CHAPTER 1

INTRODUCTION

SPARC

SPARC structure and expression

SPARC (secreted protein acidic and rich in cysteine) is a 43 kDa glycoprotein that is secreted by many different types of cells. The various names given to the protein have reflected the protein size (43 kDa) [1], tissue source in basement membrane (BM-40) [2], and biochemical characteristics, such as affinity for bone matrices (osteonectin) [3]. SPARC was first purified as a major non-collagenous component of bovine bone, with binding affinity to hydroxyapatite and collagen I [3]. Human SPARC consists of 286 residues which, under current classification [4], is divided into 3 domains; the acidic, amino-terminal domain, the follistatin-like (FS) domain, and the extracellular calcium-binding (EC) domain. As such, SPARC is a member of the FS-EC family of proteins.

(i). N-terminal domain I (residues 1-52) is highly acidic and binds several calcium ions with low affinity. This domain contains the major immunological epitopes of SPARC. It also exhibits the most divergent sequence among the family of SPARC-like proteins. Therefore, antibodies against SPARC have not been found to cross-react with or recognize SPARC-like proteins [5].

(ii). Follistatin-like (FS) domain (residues 53-137) contains 10 cysteins and is homologous to a repeated domain in follistatin, an inhibitor of the TGF-β-like cytokines activin and inhibin, and agrin, which induces aggregation of nicotinic acetylcholine receptors [6].

(iii). Extracellular calcium binding (EC) domain (residues 138-286). This domain contains two EF-hand motifs, which consists of an α-helix-loop-α-helix conformation, each with a high affinity calcium binding site.

Before this classification, Engel *et a*l [7] proposed that the protein contained 4 distinct domains. Evidence was later provided for a close interaction between domain III and IV, and indicated that these 2 domains are not independent but represent one domain, for which Maurer *et al.* proposed the name EC domain [4]. More recently, Busch *et al.* [8] have shown considerable interaction between the FS and EC domains with respect to calcium binding.

SPARC occurs as a single copy gene per haploid human genome. There are 2 species of SPARC mRNA in human tissue with lengths of 2.3 and 3.0 kb. The larger species of message is consistently less abundant and probably arises from alternative poly A+ attachment site utilization [6, 9]. Although the major SPARC mRNA encodes a 32 kDa protein, the secreted form migrates at 43 kDa on SDS-PAGE, in part due to glycosylation.

The SPARC gene is highly conserved among vertebrate species, suggesting that the protein has an important physiological role. Human and bovine SPARC exhibit 99% identity in amino acid sequence [6, 9], and 93% between human and mouse SPARC [6, 10]. The major divergence was observed in the amino terminus of the mouse protein located specifically between amino acid residues 5-23, so called peptide 1.1. However, while considerable divergence exists in this region, the character of the amino acids does not change substantially with the substituted amino acids being primarily acidic in nature [9]. Purification of SPARC from fetal porcine bone has also been reported [11]. Unlike bovine bone SPARC, porcine SPARC is

reported to have no affinity for either native or denatured collagen, nor does it selectively bind hydroxyapatite.

Platelets treated with varying concentrations of collagen and thrombin release SPARC in a concentration-dependent manner [12, 13]. Using automated Edman degradation, the amino terminal sequence of platelet SPARC is shown to be identical to the sequence of human bone SPARC. However, some structural differences were found between bone and platelet SPARC. Monoclonal antibodies have been produced that distinguish bone and platelet SPARC [14]. Platelet SPARC has a greater apparent molecular weight than bone SPARC when analyzed on SDS-polyacrylamide gel due to the presence of N-glycosylation, which reduces the binding capacity of platelet SPARC to collagen type V [13, 15, 16]. Kelm and Mann [15] showed that bone and platelet SPARC have different oligosaccharide chain structures, with bone SPARC possessing a high mannose-type and platelet SPARC a complex-type structure. They are also different in sensitivity to specific glycosidases. Bone SPARC was susceptible to cleavage by endo H but not neuraminidase, while platelet SPARC was susceptible to neuraminidase but not to endo H. Also concanavalin A bound specifically to bone SPARC but not platelet SPARC, while Lens culinaris agglutinin bound to platelet but not bone SPARC. Moreover, bone SPARC bound specifically to collagen type I, III and V, whereas platelet SPARC had no apparent affinity for these collagen types, suggesting further that the two proteins are functionally distinct [15].

SPARC expression in normal tissues

Since SPARC was firstly purified from bone, its biological significance was initially linked to the regulation of bone mineralization. High levels of SPARC mRNA and protein are associated with developing bones and teeth, principally

osteoblasts, odontoblasts and perichondrial fibroblasts of murine, bovine and human embryos [17]. Later studies showed that SPARC is also present in many non-mineralized tissues and platelets. It is found in gut, bone marrow, tendon, lens, skin, liver, cornea, pulp, and also in vascular smooth muscle and endothelial cells [18-20].

SPARC levels can be regulated by several growth factors and the response varies in different cell types. SPARC mRNA levels are increased by PDGF or bFGF in cultured mesangial cells [21] and TGF-β can enhance both mRNA and protein levels in fibroblasts by a nuclear post-transcriptional mechanism [22]. Basic FGF decreased SPARC synthesis by decreasing the stability of SPARC mRNA in osteoblasts [23]. In human pulp cell cultures, TGF-β was shown to increase SPARC mRNA and protein levels. Basic FGF, PDGF and EGF decreased SPARC levels, while TNF-α and IL-1β abolished SPARC synthesis [18, 24].

In the adult the expression of SPARC is limited largely to tissues undergoing repair or remodelling due to wound healing, disease, or natural processes. Normal liver expresses SPARC but increased expression was found in the initial stages of hepatic fibrosis [25]. Also, in the angiotensin II-infused animal model, SPARC was transiently expressed by interstitial fibroblasts at sites of tubulointerstitial injury and fibrosis, and by smooth muscle cells in the adventitia of injured arteries [26]. Immunohistochemistry showed enhancement of SPARC in synovial cells of rheumatoid arthritis and osteoarthritis [27].

SPARC expression in neoplasia

Cancer formation and metastasis is a process that involves tissue remodeling, and upregulation of SPARC was also found in many types of cancer including

melanoma [28], hepatocellular carcinoma [29], esophageal carcinoma [30], prostate cancer [31] and breast cancer [32, 33].

In vitro, SPARC is expressed in many cancer cell lines including glioblastoma [34], melanoma [35], ostosarcoma [5], HT1080 fibrosarcoma [5] and prostate cancer cell lines [31]. Among breast cancer cell lines, SPARC is not expressed by better-differentiated lines like T47D and MCF-7, but expressed in cell lines that have acquired mesenchymal features such as BT-549, MDA-MB-435 and Hs578T [36]. In the course of our studies, we found that the invasive MDA-MB-231 breast cancer cell line also expresses low levels of SPARC (Chapter 3). Expression of SPARC is usually increased when attachment-dependent cells are placed in culture. This phenomenon, referred to as culture shock [37] is particularly evident in cultured fibroblasts and endothelial cells, and is thought to be analogous to the enhanced synthesis of certain proteins that occurs upon tissue disruption. Expression of SPARC also diminishes after neoplastic transformation [6].

SPARC-related protein family

SPARC is a member of a family of proteins that exhibit a similar basic structure. These proteins have clusters of acidic residues imparting an overall acid pI, a follistatin-like region that is cysteine-rich, and a high affinity calcium-binding region containing two EF hands [38]. Members of this family referred to as FS-EC proteins includes the rat brain protein SC1 [39] and its human homologue hevin [40], the quail retina protein QR1 [41], the TGF-β-induced protein TSC-36/glioma-secreted follistatin-related protein (FRP) [42, 43] and the human testicular proteoglycan testican [44]. This group of proteins contains a follistatin-like module followed by the EC domain. However, the N-terminal domain I of each protein exhibits considerably

less homology, although all are acidic. With regards to functional similarities, hevin has been shown to inhibit the attachment and spreading of human endothelial cells [45] and is lost with malignancy in the prostate [46]. Both QR1 and TSC-36 are associated with cell cycle inhibition [42, 47].

SPARC and calcium

The EF-hand is a highly conserved calcium-binding motif found in a large number of intracellular proteins, including calmodulin, troponin C, and calcineurin. It is responsible for mediating calcium signal to target proteins of a variety of functions. The discovery of EF-hand motifs in extracellular multi-domain proteins such as SPARC led to speculations about their regulatory functions in the extracellular space where calcium levels are higher than in the cytosol. SPARC is calcium-dependent, and both the function and structure of SPARC are modulated by calcium ions. There are 2 calcium-binding sites of very different affinity in SPARC [8, 48]. The low affinity site (Kd≥10 mM) belongs to the N-terminal domain 1 and the high affinity site (Kd=0.6 mM) is in the EF-hand in the C-terminal.

Variations of calcium levels in the extracellular space can regulate functions of SPARC such as collagen binding, and the high-affinity calcium binding is important for stabilization, folding and secretion during biosynthesis. Depletion of calcium from the single high affinity site changes several physical properties o

f the protein dramatically. It causes a change in α-helicity, intrinsic fluorescence and rate of cleavage by α-chymotrypsin [48]. Binding of SPARC to collagen type III was only observed above 10 mM calcium [49], while calcium requirement for collagen type IV binding was found to be 0.02 mM or less [50]. Calcium concentrations in the range of 3 mM are considered to be characteristic for

the extracellular space [51]. Pottgiesser et al [52] studied mutant human SPARC with deletions including the N-terminal acidic domain, a central α-helical domain and the C-terminal EF hand domain. The results showed that only elimination of the whole EF hand domain or its single disulfide bond decreased production and secretion, indicating that the C-terminal region is essential for correct folding. Also, deletions in the α-helical domain caused a dramatic reduction in calcium binding and abolished calcium-dependent binding of SPARC to collagen type IV [52].

SPARC and the ECM

SPARC, along with other extracellular macromolecules such as thrombospondin (1 and 2), tenascin (C and X) and osteopontin, are classified as matricellular proteins, which means they bind cells to ECM but are not part of the ECM structure themselves. Although structurally unrelated, they seem to share similar antiadhesive effects, which leads to cell rounding and finally disrupts cell-matrix interactions. Matricellular proteins are different from traditional ECM proteins such as fibronectin, laminin, fibrillar collagens and vitronectin, all of which are adhesive proteins and contribute to the structural stability of the ECM [38, 53].

SPARC can bind a number of different ECM components including thrombospondin 1, vitronectin, entactin/ nidogen, fibrillar collagen (types I, II, III and V) and collagen type IV, the prevalent collagen in basement membrane [54]. Therefore, SPARC has the potential to contribute to the organization of matrix in interstitial connective tissue as well as basement membrane. Binding of SPARC to collagen depends on moderate calcium concentration [48].

Some studies have shown specific interactions between SPARC and collagen. Mov-13 mice, which are deficient in collagen type I, do not retain SPARC in the

extracellular matrix, even though they are able to transcribe and translate SPARC mRNA *in vitro* [55]. Furthermore Sasaki *et al.* [56] showed that SPARC could be cleaved by a number of matrix metalloproteinases (MMPs) including collagenase 3, gelatinases A and B, matrilysin, stromelysin I, and that this was accompanied by an increase in collagen-binding affinity.

Exogenous SPARC moderately inhibited collagen type I synthesis in the MG63 osteosarcoma cell line, and significantly decreased fibronectin and laminin synthesis in HT1080 fibrosarcoma, MG63 osteosarcoma and T-24 bladder carcinoma cell lines [5]. In contrast, Franci *et al.* showed that SPARC-null mesangial cells displayed diminished expression of collagen type I and TGF-β1 mRNA and protein. Addition of rSPARC restored both proteins, suggesting that SPARC regulates the expression of collagen type I and TGF-β1 in kidney mesangial cells [57].

SPARC modifies the interaction of cells with ECM, probably in part through the induction of proteases and their inhibitors. It increases the expression of collagenase (MMP-1), stromelysin (MMP-3) and gelatinase B (MMP-9) in rabbit synovial fibroblasts. Experiments with synthetic peptides indicated that a region in the neutral α-helical domain III was involved in the regulation of collagenase [58]. SPARC and synthetic peptide 3.2, belonging also to domain III, stimulated the production of gelatinase B (MMP-9), interstitial collagenase (MMP-1) and prostaglandin synthase (PGHS)-2 by human monocytes [59]. Addition of purified SPARC, or an N-terminal synthetic peptide, to bovine aortic endothelial cells undergoing angiogenesis *in vitro* results in a decrease in the synthesis of fibronectin and thrombospondin-1, and an increase in the synthesis of type-1 plasminogen activator inhibitor [60].

Biological functions of SPARC

SPARC is an abundant glycoprotein and has been suggested to participate in the modulation of cell-matrix interactions, bone mineralization, wound repair and angiogenesis [6]. SPARC has a role in branching morphogenesis of developmental fetal rat lung [61]. Its mRNA and protein are abundant in epithelial cells of the developing airways in early gestation lung, and in the blood vessels associated with these structures at later stages of development. Treatment of the explants with synthetic SPARC peptide 1.1 or anti-SPARC monoclonal antibody markedly attenuated airway branching, suggesting an important role of SPARC in this process. SPARC is also involved in myoblast differentiation. The expression of SPARC mRNA was markedly upregulated during myogenesis, and the treatment with an anti-SPARC antibody almost completely prevented the differentiation of myoblasts [61].

In nematodes, developmental anomalies are induced by over- expression or suppression of SPARC [62, 63]. Microinjection of SPARC RNA, protein, peptides, or antibodies interferes with Xenopus embryonic development [64, 65]. In contrast, deletion of the SPARC gene in mice resulted in a relatively mild phenotype. SPARC-deficient mice appear normal and fertile but develop severe eye pathology characterized by cataract formation and rupture of the lens capsule [66]. A study by electronmicroscopy and immunohistochemistry showed modification of the basal surface of the lens epithelial and fiber cells at the basement membrane (capsule) interface [67]. Additional potential defects, such as decreased bone formation leading to profound osteopenia, are under investigation [68]. It is suspected that homologous proteins, such as SC1, QR1, testican-1 or testican-2 (see above section) may compensate for several SPARC functions during embryonic development of SPARC-deficient mice.

Although SPARC expression is high in bone, it is known to be a potent inhibitor of hydroxyapatite formation and may play a role in preventing excessive mineralization in bone [69]. The ability of SPARC to initiate the precipitation of calcium and phosphate from stable solution in the presence of collagen, along with the ability of SPARC to interact with hydroxyapatite, collagen and calcium, suggest an important role in bone formation and the regulation of bone growth [3, 69]. In adults, SPARC is highly expressed in the eye, suggesting a role in maintenance of ocular physiological functions [38].

In vitro, the 2 major effects of SPARC on mesenchymal cells are anti-adhesion and anti-proliferation. SPARC, along with tenascin (TN) and thrombospondin (TSP), is known to have antiadhesive properties that lead to cell rounding and partial detachment from the substratum [70]. Exogenous SPARC has been shown to induce a round morphology in confluent monolayers of bovine smooth muscle cells, fibroblasts and endothelial cells. It also maintained the round morphology of newly plated (trypsinized) cells and inhibited their spreading [49]. A study with SPARC peptides showed that peptide 1.1 from the N-terminal, and peptide 4.2 from the C-terminal, can each inhibit endothelial cell and fibroblast spreading [71]. This finding that 2 separate regions of SPARC contribute to its anti-spreading activity suggests that multiple regions of the protein act in concert to regulate the interaction of cells with their extracellular matrix. Mesangial cells from SPARC-null mice exhibited a flat morphology and an altered actin cytoskeleton. SPARC-null fibroblasts did not display any overt differences in cell morphology, but they responded to exogenous SPARC by rounding up in a manner similar to wild-type fibroblasts.

The effects of SPARC on cell proliferation are cell type-specific. SPARC does not effect proliferation of melanoma [35], fibrosarcoma [72] or prostate cancer cell lines [73], but inhibits the growth of fibroblasts, endothelial cells [74], mesangial cells [21] and ovarian cancer cell lines [75]. In the course of our studies, we also found it to inhibit human breast cancer cell proliferation. SPARC-null mesangial cells, fibroblasts and aortic smooth muscle cells were shown to proliferate faster than their respective wild-type counterparts [76].

SPARC can also associate with certain growth factors and modify their effect on cells. It can bind to vascular endothelial growth factor (VEGF), inhibits VEGF-stimulated proliferation of human microvascular endothelial cells [77], and counteracts the proliferative effect of basic fibroblast growth factor on smooth muscle cells [54]. rSPARC or its synthetic peptide 2.1 inhibited PDGF-induced mesangial cell DNA synthesis *in vitro* [21]. SPARC can bind PDGF-AB, PDGF-BB, but not PDGF-AA, and inhibits their binding to cell surface receptors on human fibroblasts [78]. Hasselaar and Sage showed that exogenous SPARC could inhibit the migration of BAE cells induced by bFGF. Without bFGF, the migration of endothelial cells was unaffected by SPARC. A molecular interaction between SPARC and bFGF was not detected, and SPARC did not interfere with the binding of bFGF to high affinity receptors on endothelial cells [79]. SPARC also modulated the mitogenic activity of VEGF on human microvascular endothelial cells (HUMEC) through a direct binding interaction and reduced the association of VEGF with its cell-surface receptor [77].

An effect of SPARC on cell cycle has been reported. Exogenous SPARC or synthetic peptide 2.1 repressed DNA synthesis and delayed the entry of bovine aortic endothelial (BAE) cells into S-phase [74]. Similar effects of SPARC and peptide 2.1 on DNA synthesis were also shown for human umbilical vein endothelial (HUVEC)

cells, for a transformed fetal BAE cell line, and for bovine capillary endothelial cells [80]. Interestingly, human foreskin fibroblasts and fetal bovine ligament fibroblasts exhibited a biphasic response to peptide 2.1. Whereas lower concentrations of peptide 2.1 increased [3H]-thymidine incorporation, inhibition was observed at concentrations in excess of 0.4 mM [80].

SPARC and cancer

Evidences show that most invasive malignant tumours, with either mesenchymal or epithelial origin, exhibit overexpression of SPARC. SPARC is overexpressed during neoplastic progression of human melanoma [28], and is highly expressed in invasive meningiomas regardless of the grade, where it may serve as a potential diagnostic marker of invasive meningiomas [81]. Also in glioma, increased SPARC expression was found in infiltrating tumors at the brain-tumor interface, where it may promote diffuse tumor cell infiltration into adjacent brain and may serve as a cellular marker of the invasive glioma phenotype [82]. In primary colorectal cancers and their liver metastases, SPARC and the matrix metalloproteinase stromelysin were found to be overexpressed, and were localized in fibroblasts adjacent to the neoplastic foci [34]. Overexpression of SPARC was also seen in esophageal carcinogenesis [30]. Immunohistochemical staining of human mammary tumours has shown higher expression of SPARC in invasive breast carcinoma compared to benign lesions and adjacent normal breast tissue [33]. The SPARC gene was found to be expressed in all invasive breast carcinomas, metastatic lymph nodes and mouse mammary tumors. Its transcripts were specifically detected in fibroblast cells in the stroma surrounding cancer cell islands [32]. In breast tumor biopsies, an inverse correlation was seen between SPARC mRNA expression and estrogen

receptor levels, but not with SPARC protein [83]. Thomas *et al.* showed low to moderate levels of SPARC mRNA and protein in glandular epithelial cells of normal tissue as well as a few primary prostate cancers. However, high level of expression was seen in most of the prostate cancer metastatic foci, both osseous and nonosseous [31].

In vitro, SPARC effects on several cancer cell lines tend to associate with increase aggressiveness. SPARC has been shown to induce activation of MMP-2, an ECM degrading enzyme important for tumour formation and progression, in invasive breast cancer cell lines [36] and prostate cancer cell lines [73]. U87 glioblastoma cells transfected with SPARC using tetracycline-inducible gene expression (Tet-Off) showed altered adhesion and increased invasion *in vitro* [84]. In a melanoma cell line, suppression of SPARC expression using antisense RNA significantly decreased *in vitro* adhesive and invasive capacities, and completely abolished *in vivo* tumourigenicity [35]. Additionally, a positive role of SPARC in process of angiogenesis has been indicated [85].

SPARC can also affect cell migration. Jacob *et al.* [73] showed that SPARC could induce prostate cancer and breast cancer cell line migration and invasion through collagen type IV or Matrigel coated membranes, respectively. Also, SPARC increase the migration of human renal carcinoma cells through collagen type IV coated membranes [86]. Soluble SPARC itself did not show motility-promoting activity when noncoated filters were used. These results suggest an important interaction between SPARC and collagen type IV for cell migration.

However, a positive role of SPARC for the host has also been reported in some tumour types. Transfection of SPARC into an ovarian carcinoma cell line reduced the growth rate in culture and reduced the cells ability to form tumours in

nude mice [75]. We also found that the induction of SPARC in MDA-MB-231 human breast cancer cells reduced their proliferation (chapter 3). *In vitro* transformation of primary cultures of chick embryo fibroblasts by the oncoprotein, vSrc or vJun is correlated with a downregulation of SPARC expression. This low level of SPARC facilitates *in vivo* tumourigenesis. It has been proposed that SPARC downregulation and upregulation might contribute to distinct stages in carcinogenesis [87].

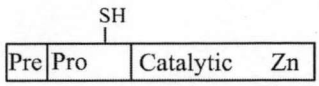
MMPs

MMPs family, structures and regulation

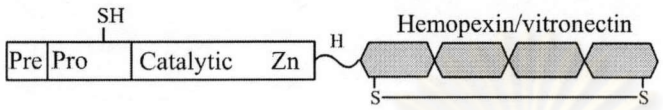
Matrix metalloproteinases (MMP/ matrixins) comprise a family of endopeptidases that are able to degrade extracellular matrix and basement membrane under physiological conditions. Up to present, there are at least 26 members of this enzyme groups in vertebrates as shown in figure 1.1 [88, 89].

MMPs have been classified into subfamilies based on their substrate specificity and domain homology. They are collagenase, gelatinase, stromelysin, membrane type (MT) MMP and others. The collagenases, comprising interstitial collagenase (MMP-1 or collagenase1), neutrophil collagenase (MMP-8 or collagenase2), collagenase 3 (MMP-13) and also collagenase 4 (MMP-18) are the major enzymes for degradation of fibrillar collagen, the major types being I, II, III. The gelatinases, including gelatinase A (MMP-2) and gelatinase B (MMP-9), degrade denatured collagens (gelatin) as well as type IV collagen, which is the major structural component of the basement membrane, and hence their historical name, type IV collagenases. The stromelysins, made up of stomelysin-1 (MMP-3), stromelysin-2 (MMP-10), matrilysin (MMP-7) and matrilysin-2 (MMP-26) have the widest range of substrate specificities, which include proteoglycans, gelatin, laminin, fibronectin,

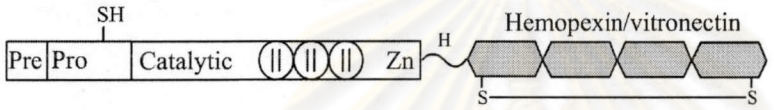
Minimal domain MMPs
(MMP7/matrilysin, MMP26/endometase)



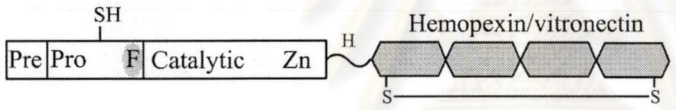
Hemopexin/vitronectin domain MMPs
a) Simple
MMP1/collagenase-1, MMP8/collagenase-2, MMP13/collagenase-3,
MMP18/collagenase-4, MMP3/stromelysin-1, MMP10/stromelysin-2,
MMP12/metalloelastase, MMP19/RAS-1, MMP20/enamelysin, MMP22/CMMP



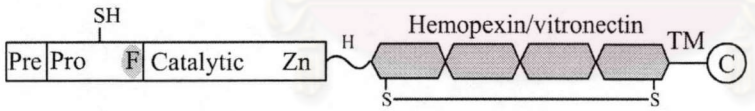
b) Gelatin-binding
(MMP2/Gelatinase A, MMP9/Gelatinase B)



c) Furin-activated
(MMP11/Stromelysin-3, MMP21/XMMP



d) Membrane-anchored-furin-activated
(MMP14/MT1-MMP, MMP15/MT2-MMP, MMP16/MT3-MMP, MMP17/MT4-MMP,
MMP24/MT5-MMP, MMP25/MT6-MMP)



Cysteine/proline-rich IL-1 receptor-like domain MMPs
MMP23

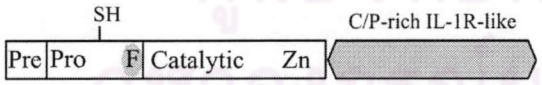


Figure 1.1: MMPs family and structure (adapted from Sternlicht & Bergers [88])

elastin and a variety of collagens. The membrane type MMPs are a subgroup of MMPs that are not secreted, but instead stay on the surface of certain cells. Up to present there are 6 members of this supgroup. They are MT1-MMP (MMP-14) [90], MT2-MMP (MMP-15) [91], MT3-MMP (MMP-16) [92], MT4-MMP (MMP-17) [93], MT5-MMP (MMP-24) [94] and MT6-MMP (MMP-25) [89]. Other members of the MMP family for which substrate specificity has been examined include macrophage metalloelastase (MMP-12), a macrophage enzyme that degrades elastin [95], stromelysin-3 (MMP-11), which has relatively weak catalytic activity against matrix substrates but degrades α1-antitrypsin [96].

Their structures, in general, are quite similar [88]. The common domains that can be found in almost all members are pre-domain, pro-domain, catalytic domain, hinge region and hemopexin domain (figure 1.1). The pre-domain is an amino-terminal signal sequence that directs their synthesis to the endoplasmic reticulum. The pro-domain has a highly conserved PRCGXPD motif, containing a cysteine residue, which is critical for maintaining enzyme latency. The catalytic domain has the zinc binding sites and regions with high affinity for calcium. Connecting the catalytic and hemopexin-like domain is a proline rich hinge region, the actual function of which has not been fully defined. This connects the catalytic domain to the hemopexin-like domain, except in MMP-7 and MMP-28. The hemopexin domain has been shown to play a functional role in substrate binding, except for gelatinases, where it rather shows interaction with the tissue inhibitor of matrix metalloproteinases (TIMPs), a family of specific MMP inhibitors [97]. This hemopexin-like domain in MMP-2 is also required for cell surface activation by MT-MMPs [98, 99]. The exceptions are for the matrilysins (MMP-7, MMP-26) which lack both the hinge region and hemopexin-like domain, MT-MMPs which contain a transmembrane domain and short

cytoplasmic tail, gelatinases which contain 3 fibronectin-like repeats in a gelatin-binding domain, and stromelysin 3 and MT-MMP which have a 10 amino acid, furin-cleavable insert between the pro and catalytic domains.

With the exception of the MT-MMPs and stromelysin 3, MMPs are secreted as proenzymes and require the proteolytic removal of the propeptide for their activation. Thus, the net activity of MMP can be regulated at 3 major levels [100, 101]. The first is gene transcription. Expression of MMPs is regulated principally at this level, with the exception of neutrophil collagenase (MMP-8), which is additionally controlled at the level of release from storage granules. MMPs expression can be modulated by a number of agents including tumour promotors, growth factors and oncogene products. They can be upregulated by interleukin-1 (IL-1), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF-α) and tumour necrosis factor alpha (TNF-α), and can be downregulated by transforming growth factor beta-1 (TGF-β1), interferon γ, and the steroid hormones, including glucocorticoids and retinoids [100, 101]. In addition to growth factors, components of the extracellular matrix have been shown to modulate synthesis of MMP. The RGD peptide sequence in fibronectin can stimulate stromelysin secretion by fibroblasts [102], and SPARC was shown to induce MMP 1 and 9 production in human peripheral blood monocytes [59], MMP 1, 3 and 9 in cultured rabbit synovial fibroblasts [58].

The second mode of regulation is proenzyme activation. Latency of proMMPs is maintained by the interaction of an unpaired cysteine in the propeptide domain with a zinc atom in the catalytic domain. The mechanism of MMP-activation, referred to as the "cystein switch" [103], is to interrupt this bond which will initiate a cascade of events that alters the conformation of the protein, allowing the protein to unfold and

exposing the active site to its ligand. *In vitro*, latent MMPs can be artificially activated by a number of agents including organic mercurials like the 4-aminophenylmercuric acetate (APMA), detergent, oxidants, and cleavage by trypsin and other proteinases. All of these agents destabilize the cysteine-zinc non-covalent bonding. *In vivo*, proteolytic cleavage of the pro-domain represents the most likely physiological activation mechanism. These proteinases include serine proteinases and also activation cascade by MMP family members such as MT-MMP and stromelysin have been found [100].

The last level of MMP regulation is inhibition of MMPs by specific inhibitors referred as tissue inhibitors of metalloproteinases (TIMPs). To date, four distinct TIMPs have been isolated. TIMP-1 and TIMP-2, which are secreted, and the ECM-bound TIMP-3, are found in a wide variety of tissues and are produced by many cell types. In contrast, TIMP-4 expression in normal tissues seems to be restricted to the heart. TIMP synthesis is regulated at the transcriptional level by a variety of cytokines and growth factors such as TGF-β and retinol. TIMPs form a 1:1 stoichiometric complex with activated MMPs [104], which results in protease inhibition. In addition, TIMP-1 and 2 are also able to bind pro-MMP-9 and pro-MMP-2 respectively [105], and in the case of MMP-2, this facilitates proenzyme activation. So the balance between TIMPs and MMPs precisely regulates the pattern of ECM degradation or accumulation.

Roles of MMPs

MMPs are key enzymes involved in the remodelling of extracellular matrix. They are involved many normal and pathological tissue-remodelling processes such as implantation, embryonic development, wound repair, inflammation, angiogenesis

and also tumour formation, invasion and metastasis [100]. Several MMPs were initially cloned as cancer-related genes and most have been detected in one cancer cell line or another [88]. MMPs are almost always present in higher amounts and activated more often in and around malignant cancers than in matched normal tissues [88]. Initially, it was believed that MMPs played a major structural role in metastasis by facilitating the breakdown of physical barriers, thus promoting tissue invasion and passage into and out of blood or lymphatic vessels (intravasation, extravasation). Later studies showed that MMPs have more complex roles, particularly in the cleavage of cytokines, growth factors, and their receptors. Thus, they can contribute to many aspects of cancer, including angiogenesis and the initial stages of tumour development in both primary and metastatic sites [88, 106].

MMP-2

Among the MMPs, MMP-2 and 9 have been closely linked to tumour progression since they are able to degrade collagen type IV, a major constituent of basement membrane, and thus can be important for tumour cell invasion and metastasis [107, 108]. We have focussed on MMP-2, the 72 kDa gelatinase/ type IV collagenase. MMP-2 is primarily expressed in mesenchymal cells (mainly fibroblasts) during development and tissue regeneration. It was originally isolated from a malignant mouse tumour and was found to be highly expressed in stromal cells surrounding the invading front of metastasizing tumours [109-112]. Many evidences have shown an involvement of MMP-2 in cancer. MMP-2 mRNA has been detected in colorectal neoplasia [111], squamous and basal cell carcinoma [112], pancreatic carcinoma [113] and mammary carcinoma [101]. In primary breast carcinomas, MMP-2 was more often expressed at high levels in carcinomatous than in normal

breast tissues. High levels of MMP-2 mRNA were inversely associated with survival [114]. In epithelial cancers, most MMPs are expressed by the surrounding stromal cells rather than by the carcinoma cells themselves. Still, MMPs are generally upregulated in and around malignant cancers and their overexpression often correlates with more aggressive pathologic behaviour and a poor clinical outcome [88, 100]. But since there is abundance of this latent proenzyme in normal tissues and fluids, an important role of MMPs activation rather than MMPs production has been emphasized, especially for MMP-2. Increased activities of MMP-2 was found in many human carcinomas including stomach [115], lung [116], breast [117, 118], thyroid [119] and oral squamous cell carcinoma [120]. A study of MMP-2 deficient mice showed reduced angiogenesis and reduced tumour progression [121].

MMP-2 activation

MMP-2 activation is a 2-step process. ProMMP-2 is 72 kDa. Through proteolytic activation, it is first converted to the 64 kDa intermediate form, and after further autocatalytic activation, becomes the 62 kDa fully active form. Unlike many other MMPs, proMMP-2 does not appear to be activated by plasmin or stromelysin [122]. Numerous studies of physiological activation have shown that MMP-2 activation occurs at the cell surface. It has become well-accepted that MMP-2 activation requires MT-MMP and TIMP-2, the latter being an inhibitor of both MMP-2 and MT-MMP (figure 1.2). First, cell surface MT-MMP forms a complex with free TIMP-2. This complex acts as a receptor for the C-terminus of proMMP-2, which then can be cleaved within the pro-domain by adjacent free MT-MMP [99, 123, 124] to yield a 64 kDa intermediate form. The second cleavage generates the mature active MMP-2 (62 kDa), which lacks the entire pro-domain. This second step is an

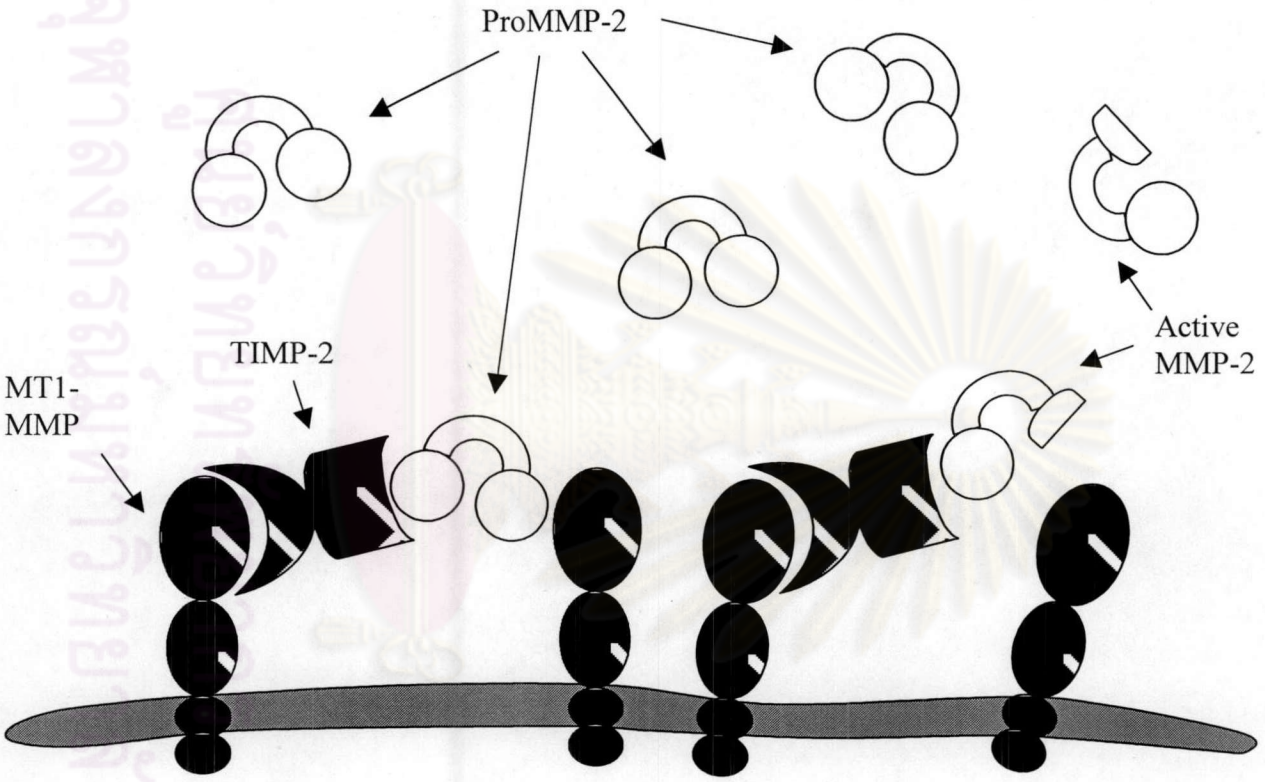


Figure 1.2: MMP-2 activation on the cell surface

autocatalytic process by another MMP-2 molecule, and depends on the MMP-2 concentration at the cell surface [124]. Some studies showed that it might involve another cell surface receptor, such as $\alpha_v\beta_3$ integrin [125]. TIMP-2 concentration is also very important to the MMP-2 activation process. If there was excess TIMP-2 in the environment, it would bind to all MT-MMP molecules and leave no free MT-MMP to activate MMP-2. On the other hand, without TIMP-2, the complex cannot be formed, and no activation will occur.

Eventhough many breast cancer cell lines express MT1-MMP, they are not able to activate MMP-2 constitutively and require additional stimulation. *In vitro* MMP-2 activation can be induced by concanavalin A (Con A), 12-o-tetradecanoylphorbol-13-acetate (TPA) and cytochalasin D. Con A, a plant lectin, can increase MT1-MMP mRNA and protein level [126]. It also has a non-transcriptional effect, which can rapidly activate MMP-2, possibly by influencing pre-existing MT1-MMP [126]. TPA was shown to stimulate activation of MMP-2 in HT-1080 fibrosarcoma, A2058 melanoma cells and some breast cancer cell lines [127-130]. Cytochalasin D upregulates MT1-MMP and induces MMP-2 activation in mesangial cells [131].

Fibrillar collagens are also able to induce MMP-2 activation and may represent a physiological counterpart to Con A. Culture of fibroblast from normal and neoplastic human breast, as well as the sarcomatous human HS578t and HT1080 cell lines on three- dimensional gels of type I collagen resulted in activation of endogenous MMP-2 [132]. In human breast cancer cell lines, collagen I-induced MMP-2 activation is restricted to highly invasive estrogen receptor negative, vimentin positive lines and is associated with metastatic potential [130, 133, 134]. Like Con A, this process involves upregulation of MT1-MMP mRNA and protein and a non-

transcriptional component [135]. Collagen-induced activation has also been reported by others in fibroblast [132, 135, 136], other cancer cells, endothelial cells, and platelets [133, 135, 137, 138].

TIMP-2

TIMP-2 is expressed in many cell lines including melanoma, fibrosarcoma, breast cancer [36] and a fetal lung fibroblast cell line [139, 140]. In melanoma cell lines, production of TIMP-2 [139] was about 10 fold higher in low invasive lines as compared with high invasive cells. Also, the MMP-2: TIMP-2 expression ratio was found to correlate well with the invasion and metastasis of renal cell carcinoma cells [141]. *In vivo*, high levels of TIMP-2 correlate with both shortened disease-free interval and overall survival of breast cancer [142]. However, in primary breast cancer, TIMP-2 levels showed no significant relationship with either tumour size or axillary node status but correlated inversely with estrogen receptor (ER) levels [142]. In murine melanoma cells, TIMP-2 overexpression caused down-regulation of metastasis and angiogenesis, but a possible involvement in tumour cell survival was indicated. Two separate studies showed that TIMP-2 deficient mice were normal, viable and fertile, but failed to activate MMP-2 *in vivo*. This can be restored *in vitro* by adding exogenous TIMP-2 [143, 144].

MT1-MMP

MT1-MMP is the first member of the MT-MMPs, a cell surface associated MMP subfamily identified recently [90-94, 145]. Up to present, there are 6 members of this subfamily, 4 of which (MT-MMP-1-3 and 5) contain an approximately 25

amino acid transmembrane domain. MT4- and MT6-MMP are cell surface associated but anchored through a glycoprotein linkage instead [89, 94].

The mechanism of MT1-MMP activation, intracellularly or extracellularly, is still controversial. In contrast to other MMPs, all MT-MMPs and stromelysin-3 [146] have an insertion of 11 amino acids between the pro-domain and the catalytic domains. The conserved Arg-Arg-Lys-Arg sequence precedes the potential processing sites of these MMPs. This sequence is recognized by furin-like, a proprotein convertases present in the Golgi apparatus, which are able to activate recombinant MT1-MMP [147], and presumed to be essential for the processing of pro-MT1-MMP intracellularly. On the other hand, plasmin was also found to activate pro-MT1-MMP [148], suggesting that pro-MT1-MMP can be transported to the plasma membrane before being activated by plasmin outside the cell. Furthermore the prodomain of MT1-MMP has been shown to function as an intramolecular chaperone, facilitating protein folding and trafficking to the cell surface [149]. Studies using COS-1 cells transfected with MT1-MMP indicated that latent membrane-bound MT1- MMP does not appear to be cleaved by furin, but it is cleaved and activated when secreted as a C-terminal truncated pro-enzyme [150, 151]. Although secreted MT-MMPs are of unknown biological significance, pro-MMP-2 activation can be induced by the latent form of MT1-MMP so that furin induced processing of MT1-MMP is not a prerequisite for proMMP-2 activation [150]. That result contrasts the report by Maquoi *et al.*. [152], which supports the concept in which the pro-MMP-2 activation process requires the mature form of MT1-MMP processed via a furin-dependent mechanism. Later on, Cao *et al.* [153] demonstrated the requirement of the propeptide domain of MT1-MMP in maintaining the biological function of the enzyme in the activation of proMMP-2 on the cell surface. In addition the C-terminal domain of

MT-1 MMP, a hydrophobic amino acid sequence, which acts as a transmembrane domain, has been considered as a functional domain required for pro-MMP-2 activation [154].

MT1-MMP is expressed on the surface of various tumour cells. It is found in human gastric carcinoma [155] and stromal cells of human colon, breast, and head and neck carcinomas [156]. Co-localization of MT1-MMP and MMP-2 have also been correlated with melanoma progression [157]. In breast cancer cell lines, MT1-MMP expression was restricted to vimentin-positive, invasive lines and thus may play a key role in the invasiveness and metastatis of breast carcinoma [126, 133, 158].

In addition to its role in activating proMMP-2, MT1-MMP can also act as an extracellular matrix-degrading enzyme and the importance of this is emerging. A study with transmembrane-deletion mutants of the MT1-MMP (soluble form) showed that it is able to express proteolytic activity against extracellular matrix components such as gelatin, fibronectin, the laminin B chain, vitronectin and dermatan sulfate proteoglycan [159]. Those transmembrane-deletion mutant and native MT1-MMP secreted from breast cancer cell line, MDA-MB-231, are active without any treatment for activation and digest type I (guinea pig), II (bovine), III (human) collagens into characteristic ¾ and ¼ fragments [160]. Thus, MT1-MMP appears to play a dual role in ECM remodelling through direct cleavage of ECM components and activation of MMP-2 and also procollagenase 3.

Breast cancer

Breast cancer is one of the most common forms of cancer in women. The major cause of death in patients is the development of multiple metastatic lesions, which are resistant to therapy [161]. If breast tumours did not metastasize, a high

proportion of all patients could be cured by local therapies. Cancer invasion is a multistep process [162], and can be delineated into attachment to the extracellular matrix (ECM), degradation of the structural components, and migration through the ECM. Metastatic process includes the following: escape of cells from the primary tumour, intravasation (entry of cells into the lymphatic or blood circulation), survival and transport in the circulation, arrest in distant organs, extravasation (escape of cells from the circulation), and growth of cells to form secondary tumours in the new organ environment. Angiogenesis, the recruitment of new blood vessels, is required for the primary and metastatic tumours to grow beyond minimal size, and evasion of immune destruction is necessary at various steps throughout the process [106]. Metastasis can thus be considered a multi-factorial process, and competence in each aspect is critical for successful metastasis. Another important feature of metastasis is the specific preferences of certain tumours to spread to certain organs. Breast cancer in particular has a high propensity for metastasis to bone, liver, and brain [163]. It is thought that such target organs contain appropriate factors for the specific retention, growth and survival of cells from the mammary gland and this has been termed the "Seed and Soil" hypothesis over 100 years ago.

Human breast cancer cell lines

A number of human breast cancer cell lines have been established *in vi*tro. The utilization of these cells for *in vitro* assays as well as growing as tumours in athymic nude mice have proved a powerful model for the study of human tumour biology. Breast cancer cell lines are generally considered in terms of their ER content- that is, whether they are estrogen receptor-positive (ER+) or estrogen receptor-negative (ER-). This classification largely reflects the clinical value of steroid hormone receptor

expression in predicting response to endocrine therapy. The ER+ cell lines are characterized by a dependence on estrogens for growth *in vitro* or *in vivo* and by sensitivity to the growth-inhibitory effects of antiestrogenic and progestational drugs. In general, steroid-dependent cell lines are poorly invasive and nonmetastatic in athymic nude mice [164]. The majority of human breast cancer cell lines are ER-. These ER- cell lines will form proliferating tumours in the presence or absence of estrogen supplementation, and do not respond to estrogenic stimulation or antiestrogenic inhibition [165]. They are generally more rapidly growing, more aggressive, and exhibit a poorer prognosis. Also, the ER- cell lines tend to produce rapidly growing tumours in nude mice, several of which are highly invasive and some of which can produce distant metastases. However, the presence of the intermediate filament glycoprotein vimentin (VIM) has also shown prognostic significance in some studies. Vimentin expression is usually characteristic of mesenchymal rather than epithelial cell phenotypes, and has been found to associate with lack of ER, high growth fraction, and poor nuclear grade in human breast cancer [166-168]. When inoculated into nude mice, local invasiveness or hematogenous dissemination is apparent over a 60-day time period only in the vimentin positive subset of ER- cell lines [169].

Both of the human breast cancer cell lines used here in this study, the MDA-MB-231 and BT-549, are highly invasive ER-, and VIM+ [169]. The MDA-MB-231 is among the most widely used and is frequently used as a negative control in many laboratories studying the endocrine regulation of breast cancer cell growth. The cells were established from a 51-year-old woman with stage IV breast cancer by pleural effusion [170]. The MDA-MB-231 cells are highly tumourigenic and can produce lung metastases from mammary fat pad tumours in nude mice [171, 172]. The BT-549

is also a highly invasive line and was established from a 71-year-old woman who developed stage II breast cancer. It does not grow *in vivo*, but if stimulated to do so, show signs of invasiveness and metastasis [173].

In vitro study of breast cancer

Basement membranes are thin sheets of extracellular matrix, which surround epithelial tissues, nerves and muscles, providing both physical support and morphogenic signals to these tissues. The ability to traverse basement membranes, which limit the passage of normal epithelial cells, has emerged as a critical aspect of the metastatic phenotype. The invasive potential of cells can be examined *in vitro* using a reconstituted basement membrane extract (Matrigel) obtained from murine Engelbreth-Holm-Swarm (EHS) tumour [174]. These techniques include the Boyden chamber chemoinvasion assay [175] and the Matrigel outgrowth assay [175, 176]. Human breast cancer cell lines fall into two distinct groups of activities. Cells expressing vimentin (all of which are ER-negative) exhibit high activity in the Boyden chamber assay, while those without vimentin expression irrespective of ER are poorly invasive or inactive [169]. These differential activities are also reflected in different morphological responses to Matrigel in the outgrowth assay. While all VIM+ cell lines form stellate, invasive colonies in Matrigel, cell lines from the ER+/VIM- and ER-/VIM- groups show either a non-invasive, spherical, organoid-like morphology, or grow as non-invasive clusters of rounded cells [177].

MMPs & Breast cancer

Metastatic breast cancer cells encounter and traverse numerous basement membranes as they exit the primary tumour, intravasate into the lymphatics or

vasculature, and disseminate into the preferred sites of lung, liver, bone and brain [178]. Extracellular matrix degrading enzymes, especially basement membrane degrading MMP-2, are required to break down these structural barriers. *In vitro* study has showed that Matrigel invasion by tumour cells is regulated by optimal levels of active MMP-2 [107]. MMP-2 appears to be expressed very early in breast cancer but not in normal, resting breast tissue [179]. MMP-2 can be detected in some fibroadenomas and benign tumours [180, 181]. It is also found in most mammary gland carcinomas [180, 182] and its expression becomes more consistent with increased tumour grade [179]. *In situ* hybridization showed that MMP-2 is synthesized by stromal fibroblasts, immediately surrounding the clusters of tumour cells [179, 180].

Recent studies showed that MMPs have more complex roles than facilitating the breakdown of physical barriers for cancer metastasis. They also contribute to other aspects of cancer, including angiogenesis and the initial stages of tumour development [88]. Studies using intravital microscopy as well as other studies in which levels of MMPs and their inhibitors were manipulated suggest that MMPs are key regulators of the growth of tumours, in both primary and metastatic sites. It is believed that MMPs are important for creating and maintaining an environment that supports the initiation and maintenance of growth of primary and metastatic tumours [106].

Problem and Hypothesis

SPARC has been shown to play important roles in many cancers, where in most cases it is associated with poor prognosis. However, the study of SPARC in breast cancer is still limited. Previous study showed that recombinant human SPARC could induce MMP-2-activation in BT-549 and MDA-MB-231 breast cancer cell lines. This process was not associated with any changes in MT1-MMP mRNA or protein levels. Reduction of TIMP-2 in the conditioned media was detected without changes in its mRNA levels. Using synthesized peptide, it was found that peptide 1.1, from the acidic amino terminal domain, was responsible for those effects. The question remained as to how SPARC reduces TIMP-2, and whether TIMP-2 reduction is a cause or a consequence of SPARC-induced MMP-2 activation. Also, since the role of SPARC in breast cancer is still not known, this study would explore other biological functions of SPARC in breast cancer.

The hypothesis of the study are

- That reduction of TIMP-2 in the media after SPARC treatment is critical for its induction of the MMP-2-activation process.
- SPARC produced endogenously by the MDA-MB-231 cell line should cause constitutive activation of exogenous MMP-2 and reduction of TIMP-2 in the culture medium

Specific Aims

The specific aims of this study were:

1. To study the potential role of TIMP-2 in the SPARC-induced MMP-2 activation process.
2. To investigate the effect of SPARC transfection on MMP-2 activation in the MDA-MB-231 breast cancer cell line.

The overall analysis of SPARC effects in breast cancer was performed with two distinct approaches. First, the BT-549 breast cancer cell line was treated with various preparations of SPARC from human, cow and mouse, and MMP-2 activation and TIMP-2 levels were examined. SPARC deletion mutants were also used to confirm the importance of domain I. Second, SPARC was transfected into the MDA-MB-231 breast cancer cell line using an inducible Tet-On transfection system. Apart from MMP-2 aspect, other biological consequences were studied in this transfected cell line.