CHAPTER 4

GENERAL DISCUSSIONS AND FUTURE STUDIES

SPARC

The extracellular matrix (ECM) is composed of structural proteins, proteoglycans, growth factors and matricellular proteins [53]. Collectively, the ECM provides both important structural cues to the cells its supports, but also potent functional signals [53]. SPARC, secreted protein acidic and rich in cysteine, belongs to a group of matricellular proteins, which bind cells to the ECM but are not part of the structural ECM, and do not contribute to structural stability. Instead, SPARC tends to modulate cell-matrix interactions, and is often expressed in tissues during events such as remodelling, development and repair [54]. Other matricellular proteins include thrombospondin 1 and 2, tenascin C and X, and osteopontin. Although structurally unrelated, they all exhibit anti-adhesive effects leading to changes in cell shape that finally result in disruption of cell-matrix interactions [38].

Like ourselves, many studies have focused on SPARC and cancer. Accumulated evidence has shown increased expression of SPARC in many cancers including melanoma [28], hepatocellular carcinoma [29], esophageal carcinoma [30], prostate cancer [31] and breast cancer [32, 33]. In most cases, SPARC tends to be associated with poor prognosis. The only exception so far is in ovarian cancers, where SPARC was found to reduce the growth rate of transfected cells *in vitro* and reduce their ability to form tumours in nude mice [75]. In general, it appears that the cancer systems in which SPARC is associated with aggressiveness are predominantly mesenchymal in nature. Indeed, SPARC is primarily expressed by mesenchymal cells [20], and is usually expressed by stromal cells surrounding epithelial tumours rather than by the tumour cells *per se*. Mesenchymal tumours, such as glioma and

melanoma, show expression of SPARC by the cancer cells [28, 82], and it is these tumours that show positive correlations between SPARC levels, aggressive behaviour, and poorer outcomes. In these cases, SPARC may assist individual cell interactions with the ECM. Cancer cells metastasize by invading through basement membranes, crossing underlying ECM, penetrating the vascular and/or lymphatic circulation, extravasating, and growing at a secondary site. At each step, the interaction between cancer cells and the ECM molecules is important for the final outcome of the disease. As an important link between cells and the ECM, SPARC could potentially influence any or all of these steps in cancer progression.

Breast cancer is a cancer of epithelial origin. Studies of SPARC in breast cancer have shown increased expression of SPARC in invasive breast carcinomas [32, 33]. An inverse correlation was also seen between SPARC mRNA expression and ER level [83]. By *in situ* hybridization, SPARC transcripts were specifically detected in fibroblastic cells in the stroma surrounding cancer cell islands in breast cancer [32], and this has also been reported in hepatocellular carcinoma [29]. Again, SPARC would be well positioned to help cancer cells interact with ECM, but in most cases this SPARC would be derived from the stromal cells. One caveat to this, however, is the unexpected expression of SPARC by a number of breast cancer cell lines, especially those which are invasive, metastatic, and displaying mesenchymal properties. Since these cell lines represent a form of breast cancer progression, these cells may be expected to behave somewhat like glioma or melanoma cells. Indeed, SPARC was shown as induce migration of MDA-MB-231 breast cancer cells through collagen type IV or Matrigel coated membranes [73].

The interest of our laboratory in SPARC was initiated with the study by Gilles *et al.* [36], where SPARC was found to induce MMP-2 activation by the breast cancer

cell lines BT-549 and MDA-MB-231. The MMP-2 activation mechanism as currently understood occurs on the cell surface through a tri-molecular complex of MT-MMP, TIMP-2 and MMP-2 [99]. Being both inhibitor and activator of MMP-2, an optimal level of TIMP-2 is required for the MMP-2 activation process to proceed. Gilles *et al.* could not detect any alterations in the levels of MT1-MMP mRNA or protein, or TIMP-2 mRNA, but showed a reduction in the TIMP-2 levels in the culture medium after treatment with SPARC. The relationship between TIMP-2 reduction and MMP-2 activation was not clear, however, other studies [125, 131] have reported paralell findings. The question also remained as to how SPARC caused reduced TIMP-2 levels, and whether the MMP-2 activation by SPARC is a cause or an effect of TIMP-2 reduction. The initial primary aim of this study was to extend our understanding of this process

Cell lines

Two human breast cancer cell lines, BT-549 and MDA-MB-231, were used in the present study. Both of them are highly invasive, estrogen receptor negative (ER-), vimentin positive (VIM+) lines, and it is this subset of invasive human breast cancer cell lines which in general have been shown to (i) activate MMP-2 in response to certain stimuli (ii) express MT1-MMP and upregulate MT1-MMP in response to the stimuli which cause MMP-2 activation [135, 158], (iii) tend to express SPARC [36] and (iv) respond to SPARC with increased MMP-2 activation [36]. Although similar in these regards, the BT-549 and MDA-MB-231 cell lines differ in a few aspects relevant to my studies. BT-549 cells express quite high levels of endogenous SPARC compared to MDA-MB-231 cells. This may explain the better response to lower concentrations of exogenous SPARC treatment than the MDA-MB-231, as seen both

in the studies described herein and in the previous study [36]. This line is thus amenable for studies of exogenous SPARC treatment, as it requires lower concentrations of added SPARC to stimulate MMP-2 activation. BT-549 cells also perform better in MMP-2 activation in general, as shown by their ability to activate some of the exogenous MMP-2 to a fully active form after 72 hours culture without additional treatment. The MDA-MB-231 lacks endogenous SPARC to the extent that it could not be seen by Northern analysis in the previous study [36], and was also not detected in large-scale gene array analysis [211, 212]. For this reason, I chose this line for a transfection study of SPARC. In the course of my study, I found low levels of SPARC expression by MDA-MB-231 by Western analysis of the culture media after 72 hours culture, however the level is still considered very low when compared to BT-549 and other invasive breast cancer cell lines.

Effect of exogenous SPARC on BT-549

A previous study showed that recombinant human SPARC could induce MMP-2 activation and reduce TIMP-2 level [36]. In an attempt to further characterize this response, I looked at the effects of different preparations of SPARC on MMP-2 activation and TIMP-2 reduction. These preparations included recombinant human SPARC, SPARC purified from human platelets or bovine bone, and different preparations of purified and recombinant mouse SPARC. SPARC is highly conserved among vertebrates [6], with only a few differences existing between species and tissues of origin (bone and platelet), as described in Chapter 1. For this reason, it was surprising to find that all the preparations of SPARC used showed different effect on MMP-2 activation, from a strong activation to a slight inhibition. However, all full-length SPARC preparations tested were able to reduce TIMP-2 levels. No changes

were detected in the mRNA levels of TIMP-2 or MT1-MMP, nor in the levels of cell-associated TIMP-2, leading us to conclude that the TIMP-2 reduction by SPARC is not a consequence of its effect on MMP-2 activation, but possibly causal. The interesting mechanism of how SPARC reduces TIMP-2 is still unclear. It appears not to be through binding to the cell surface, as no change in cell-associated TIMP-2 was detected, and this also rules out reduced secretion into the media since that would also lead to an accumulation inside the cell, which was not seen. Other possible explanations for our observations include a reduced translation rate, since mRNA levels are not decreased, or increased degradation. For the former, it has been reported that TIMP-2 mRNA levels are often discordant with the amount of protein produced [213] , whereas for the latter, Maquoi *et al.* [199] have reported internalization and degradation of 125 I-rTIMP-2 as a consequence of MT1-MMP-mediated activation of MMP-2. However, since the TIMP-2 reduction was not always accompanied by increased MMP-2 activation, degradation seems a less likely explanation for our results.

Although full elucidation of this mechanism requires further work, we have made progress in identifying the important domains of SPARC required for this effect. Using a synthetic peptide and different human SPARC deletion mutants, we found that peptide 1.1, from domain I, was capable of causing this effect on TIMP-2. Domain I of SPARC is highly acidic and binds calcium with low affinity [48]. Peptide 1.1 was reported to bind hydroxyapatite [69], and also to inhibit the spreading of newly plated endothelial cells and fibroblasts [71].

The putative role of peptide 1.1 is supported by observations that mutants lacking domain I (Del I, Del I-II) were incapable of inhibiting TIMP-2, and further that reduced/alkylated SPARC, which has an intact domain I but disrupted secondary

structure in domains II and III, was capable of reducing TIMP-2. The exact attributes of peptide 1.1 which are required were not explored further, largely because peptide 1.1 is very difficult to prepare, and consequently very expensive. Mutation analysis of peptide 1.1 would otherwise have been a possible means to further explore the actual features required. MMP-2 activity is dependent on calcium for structural integrity, and it is possible that this could be involved. Interestingly, peptide 1.2 [71] which also binds hydroxyapatite, was less active in previous study (Gilles, personal communication). It is notable that the effect on TIMP-2 reduction is due to this small segment of SPARC protein. This indicates that the effects do not require the multivalent potential of SPARC in bridging important players together, and may suggest a cell surface receptor specific for this region of the SPARC molecule.

The lack of concordance between reduced TIMP-2 and MMP-2 activation is puzzling, but instructive with respect to the temporal relationship between these two events. Previous workers showing both reduced TIMP-2 and increased MMP-2 activation have suggested that the reduction in TIMP-2 is due to sequestration of TIMP-2 to MT1-MMP after the levels of MT1-MMP increase on the cell surface. However, our data suggest that these two events may be unrelated. One possible reason is that TIMP-2 has a biphasic effect on MMP-2 activation, with inhibition seen with either too little or too much TIMP-2. This was also evident in our cells with neutralization studies. Neutralizing TIMP-2 with anti-TIMP-2 antibodies showed either of two different effects on MMP-2 activation; It stimulated MMP-2 activation at low concentrations, but inhibited this at higher concentrations. Thus, the disparate effects of each SPARC preparation may be due to the delicate balance required for the appropriate amount of TIMP-2 to support the activation process. Nonetheless we were

never able to find more permissive concentrations of SPARC preparations which did not stimulate MMP-2 activation.

Transfection of SPARC into MDA-MB-231 cells

Studies employing exogenous SPARC are subject to limitations with respect to the source, purity and preservation of the protein. Another approach to analysis of the function of a given protein is through gene transfection of the specific cDNA, in either the sense or antisense direction, to examine either gain of function or loss of function consequences. Although antisense experiments can be more definitive, it is more difficult to effectively reduce mRNA levels than it is to enhance these through sense transfection. The best-controlled transfection studies are those in inducible systems. To this end, I transfected SPARC into MDA-MB-231 cells using the Tet-On transfection system [203, 214]. With this system, the transfected gene is expressed in the presence of doxycyclin, but silent in its absence. When examined in the presence and absence of doxycyclin, the same cell can be used as its own control, avoiding the problem of clonal variation. Also, the amount of protein expression is dependent on the concentrations of doxycyclin used (as shown in Chapter 2, figure 2.1), allowing the possibility of dose-effect studies. In our study, SPARC expression was highly induced by doxycyclin in a concentration-dependent manner. However, I was unable to see any MMP-2 activation effect, which is what I originally expected to see.

A potential explanation for this could be in the levels of SPARC we are able to achieve with transfection. In comparison to exogenous SPARC standards on Western blot, we estimated the concentrations of SPARC secreted by MDA-MB-231 after 72-hour culture in the presence of maximal doxycyclin to be approximately 5 μg/ml. In contrast, the concentrations of exogenous SPARC required to stimulate MMP-2

activation, as shown both in this work and in a previous study [36], was around 50-100 μg/ml. Thus, despite potent induction in our trasfected cells, we may simply have insufficient SPARC to effect TIMP-2 reduction and/or MMP-2 activation. One may then question the physiological relevance of such high concentrations required for these SPARC effects. However, such concentrations might be possibly to achieved *in vivo* through binding to hydroxyapatite or other ECM component, especially in bone. Moreover, since I was also unable to induce MMP-2 activation or TIMP-2 reduction effects with exogenous SPARC in this cell line, another possibility could be due to the difference of the MDA-MB-231 cell line used in different labolatories. The MDA-MB-231 from different labs is known to be slightly different. For example, some study show endogenous expression of MMP-2 in MDA-MB-231 [215], while others, including the one that I used in this study, has no endogenous MMP-2. Thus it would appear that the MDA-MB-231 line that I used in this study is not as responsive to SPARC as the one used previously [36]. One opportunity for important differences is the endogenous levels of TIMP-2. As shown in Chapter 2 figure 2.1, my MDA-MB-231 cells already have a low level of endogenous TIMP-2, and were able to tolerate a number of concentrations of added rTIMP-2 before MMP-2 activation was inhibited. If SPARC stimulates activation of MMP-2 by reducing TIMP-2 levels, which are already too high, it is not likely for SPARC to have this effect on this cell line. Whatever the reason, I was unable to study MMP-2 activation and TIMP-2 reduction responses to SPARC in this transfected cell system.

Nonetheless, the transfection system did provide an opportunity to further examine other implications of SPARC in the breast cancer context, with concentrations that are likely to be similar to those achieved in the primary tumour environment, produced there by peritumoural stromal cells [29, 32]. Thus other

biological effects of SPARC on the MDA-MB-231 cells were examined. Since SPARC is known to have prominent anti-adhesive and anti-proliferative effects [54], we first looked at these. We did not observe any changes in cell shape, either with the transfected SPARC in these cells or with even higher levels of exogenous SPARC, suggesting that SPARC has no anti-adhesive effect on this cell line. We also found that cells pretreated either with or without DOX (with or without SPARC) showed no difference in adhesion to a variety of substrates when harvested and tested in cell adhesion assays. However, it is possible that this assay is not appropriate for these transfected cells, since it may lack the time needed to produce and accumulate SPARC to a sufficient concentration to influence adhesion. Since the cells were trypsinized and let attach for only 1 hour, it is possible that they are unable to carry their own SPARC into the assay.

The other major reported effect of SPARC is on cell proliferation. SPARC is well known for its counteradhesive function, achieved in part by the dissolution of focal adhesion complexes and reorganization of actin stress fibers [6]. It was also reported to inhibit growth in ovarian cancer cell lines [75]. The transfected MDA-MB-231 cells were subjected to standard monolayer growth analysis using 96-well plate assays. Reproducible, albeit modest, anti-proliferative effects of SPARC were seen both in the 96-well proliferation assay and also in the monolayer wound healing assay. In the latter, delayed wound healing was seen when DOX was added. This activity is a combination of both migration and proliferation, since the wound closure is measured over a 4-day period. SPARC has been shown to promote the migration of breast and prostate cancer cell lines through a chemotactic mechanism whereby cells migrate towards the source of SPARC, and this is potentially important for bone metastasis [73]. To examine migration in isolation, we included 10 mM thymidine,

and saw that this abrogated the inhibitory effect of SPARC on wound closure. Thus, by inference, we concluded that the effects of SPARC are primarily due to delayed proliferation rather than reduced migration. In support of this conclusion, DOX-treated cells showed no difference in cell migration assays in 48-well microchemotaxis chambers, even when their conditioned media were carried through into the assay.

The effects on cell proliferation were further analyzed at the cell cycle level, where cells were grown in the presence or absence of DOX for 48 hours before being collected and stained for DNA by propidium iodide. SPARC was found to inhibit progression of cell cycle to S-phase, and this is consistent with its anti-proliferative effect. The lack of progression to S phase is consistent with the observation of Funk *et al.*, who showed arrest of bovine aortic endothelial (BAE) cells in G_1 after treatment with 20 μg/ml of SPARC [74].

In addition to these assays of adhesion, morphology, migration and proliferation, we examined anchorage-independent growth, Matrigel morphology and radial outgrowth assay, none of which showed any response to SPARC/DOX. Thus, the biological consequences of SPARC on breast cancer cells, as modelled in MDA-MB-231, appear different to the dramatic effects seen in both glioblastoma [84] and melanoma [35] systems. Since SPARC has been shown by *in situ* hybridization to be expressed by stromal cells around breast tumours [32] and hepatocellular carcinomas [29], it is possible that it is part of the host mechanism to suppress the growth of tumour cells. This might implicate a more conservative role of SPARC in breast cancers, similar to that found in ovarian cancer cell lines, where SPARC also inhibited growth [75]. These tumours are similar by being epithelial origin, and further work is required to compare the actions of SPARC in epithelial versus

mesenchymal tumours. My studies, however, do serve an important role in defining that SPARC would be inhibitory to breast carcinoma since this is against the current dogma.

Concluding Remarks

The ability of breast cancer cells to invade and metastasize depends on many factors, among which interactions with the ECM component are very important. SPARC is known to play important roles in cancers and thought to play important roles in all tissue rearrangements, by inference. However, the study of SPARC in breast cancer is very limited, especially in the aspect of MMP-2 activation. Although I originally planned to focus only on the effect of SPARC on MMP-2 activation in this study, the limitation found in the transfected cells led to the study of other biological effects of SPARC. This, in turn, showed 2 different ways by which SPARC, a small matricellular protein, may affect breast cancer cell lines. In the first instance, a specific region (peptide 1.1) of the amino-terminal acidic domain I, was found to reduce extracellular TIMP-2 levels and this, in some cases, caused MMP-2 activation. It is interesting to find that only a small portion of the SPARC molecule, in this case peptide 1.1, is responsible for its TIMP-2 reduction effect. The full implications of this effect require further work and the context of this effect may be confined to bone where relatively high concentrations of SPARC may be found. In the second instance, use of the well-controlled Tet-On system allowed us to identify an anti-proliferative effect of SPARC and also explore other biological effects of SPARC *in vivo* (ongoing). It is likely that the concentration requirements of SPARC for growth-inhibition reflect the consequences of SPARC in the primary tumours since the amounts induced appear similar to that secreted by cultured fibroblasts. The

abundance of SPARC production by the relative stroma around breast cancers [32] and increased levels associated with more aggressive tumours [53] has always been interpreted to suggest that it may promote tumour aggressiveness. This is a natural assumption because it has been well documented to potentiate invasion in melanoma and glioblastoma [84]. The combined data from this thesis, however, would suggest a rather minor role of SPARC in breast cancer in general. SPARC remains, however, a fascinating molecule with an uncharacterized receptor, and the further elucidation of its various functions will continue to interest cancer researchers in the future.

Future studies

Although outside the scope of this thesis, a number of new directions arise, as follows:

1. The mechanism by which SPARC reduces extracellular TIMP-2. Reduced translation/secretion, increased degradation, and/or covalent complex appear possible candidates. Pulse-chase labeling experiments would shed light on the possibility of altered translation/secretion of TIMP-2 after SPARC treatment. ^{125}I-rTIMP-2 could be used to examine binding and degradation of TIMP-2, as described by Maquoi *et al.* [199], after SPARC treatment.

2. Explore roles of peptide 1.1 in terms of its receptor, critical amino acids and also examine peptide 1.2, which is also part of the amino terminal domain I and shares the hydroxyapatite-binding capacity of peptide 1.1 [69]. Since calcium is known to be important for SPARC structure and function [48], it would be interesting to look at effect of calcium on SPARC-reduced TIMP-2 function.

3.Confirm the anti-proliferative effect and study other biological effects of SPARC *in vivo*. The Tet-On system allows us to explore this by adding doxycyclin in drinking

water. MDA-MB-231 cells grow well in the mammary fat pads of immunocompromised mice [169] and spread to the lymph nodes and lungs [172]. I have performed the Dox-inducible transfection in MDA-MB-231-BAG cells, which are genetically tagged with bacterial ß-galactosidase, enabling their tracking after metastasis in nude mice. I also have participated in the development of a "Real Time PCR (TaqMan) method for measuring these cells [216]. Thus, the potential effects of SPARC, either positive or negative, could be determined using these methods, and these studies have been initiated.