPARC (data not shown). DNA sequence analysis revealed that the insert contained 3400 base pairs upstream of the start of translation. Sequencing of the 5' RACE product yielded the sequence 5'TTTCTCCAGACCATCTAGGAGCCTGAAGTTTTTCTCCTCGACAGGCGTTTGAC TTCAGAATGAGGGTCTGGGTCTTC (Fig 17, exon1 and first 31 bases of exon 2). The
Figure 16. SPARC expression in animal cap explants requires cell-cell contact.

Analysis of NCAM (panel A, 440 bp) and SPARC (panel B, 835 bp) expression in intact (cap), dissociated animal cap cells (dis) and stage 22 embryos (22) was measured by RT-PCR. Panel A shows intact animal cap explants do not express NCAM, whereas dissociated cells and whole embryos do. In contrast (panel B) dissociated animal cap cells did not express SPARC transcripts, whereas expression is detected in intact animal caps and stage 22 embryos.

Differences between the quantity of PCR product do not reflect levels of expression.
start of transcription was found to be separated from the start of translation by a 1800 bp intron (Fig. 17). The site of the start of transcription was confirmed by S1 mapping (Fig 18). Like its mammalian counterparts (Young et al, 1989), the first two exons were short, 45 and 70 bp respectively. However, no significant sequence homology was found between the 5' flanking region or first intron of the *Xenopus* and mammalian SPARC genes. PCGENE (Intelligenetic, Inc.), DNASIS (Hitachi Software) and MatInspector (Quandt et al, 1995) analysis of 1420 bp upstream of the start of transcription (Fig. 17, Table 3) revealed the presence of a TATA-box, -34 bp from the start of transcription, but no consensus CAAT promoter element. While most of the potential cis-acting elements identified by these programs are unlikely to represent *bona fide* targets of transcription factors, several deserved special consideration. For example, SPARC has been shown *in vitro* to be upregulated by retinoic acid, glucocorticoids, and heat shock (Mason et al., 1986, Nomura et al., 1989). Putative response element binding sites for a number of regulatory factors, such as for retinoic acid at positions -1118, and glucocorticoid at position -659, were identified (Table 3).

Moreover, several DNA elements also exist that have homology to the target sites of transcription factors that regulate expression of genes in tissues where SPARC is expressed. For example, two potential myo D response elements, critical for activation of muscle-specific genes, are found at positions -365 and -810.
Figure 17. Sequence organization of the 5' region of the Xenopus SPARC gene

A 4800 bp HindIII restriction endonuclease fragment was subcloned and sequenced. S1 mapping (Fig 18) confirmed the 5' RACE results indicating the transcription start site is 1420 bp from the 5' end the DNA fragment. A consensus TATA box is present at -34. Exon 1 (+1 to +45) is separated from exon 2 (+1849 to +1919) by a 1805 bp intron. Exon 2 contained the encoded the start of translation (+1863) and the entire leader peptide sequence (+1863 to +1919). The position of potential cis-acting genetic elements (presented in Table 3) are underlined.
Figure 18. S1 mapping of the transcription start site.

A 5' end $^{32}$P-labelled 152 base TaqI-NlaIII restriction fragment overlapping the transcription start site was hybridized to *Xenopus* heart poly(A)$^+$ RNA (known to be enriched in SPARC) and digested with S1 nuclease. Urea-PAGE analysis revealed the protected fragment (S1 lane, arrow) was 38 bases long (compared to the known sequence run adjacent to it), confirming the transcription start site indicated by the sequence of the 5' RACE clone.
Table 3: Potential cis-elements identified within the 5' flanking region and intron 1 of *Xenopus* SPARC genomic DNA.

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Position and Consensus Sequence</th>
<th>Potential role</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA Factor</td>
<td>-1299 WGATAR</td>
<td>erythroid transcription factor</td>
</tr>
<tr>
<td>Retinoic Acid (RARα)</td>
<td>-1118 TCAGGTCA</td>
<td>often plays role in hormone response</td>
</tr>
<tr>
<td>Hepatocyte Nuclear Factor 1 (HNF-1)</td>
<td>-1042 TRTTTGY</td>
<td>common transcription factor</td>
</tr>
<tr>
<td>Activator Protein 1 (AP-1)</td>
<td>-996 TGACT</td>
<td>general transcription factor</td>
</tr>
<tr>
<td>Activator Protein 1 (AP-1)</td>
<td>-871 TGACT</td>
<td>general transcription factor</td>
</tr>
<tr>
<td>GATA Factor</td>
<td>-850 WGATAR</td>
<td>erythroid factor</td>
</tr>
<tr>
<td>MyoD</td>
<td>-819 CANCTGNY</td>
<td>myogenic factor</td>
</tr>
<tr>
<td>Activator Protein 2 (AP-2)</td>
<td>-727 CCSCRGGC</td>
<td>general transcription factor</td>
</tr>
<tr>
<td>Activator Protein 1 (AP-1)</td>
<td>-710 TGCAT</td>
<td>general transcription factor</td>
</tr>
<tr>
<td>Glucocorticoid (GRE)</td>
<td>-660 AGAWCAGW</td>
<td>hormone response element</td>
</tr>
<tr>
<td>Nuclear Factor 1 (NF 1)</td>
<td>-580 AGCCAAAT</td>
<td>general transcription factor</td>
</tr>
<tr>
<td>cAMP Response Element</td>
<td>-554 ACGTCA</td>
<td>often plays role in hormone response</td>
</tr>
<tr>
<td>Octamer Binding Protein (OCT-1)</td>
<td>-541 AAATTCC</td>
<td>general transcription factor</td>
</tr>
<tr>
<td>MyoD</td>
<td>-371 CANCTGNY</td>
<td>myogenic factor</td>
</tr>
<tr>
<td>GATA Factor</td>
<td>-252 WGATAR</td>
<td>erythroid factor</td>
</tr>
<tr>
<td>Hepatocyte Nuclear Factor 1 (HNF-1)</td>
<td>-119 TRTTTGY</td>
<td>common transcription factor</td>
</tr>
<tr>
<td>Heat Shock Factor</td>
<td>-90 CNNGAANNTTCNNNG</td>
<td>stress response element</td>
</tr>
<tr>
<td>TATA element</td>
<td>-34 TATATAA</td>
<td>transcription initiation</td>
</tr>
<tr>
<td>Negative Response Element (NRE)</td>
<td>+826 ANTCTCCTCC</td>
<td>found with hormone regulated genes</td>
</tr>
<tr>
<td>cAMP Response Element</td>
<td>+1250 ACGTCA</td>
<td>often plays role in hormone response</td>
</tr>
<tr>
<td>CArG</td>
<td>+1355 CCWWWWWG</td>
<td>element associated with myogenic genes</td>
</tr>
</tbody>
</table>
Expression of the reporter β-galactosidase gene in *Xenopus* embryos

Co-injection of XG6 (containing 1400 bp 5' to the SPARC transcription start site) and rhodamine dextran sulfate into one blastomere of a 2 cell embryo resulted in reporter β-galactosidase being expressed in approximately 50% (23/50) of injected embryos. β-galactosidase-positive staining was evident by stage 15 (Fig 19, B, top embryo) along the developing dorsal axis. A more mosaic distribution within the trunk was visible in later embryos (Fig. 19, B, bottom embryo). In particular, strong staining was observed within the notochord and the somites, tissues which are known to express high levels of SPARC (Fig. 1 B). Embryos that were β-galactosidase-negative were also found to lack rhodamine dextran sulphate, suggesting that an injection error occurred in delivering the reagents into the embryo. No β-galactosidase activity was detected in control β-galactosidase plasmid constructs which did not contain SPARC promoter DNA (Fig. 19, A). Injection of a construct with the promoter reversed (XG10), also did not result in the expression of β-galactosidase (data not shown). Microinjection of a deletion construct (XG6-K) containing only 868 bp upstream of the start of transcription generates similar results as observed with XG6, indicating that the 868 bp of upstream sequence are sufficient to drive the expression of a reporter gene in a spatiotemporal pattern similar to endogenous SPARC.
Figure 19. Analysis of the Xenopus SPARC regulatory domains using β-galactosidase reporter constructs.

(A) Injection of a control β-galactosidase containing plasmid resulted in no detectable β-galactosidase activity in any tissue during neuralation or organogenesis (anterior to left, dorsal is up). (B) When a Xenopus SPARC genomic construct containing 1420 bp of 5' flanking DNA was added upstream of the β-galactosidase reporter, β-galactosidase was activity was detected. At stage 17, β-galactosidase staining was present within the dorsal axis (top embryo, anterior to left, n=neural fold). By stage 30, the staining patterns while more mosaic, was restricted to areas which express SPARC transcripts, such as the dorsal axis.
Discussion

In previous studies, we observed that embryonic expression of SPARC by presumptive mesoderm cells begins at the end of gastrulation. In this study, we used animal cap explants to determine whether growth factors, known to induce mesoderm patterning could directly activate SPARC expression (Table 1). Without exposure of exogenous factors, SPARC was expressed by animal cap explants, albeit at a later time than observed with sibling embryos.

Addition of activin, a potent dorsal mesoderm inducer, resulted in SPARC expression by animal cap explants at an earlier time. However, the 11 hour delay between the addition of activin and the detection of SPARC transcripts indicated that activin does not directly activate SPARC expression. Genes considered to be direct targets of activin induction, such as noggin, Xbra, X-not, goosecoid, X-lim, are expressed within two hours of activin addition to animal cap explants (Green et al., 1992; Steinbeiser et al., 1993). Another potential inducer examined was bFGF, a growth factor that induces ventral mesoderm. Although SPARC expression was upregulated by bFGF, the effect was not nearly as pronounced as observed with activin. While activin can induce a spectrum of mesodermal tissues in animal cap explants, recent studies have shown that full induction also requires the presence of bFGF (Cornell and Kimelman, 1994). We therefore tested the effect of co-incubating animal cap explants with both activin and bFGF. This resulted in a synergistic effect on SPARC expression, consistent with the proposed dependence of bFGF for activin signalling in vivo. However, the temporal expression of SPARC in the presence of both factors was the same as with activin alone, indicating that unknown intermediates acting downstream of mesoderm induction activate SPARC expression.
The question still remains as to why untreated animal cap explants eventually express SPARC. A partial explanation may lie in the fact that untreated animal caps over a period of time adopt an epidermal phenotype. SPARC is expressed in the epidermis later in development. Therefore, mesoderm induction may not be necessary for SPARC expression within surface ectodermal tissues.

Another important consideration is that expression of cell-type specific gene products by animal cap cells is in part dependent on cell-cell contact. Dissociated animal cap cells cannot be induced to express mesoderm cell markers, such as Myo D (Kato and Gurdon, 1994). Instead the dissociated animal cap cells adopt a more neural character, as evident by NCAM expression. I found that dissociated animal cap cells do not express SPARC transcripts even when cells were incubated for two days, after activin treatment. It therefore appears that, like myo-D, SPARC expression depends on cell-cell interactions, the nature of which remain unknown. Another possibility is that progression toward a neural phenotype by dissociated cells leads to the production of molecules that inhibit SPARC expression. Indirect support for this hypothesis is that in early embryos, SPARC transcripts are only detected within the mesoderm-induced floor plate of the developing neural tube (Figs. 1, 3). Since the floor plate has a distinct phenotypic character from the rest of the neural tube, the possibility exists that inhibitors of SPARC expression could be expressed by the neural tube, but these inhibitors are absent from the floor plate.

Several studies have demonstrated that the activity of inducing agents are modulated or delayed by binding to ECM glycoproteins. For example, TGF-β binds tightly to fibronectin (Fave and McClure). SPARC has been shown to bind and inhibit PDGF signalling in vitro
(Raines et al., 1992). Moreover, domain II of SPARC bears strong homology to the cysteine-rich repeats of follistatin, a secreted peptide hypothesized to inhibit activin signalling \textit{in vivo} (Hemmati-Brivanlou and Melton, 1994). I sought therefore to examine if SPARC could modulate the inducing activity of activin on animal cap explants.

Preincubation of activin with purified SPARC or SPARC peptides, demonstrated to have biological activity \textit{in vivo} and \textit{in vitro} (reviewed by Lane and Sage, 1994), had no effect on activin signalling. Therefore, within the limited context of these experiments, it does not appear that domain II of SPARC has follistatin-like activity \textit{in vitro}. Addition of exogenous SPARC or synthetic peptides also did not affect SPARC expression by untreated animal cap explants, supporting the idea that SPARC (1) does not interfere with the progression of animal cap cells toward an epidermal fate, (2) nor does it modulate its own expression. This was a concern because the floor plate of the neural tube expressed high levels of SPARC mRNA, but was devoid of protein. In contrast, SPARC was abundant within the remainder of the neural tube, which did not express SPARC mRNA (Damjanovski et al, 1994).

Amino acid sequence analysis has revealed a 78% sequence similarity exists between amphibian and mammalian SPARC, despite the evolutionary divergence of these organisms approximately 200 million years ago. Moreover, the widespread pattern of SPARC expression among these organisms is also conserved during embryonic and adult development. For example SPARC is expressed at high level within \textit{Xenopus} and mouse embryos within the developing somites, eyes and epidermis. Molecular analysis of the 5' regulatory domains of mouse, human and bovine SPARC genes also revealed a striking degree of sequence conservation. In light of the above, we sought to determine if there was
also a conservation of cis-acting genetic elements regulating SPARC expression between mammals and *Xenopus*. DNA sequence analysis revealed that there is no sequence homology between the 5' end of *Xenopus* and mammalian SPARC genes. Despite the sequence divergence, some similarities do exist in their overall organization. For example, the molecular organization of the first and second exons are conserved (Hafner et al., 1994; Young et al., 1989). Exon I (ranging from 40-70 bp) represents the majority of the 5' untranslated region, while exon II (ranging from 60-90 bp) contains the remainder of the 5' untranslated region and encodes the entire signal peptide. However, intron I in *Xenopus* is less than 2 kb, compared to greater than 10 kb in mammals. In addition, 4-8 CCTG direct repeats exist within exon I in mammals, whereas only one CCTG element is found in *Xenopus*. Interestingly, the 3' acceptor site (TGTTTTTTTTTAGA/G) of intron I is exactly duplicated 120 base pairs upstream, even though Northern suggests no alternative splicing.

The most striking differences between the 5' regulatory regions of *Xenopus* and mammalian SPARC genes were found within the first 250 base pairs upstream from the start of transcription. Mammalian promoters lack both a consensus CAAT-box and TATA-box, elements usually associated with developmentally regulated genes. In contrast, a perfect consensus TATA-box is found at position -34 in *Xenopus*. The repeated GGA-box elements found associated with the mammalian SPARC gene promoter-proximal domain (between -70 to -250 bp) are also absent in *Xenopus*. Moreover, both the promoter and promoter-proximal regions of the *Xenopus* SPARC gene are AT-rich (65%), compared to a GA-rich (65-70%) content found in mammals.

Data accumulated from the analysis of the 5' end flanking regions of eukaryotic genes
indicate that there is no conservation of the position of cis-acting elements among genes. Therefore, even though there is no sequence homology between the 5' flanking regions of *Xenopus* and mammalian SPARC genes, they share common cis-acting elements which may contribute to the conserved pattern of SPARC expression. For example, *Xenopus* and mammalian SPARC genes contain potential heat-shock, retinoic acid, cAMP, NF1, MyoD, AP-1, and AP-2 response elements found at different locations (Hafner, et al., 1994; Nomura et al., 1989; Young et al., 1989). While it remains to be established if these putative elements represent *bona fide* targets of transcription factors, SPARC expression *in vitro* is upregulated by heat shock and retinoic acid (Nomura et al., 1989; Young et al., 1989). In addition, SPARC is expressed at high levels within the developing somites, cells which depend on MyoD to initiate expression of muscle-specific gene products (Mohun et al., 1989).

A major limitation of identifying cis-acting genetic elements by a computer search of data banks is that far too many putative elements are identified to be realistic. Therefore, as a first step to determining which of the putative cis-acting elements of the *Xenopus* SPARC gene are operational *in vivo*, β-galactosidase reporter gene constructs were injected into two cell embryos. Although microinjection of a reporter construct in *Xenopus* results in mosaic expression, the pattern of expression can be deciphered by doing multiple injections. Using this approach, I demonstrated that a construct containing 868 bp upstream of the start of transcription (including the first intron) was sufficient to generate a β-galactosidase expression pattern similar to that observed for endogenous SPARC transcripts. Deletion studies are now being conducted in the laboratory to more precisely map the position and identity of these cis-acting regulatory elements.
Overall Conclusions

SPARC is a secreted calcium-binding glycoprotein which is evolutionarily conserved among organisms ranging from C. elegans to mammals. A distinguishing feature of SPARC, when compared to other calcium-binding ECM glycoproteins is that it has two distinct extracellular matrix calcium-binding domains; a low affinity, high capacity glutamic acid-rich domain and a low capacity, high affinity EF-hand related motif. Prior to this investigation, SPARC was found to be associated with a wide range of tissues in mouse embryos. However, its potential contribution(s) to the development of these tissues could only be inferred from its effects on tissue culture cells. In order to gain a better understanding of the precise morphogenetic contribution(s) of SPARC to embryonic development, I sought to make use of the several biological and technical advantages afforded by Xenopus.

As a first step, I used a combination of whole mount in situ hybridization and immunohistochemistry to examine the precise spatiotemporal distribution of SPARC from fertilization to organogenesis. The data generated led to an interesting correlation between the expression of SPARC and gap junction-mediated electrical coupling of myotomes. SPARC accumulated within the intersomitic clefts before the onset of spontaneous myotome contractions mediated by gap junctions. As innervation of the myotomes occurred, and gap junction levels decreased, so did the levels of SPARC. While this correlation may be fortuitous, SPARC expression was also found to be high in other gap junction-enriched tissues. Of special note was its enrichment in the heart, whose contractions are mediated by cardiac muscles which are also electrically coupled by gap junctions. The enrichment of SPARC within the intersomitic clefts appeared to have been facilitated by the translocation
of SPARC transcripts to the plasma membrane adjacent to the intersomitic clefts. This represented the first demonstration of SPARC transcript localization within any organism. This observation may prove to be useful in determining if gap junction-mediated electrical coupling is in part dependent on SPARC. For example, will inhibition of SPARC transcript localization interfere with myotome contractions? Attempts to interfere with SPARC transcript translocation are now being made by using the recent advent of frog transgenic procedures, which allow for non-mosaic expression of recombinant genes.

SPARC transcripts were also expressed by the floor plate of the early neural tube. However, the floor plate did not accumulate any detectable SPARC protein. In contrast, the remainder of the neural tube, which did not express SPARC transcripts, was enriched in protein. The data suggest that SPARC action is not always restricted to the site of its synthesis and that SPARC can be recruited to other locations.

The complex and transient patterns of SPARC expression in embryonic tissues indicate that a precise regulation of its expression is crucial during early development. Indeed ectopic expression of SPARC was found to generate severe developmental anomalies. Microinjection of SPARC cRNA constructs into early blastomeres, or protein into the blastocoel cavity, resulted in a variety of similar anterior defects. Synthetic peptides representing distinct domains of SPARC, demonstrated to have biological activity in vitro, were also microinjected into the blastocoel cavity to determine which regions of SPARC were active in vivo. One peptide, representing the EF-hand related motif, was found at low concentrations to generate defects similar to those observed by microinjection of RNA and native protein. Histological analyses indicated that the defects could have been generated by
interfering with the migration of mesoderm cells along the blastocoel roof during gastrulation, consistent with an anti-adhesive ability displayed \textit{in vitro}. However, increasing doses of the EF-hand related peptide resulted in unique developmental defects. The injected embryos displayed no overt signs of gastrulation, though they displayed what appeared to be three primary germ layers when analysed histologically several days later. This raised the question as to whether or not the phenotypes generated at higher doses reflect a property of native SPARC. An answer may lie in recently published data. It was demonstrated that a peptide similar to, but slightly longer than the one injected, dimerizes at high doses, while native SPARC does not (Maurer et al., 1995). These data also indicate that the dimer interacts with unidentified cell surface receptors. It is therefore conceivable that the synthetic peptide has distinct effects on \textit{Xenopus} development.

To investigate the mechanisms underlying the generation of these phenotypes, several approaches could be used. Tissue explants can be used to more directly investigate the actions of the peptides. Though addition of native protein and synthetic peptides did not interfere with the convergent extension movements of animal cap cells, nor with their induction by growth factors, other tissue explants may be more informative. Tissue-specific markers could be used to determine the molecular and cellular changes generated by ectopic expression. Tissue transplantation studies could also be pursued to examine whether a tissue is absent because the cells are no longer competent to differentiate, or can they still differentiate when transplanted into a healthy host.

Previous \textit{in vitro} studies demonstrated that a variety of factors influence the expression of SPARC. I therefore sought to examine the regulatory factors which control SPARC
expression within the more complex environment of tissue explants and intact embryos. SPARC was found to expressed by animal cap explants which maintained an ectodermal fate. However, it was not expressed by these cells if they were dissociated, indicating that cell-cell contact was necessary for SPARC expression. SPARC expression within animal caps was greatly up-regulated by the addition of mesoderm inducers such as activin. However, the upregulation was delayed by about 11 hours, indicating that unknown interdemiate activated SPARC expression. Genomic sequence analysis demonstrated that Xenopus SPARC, like mouse, human and bovine SPARC, contained an intron within its 5' untranslated region, and similarly arranged exons 1 and 2. Unlike mammalian regulatory SPARC sequences which were GA rich, Xenopus sequences were found to be AT rich. Xenopus SPARC regulatory sequences also contained a consensus TATA box which are absent in the mammals. However, there was a conservation of some regulatory domains between the Xenopus and mammalian genes. Serial deletions of the reporter constructs remain to done to determine which regulatory elements are active and how they contribute to the complex and rapid changes in SPARC expression during early Xenopus embryonic development.

In summary, this investigation has demonstrated that some of functions ascribed to SPARC from mammalian studies are likely to be conserved in Xenopus. However, some of the data generated was made possible because of the technical advantages afforded by Xenopus and the unique features of its early development. It therefore is very likely that Xenopus will continue to serve as a valuable organism to gain a more comprehensive understanding of the expression and morphogenetic contribution of SPARC to embryonic development.
Summary

1) SPARC is a matrix glycoprotein expressed early in *Xenopus* embryos shortly after gastrulation within the embryonic mesoderm.

2) As development progresses, SPARC undergoes rapid and dramatic changes in its pattern of expression within the notochord and somities.

3) SPARC is enriched within the intersomitic cleft as long as muscle contractions are mediated by gap junctions.

4) Within the developing neural tube, the distribution of the protein did not correlate with the distribution of its transcript.

5) Ectopic expression of SPARC prior to its embryonic induction results in developmental defects, particularly within the anterior of the embryo.

6) Similar anterior defects are observed with ectopic introduction of an N-terminal EF hand SPARC peptide, suggesting that this domain has important roles.

7) Use of mutant peptides demonstrate that an internal cysteine bond is necessary for the activity of the peptide.

8) SPARC transcript expression, delayed in uninduced ectodermal animal caps, is upregulated by the mesoderm inducers activin and bFGF, whose activities are synergistic.

9) Cell-cell contact is required for SPARC expression as dissociated animal caps do not express SPARC.

10) The conservation of overall genomic regulatory sequences between amphibian and mammalian SPARC is low; however, specific regulatory elements are conserved.
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Appendix A

The data in chapters one has been published and has been reproduced with permission:


All work described was performed within the laboratory of Dr. Maurice Ringuette and as such his name appears on all publications. The work described in chapter one was performed and analyzed by me, while L. Malava provided monoclonal SPARC antibodies and technical assistance on their use.

The data in chapter 2 has been submitted for publication.


The work described in chapter two was also performed and analyzed by me, however X. Karp provided control data about the (negative) effects of a protein (BSP) on development. S. Funk and E.H. Sage provided SPARC protein synthetic peptides and technical assistance on their use.