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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Angiogenic Potential of Prostate Tumor Stem Cells

A dissertation submitted in partial satisfaction of the
Requirements for the degree Doctor of Philosophy

in

Materials Science and Engineering

by

Jane Catherine Frimodig

Committee in charge:

Professor David Gough, Chair
Professor Peter Chen
Professor Shu Chien
Professor Martin Haas
Professor Joanna McKittrick
Professor Shyni Varghese

2010

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2010

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List of Abbreviations

AcLDL-Dil	Acetylated low density lipoprotein-1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate
Akt	an important signaling kinase, named for the retrovirus from which it was originally isolated
AP-1	activator protein 1, a transcription factor
ASC	adult stem cell
B+T	cells transfected to overexpress Bmi-1 and TERT
Bcl-2	B cell lymphoma 2
Bmi-1	B lymphoma Mo-MLV insertion region 1 homolog
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
CD	cluster of differentiation, nomenclature for a cell surface molecule
CDK	cyclin dependent kinase
CHO	Chinese hamster ovary (cells); in this case used it to identify glycosylation sites
CK	cytokeratin
c-Kit	CD117, first identified as the cellular homolog of the feline sarcoma viral oncogene v-kit
CM	conditioned medium
CSC	cancer stem cell
CXCR	chemokine receptor
DAPI	fluorescent cell nucleus stain
Dil	1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate
DMSO	dimethyl sulfoxide
EBM-2	endothelial cell basal medium
EC	endothelial cells
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epithelial growth factor
eGFP	enhanced green fluorescent protein
EGM-2	endothelial cell growth medium
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
EPA	erythroid potentiating activity (protein) (same as TIMP-1)
EPC	endothelial progenitor cells
ERK	extracellular signal-related protein kinase
Ets-1	avian erythroblastosis virus E26 (v-ets) oncogene homolog-1
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGF-2	fibroblast growth factor 2, same as FGF-B or bFGF
FGFa	acidic fibroblast growth factor
FSH	follicle stimulating hormone
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescent protein

GTPase	Guanosine triphosphatase
HFF	human foreskin fibroblasts
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
HSC	hematopoietic stem cells
HUVEC	human umbilical vein endothelial cells
IFN- γ	interferon gamma
IGF-1	insulin-related growth factor
Il-1 α	Interleukin-1-alpha
Il-1 β	Interleukin-1-beta
Il-8	Interleukin 8
Il-12	Interleukin 12
IP-10	CXCL10, aka 10 kDa interferon-gamma-induced protein
JAK2	Janus kinase-2
K _d	dissociation constant
LDL	low density lipoprotein
LV	left ventricular
MAPK	mitogen activated protein kinase, originally microtubule-associated protein
MDR	multi-drug resistant
MEK	mitogen activated protein kinase kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTS	a novel tetrazolium compound
NIH	National Institutes of Health
NM	nude mouse
OB-fold	oligosaccharide/oligonucleotide-binding fold
Oct-4	octamer 4
P27kip1	p27 and Kip1 are 2 names for the same protein that got rolled in together
PBS	phosphate buffered solution
PDGF	platelet-derived growth factor
Pea3	polyoma enhancer activator 3
PI3K	phosphatidylinositol-3 kinase
PIGF	placental growth factor
PIN	prostatic intraepithelial hyperplasia
PIP ₂	phosphatidylinositol-4,5-biphosphate
PMA	phorbol 12-myristate 13-acetate
PrEGM	Prostate epithelial cell growth medium
proMMP	MMP zymogen, the precursor protein
PrTuSC	prostate tumor stem cells
PSA	prostate specific antigen
Ptdins	phosphatidylinositols
PTEN	phosphatase and tensin homolog deleted from chromosome 10
PTX	pertussis toxin

Rac	small GTPase in Rho family
Raf	mitogen activated protein kinase kinase kinase
Ras	small GTPase upstream of Raf
Rho	small GTPase in Rho family
ROS	reactive oxygen species
rhTIMP-1	recombinant human TIMP-1
rTIMP-1	recombinant TIMP-1
RTK	receptor tyrosine kinase
SCID	severe combined immunodeficiency (mouse)
SDF	stromal derived factor
SDS	sodium dodecyl sulfate
SMC	smooth muscle cells
SOX-1/2	Sry-related HMG box (a transcription factor)
STAT	signal transducer and activator of transcription
TCC	transitional cell bladder cancer
TEC	tumor endothelial cells
TERT	telomerase reverse transcriptase
TGF- α	transforming growth factor alpha
TGF- β	tumor growth factor beta
TIMP-1	tissue inhibitor of metalloproteinases 1
TNF α	tumor necrosis factor alpha
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
UGM	(rat) urogenital (sinus) mesenchyme
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGF-R2	vascular endothelial growth factor receptor 2 (KDR, Flk-2)

Note: If it's an abbreviation and it's not on here, it's probably a cell line.

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Acknowledgements

I would like to thank my family, especially my mom and dad, for supporting me through years and years of school.

What really made this thesis possible was the help and the kindness of everyone in the Gough lab and the collaborators with the Gough lab, for example Peter Chen, Ruijuan Xiu, Hongwei Li, Martin Haas, Rita Finones, Isgard Hueck and my supportive fellow grad students. I am lucky to be in such a friendly lab.

I would like to give a big thank you to Hongwei for help with the cells, animal work and bead making.

Also special thanks to Zhongling Feng who trained me even though that was not in his job description.

And I cannot forget to thank all the undergraduates who worked with me in the lab: Chris Lau, Laura Restrepo, Michelle Yap, William Yuen, Tirapon Premchitt, Eli Lazarus, Suraj Kedarisetty, Phoebe Lai, and Amul Shah. I wish them all a bright and beautiful future.

Charlotte Lauve, the Materials Science grad coordinator, was one of the reasons I picked UCSD and she never let me down.

My most heartfelt thanks go to Dr. Gough, who is surely one of the few inherently good people on this planet. I was very lucky to find him.

Chapters 4, 5 and 6, in part, are currently being prepared for submission for publication of the material. Frimodig, Jane; Gough, David. The dissertation author was the primary investigator and author of this material.

Chapters 7, in part, is currently being prepared for submission for publication of the material. Frimodig, Jane; Li, Hongwei; Xiu, Ruijuan; Gough, David. The dissertation author was the primary investigator and author of this material.

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PUBLICATIONS

Hueck, I.S., Haas, M., Finones, R.R., Frimodig, J., Gough, D.A. The potential of selectively cultured adult stem cells re-implanted in tissues. Stem Cell Engineering. Eds: Artmnan, G.M., Hescheler, J., Minger, S., Springer-Verlag. In press (2010).

Dao, Q., Frimodig, J.C., Le, H.N., Li, X.Z., Putnam, S.B., Golda, K., Foyos, J., Noorani, R., and Fritz, B. Calculation of Shrinkage Compensation Factors for Rapid Prototyping (FDM 1650)", Computer Applications in Engineering Education, 7 (3), (1999).

ABSTRACT OF THE DISSERTATION

Angiogenic Potential of Prostate Tumor Stem Cells

by

Jane Catherine Frimodig

Doctor of Philosophy in Materials Science and Engineering

Professor David Gough, Chair

There is currently no successful therapy available for prostate cancer that has progressed to the androgen-deprivation-resistant metastatic stage. It is believed that after androgen-deprivation ablation of the bulk of differentiated cells that make up the tumor, the hormone-deprivation resistant tumor regrows from a subpopulation of prostate epithelial cells. The role of these prostate tumor stem cells (PrTuSC) in neovascularization is of primary interest, due to the direct correlation of angiogenesis with metastasis. *In vitro* analyses examined the angiogenic potential of the PrTuSC cells isolated from early prostate tumors. These included an angiogenic antibody array assay of media conditioned by the cells to discover which angiogenic proteins they expressed. Proteins expressed at high levels *in vitro* were Tissue Inhibitor of Metalloproteinases (TIMP-1) and Interleukin-8 (IL-8). Next, the functional angiogenic properties of these proteins were verified by endothelial cell migration and proliferation assays. ELISA was done to quantify protein levels. Development of an *in vivo* methodology to study paracrine effects of TIMP-1 and IL-8 on neovascularization was undertaken. This methodology entails the implantation of alginate disks containing the cells and proteins of interest into the dorsal skin-fold window chamber of nude mice. The window chamber allows real time *in vivo* fluorescent microscopy of angiogenic response to the proteins. Both IL-8 and TIMP-1 promoted neovascularization in the window chamber.

Chapter 1: Cancer Vasculature

1.1 Introduction

Glen Algire developed the first dorsal skin fold window chambers and used them to study vascularization of cancer tissue. In 1945 he wrote that “the rapid growth of tumor explants is dependent on the development of a rich vascular supply” (Algire, Chalkley et al. 1945). Years later Judah Folkman expounded upon this same idea (Folkman 1971). Oxygen studies of human tumors implanted in the dorsal skinfold window chamber of SCID mice confirmed that oxygen falls to hypoxic levels (less than 5 mmHg) within 200 μm of the blood supply (Figure 1-1) (Torres Filho, Leunig et al. 1994). Today it seems obvious that for the rapid growth of cancer cells, the growth of the vascular system to supply nutrients and oxygen to these cells must be equally rapid. It should also be noted the vasculature is essential for metastasis as it can transport cancer cells from the original, or primary tumor to other parts of the body, where they can leave the blood vessel (extravasate) and travel into the tissue to form a tumor in the new location.

Once these concepts were accepted by the scientific community, the race was on to develop some inhibitor of cancer tumor blood vessels, in order to ‘starve’ the tumor (Figure 1-2). But finding an efficacious inhibitor was not simple. One of the first big hopes was a drug called bevacizumab (AvastinTM, Genentech) that inhibits the receptor of VEGF, one of the main growth factors involved in angiogenesis. Animal studies looked promising but clinical trials did not live up to this promise (Loges, Schmidt et al. 2010). Subsequent studies are now revealing what could be going wrong. For one thing, even if the inhibitors effectively kill or inhibit the blood vessels, some recent findings show that this can actually stimulate metastasis, i.e. if the blood vessels cannot go to the cancer cells, the cancer cells will go to the blood vessels (Steeg 2003; Ebos, Lee et al. 2009; Paez-Ribes, Allen et al. 2009).

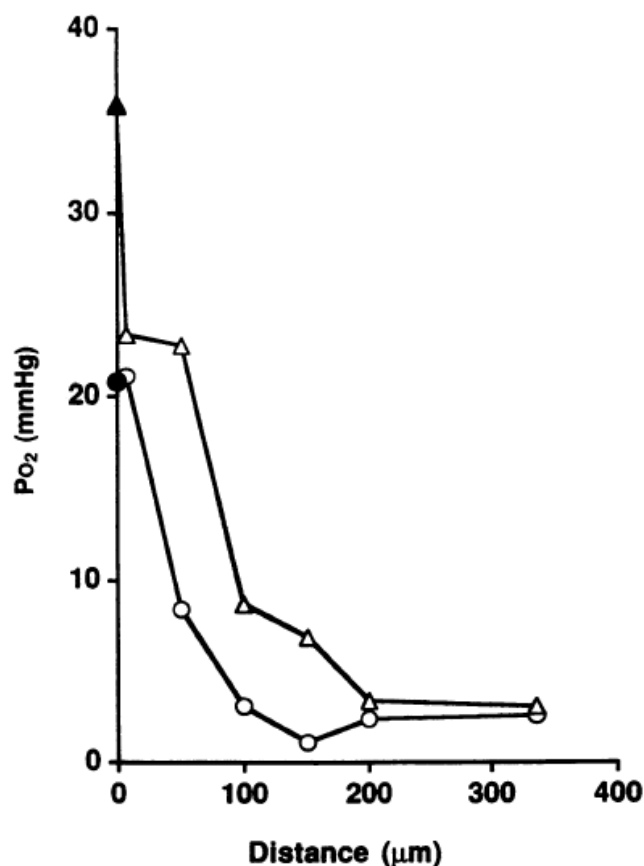


Figure 1-1.

Partial oxygen pressures measured in an LS174T(human) tumor implanted into the dorsal skin fold window chamber of a SCID mouse, relative to the distance from the nearest blood vessel. The triangles represent measurements taken under unanesthetized and the circles under anesthetized conditions. The partial oxygen pressure dropped exponentially and reached plateau values of about 3 mmHg at about 200 μm away from the blood vessel (Torres Filho, Leunig et al. 1994).

Another possible reason for the failure of anti-angiogenic therapies so far concerns MDR (multi-drug resistance), the ability of cancer cells to adapt and become resistant to chemotherapy. Tumor cells can develop resistance to various cancer therapies and the subpopulation of stem and progenitor cells is especially good at it (Donnenberg and Donnenberg 2005). What is not well known is that endothelial cells, that comprise the innermost lining of the blood vessel, completely change personality (phenotype) under the influence of tumor cells and have also been found to become drug resistant (Grange,

Bussolati et al. 2006; Meng, Henson et al. 2007; Meng, Henson et al. 2007; Langenkamp and Molema 2009; Xiong, Sun et al. 2009; Xiong, Sun et al. 2009).

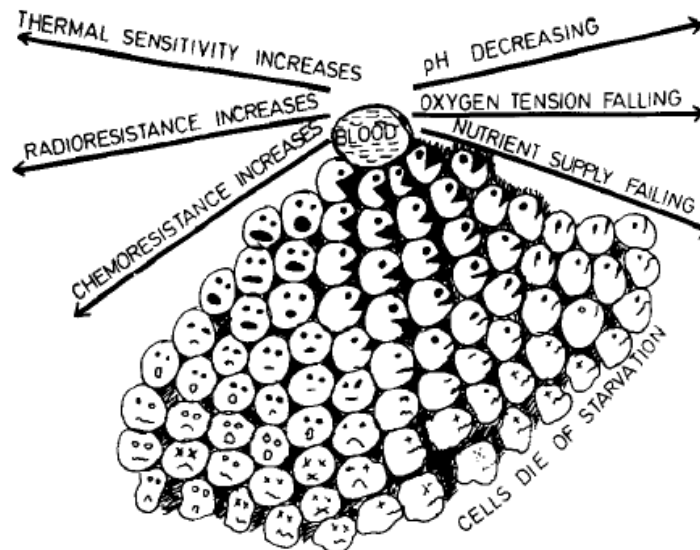


Figure 1-2.

The dependence of tumor cells and various cancer therapies on blood vessel proximity (Denekamp 1990).

Yet another interesting development in the focus on tumor vessels supports turning them into normal vessels, as opposed to getting rid of them. The blood vessels stimulated by a tumor are very distinct from regular 'healthy' blood vessels. They are leaky, tortuous, and very irregular in branching and diameter. One school of thought is that if we could 'normalize' these blood vessels, they would be much more successful at delivering chemotherapies to the tumor (Jain 2005).

The aim of all of the above is to emphasize that 1) cancer vasculature is very interesting and complicated but 2) it is really not very well understood and we will not be able to attack it therapeutically until this understanding becomes much more comprehensive. The goal of this dissertation is to contribute to that specific body of work.

1.2. Endothelial Cells, Angiogenesis, and Cancer Vasculature

Endothelial cells are the cells that line blood vessels (and also lymphatics) throughout the body. They stretch out and join together to form a single layer which comprises the inner lining of the blood vessels. Tight contacts between them maintain the blood separate from other tissues but allow for exchange of oxygen, nutrients and waste products. Endothelial cells rest on an adluminal thin membrane of extracellular matrix material known as the basement membrane.

Endothelial cells in the healthy adult are generally quiescent, with a few exceptions which will be discussed below. Of all vascular endothelial cells in the human body, less than 0.1 % are proliferating at any given time. A well-balanced array of angiogenesis stimulators and inhibitors keeps the blood vessel system healthy and efficient. In tumor vessels, however, endothelial cells are actively proliferative: 5-15% are proliferating early in tumor formation increasing eventually to as many as 40-60%. According to Denekamp, "This large difference in proliferation rates of endothelium is one of the most dramatic and consistent differences between solid tumours and normal tissues. " The only places they proliferate faster are in placental vessels and granulation tissue in wounds (Denekamp 1990).

Endothelial cells differ slightly in different parts of the body. For example, endothelial cells in the brain are sealed very tightly (the so-called blood-brain barrier) so unwanted substances are not allowed easy access to this very sensitive tissue, while in the liver they are fenestrated (fenestra is latin for window) to allow filtration of toxins from the blood. In tumors, ECs, as previously noted, do not seal tightly, and their pericytes, the supporting cells around them that help hold them together and function, are scant or irregular (Ozawa, Yao et al. 2005). Additionally, endothelial cells in tumor vasculature express different types of cell surface proteins. They are less dependent on growth factors and nutrients than normal EC (Hida and Klagsbrun 2005; Grange, Bussolati et al. 2006; Meng, Henson et al. 2007; Meng, Henson et al. 2007) and can be multi-drug resistant (Xiong, Sun et al. 2009), multipotent

(Dudley, Khan et al. 2008), and genetically unstable (Grange, Bussolati et al. 2006). In other words, they seem to become more like cancer cells due to their association with cancer cells.

Angiogenesis, the growth of new blood vessels from existing ones, occurs as a normal physiological process in wound-healing and female reproductive events. Abnormal angiogenesis is seen in diseases such as diabetes (most notably in diabetic retinopathy), atherosclerosis, macular degeneration, and cancer. Other diseases in which EC actively proliferate include rheumatoid arthritis, psoriasis, retrolental fibroplasia, hemangiomas and during the rejection of organ transplants (Fotsis, Pepper et al. 1993). Chemical and mechanical mechanisms such as hypoxia (Zweifach, Kovalcheck et al. 1981; Schmid-Schonbein 1987), high blood pressure (Zhou, Egginton et al. 1998; Egginton, Zhou et al. 2001; Egginton, Zhou et al. 2001; Xiong, Sun et al. 2009), and also low blood pressure or blood stasis as seen in shock or stroke (Hueck, Hollweg et al. 2000; Hueck, Rossiter et al. 2008), set angiogenesis into action.

To form new blood vessels, first the endothelial cells must migrate and proliferate to form a sprout. Sprouting takes place mainly in the small venules located downstream from the capillaries where blood velocity is reduced (Denekamp 1990). In the case of hypoxia, a low oxygen situation in the tissue, the regulatory protein HIF-1 α is no longer degraded and activates VEGF (vascular endothelial growth factor) signaling which initiates angiogenesis (Semenza 2008). Vascular sprouts extend, remodel and anastomose to form complete networks. VEGF concentration controls EC proliferation along the stalk of the sprout and directional blood vessel growth is controlled to a great extent by the VEGF gradient at the sprout tip (Gerhardt, Golding et al. 2003; Ozawa, Banfi et al. 2004; Chen, Silva et al. 2007), whereas tip growth is negatively regulated by Delta-like ligand and Notch signaling (Jain 2003; Claxton and Fruttiger 2004; Williams, Li et al. 2006; Hellstrom, Phng et al. 2007; Sainson and Harris 2008). A great deal of other growth factors have been found to be involved in regulation of blood vessel growth, among them FGF-2, Angiopoietins 1 and 2,

EGF, PIGF, PDGF, and TGF- β , and SDF. (Javerzat, Auguste et al. 2002; Bauer, Bauer et al. 2005; Shim, Ho et al. 2007; Al-Nedawi, Meehan et al. 2009; Song, Huang et al. 2009).

There are parallels between endothelial cells and the epithelial cells that line the prostate ducts, and are transformed into cancer cells in prostate carcinoma. Both lie on a basement membrane, although the epithelial cells are of two types: basal cells that lie directly on the basement membrane and are squamous in form (Figure 1-3) and secretory epithelial cells (more columnar in shape) that compose the inner layer on the lumen side of the basal cells. Interspersed among these cells is found the occasional neuroendocrine cell. It is thought that prostate stem cells (and prostate cancer stem cells) may originate from basal cells (Robinson, Neal et al. 1998). In the case of blood vessels, the endothelial cells are stretched and elongated out into thin sheets along the basement membrane. In a smaller vessel such as a capillary, one cell can comprise the entire diameter of a section of the blood vessel. The endothelial cells sit directly on the basement membrane and outside this generally is found a layer of pericytes, which are supporting cells for the blood vessel. Both the endothelial cells and pericytes contribute to the manufacture of the basement membrane. In the case of the prostate ducts, only one cell type (epithelial cells) generates the basement membrane. The basement membrane is a very important structure because it is the barrier that must be breached, for example, in the case of the blood vessels, for white blood cells to exit the blood vessel and migrate into the tissue and do their clean-up job. Research has shown that these cells go through in locations where the basement membrane is thinner (Voisin, Probstl et al. 2010). In cancer, the basement membrane is the barrier that metastasizing cancer cells departing the primary tumor must penetrate in order to a) gain access to the blood vessels and b) exit the blood vessel to form a new tumor in a new site. In prostate carcinoma, it is the barrier that the cancer cells must breach in order to spread beyond containment in the organ and metastasize. Usually basement membrane is composed of laminin, Type IV collagen, perlecan (heparin sulfate proteoglycan), Nidogen/entactin; plus small quantities of the

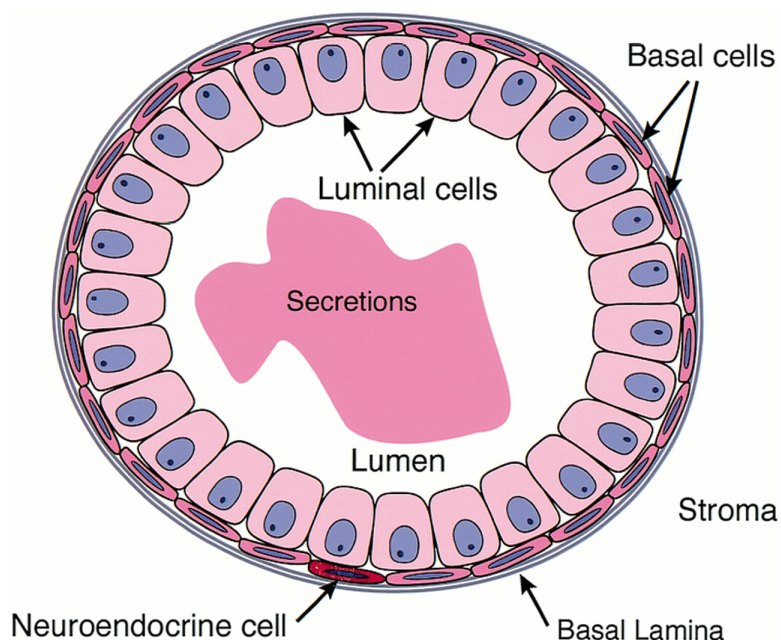


Figure 1-3.

Cross-sectional diagram of a prostate gland duct, showing the different cells types and features (Abate-Shen and Shen 2000) .

following: proteases MMP-2, MMP-9, urokinase, tissue type plasminogen activator; growth factors TGF- β , EGF, IGF-1, bFGF and PDGF; in addition to amylase, transferrin, and clusterin (Kleinman and Martin 2005). So what breaks up the basement membrane when white blood cells need to sneak through it, when a blood vessel needs to free up the endothelial cells so they can proliferate and breach the basement membrane with a sprout in order to form new blood vessels, and when cancer cells break through it in order to spread beyond the confines of their organ of origin? Matrix metalloproteinases (MMPs) do this, especially MMPs-2 and -9 (aka gelatinases A and B) which degrade type IV collagen (Rayment and Upton 2009). The importance and durability of the basement membrane is underscored by studies that show that following withdrawal of anti-angiogenic therapies, tumor blood vessels rapidly regrow along the empty basement membrane sleeves of ghost vessels (Inai, Mancuso et al. 2004; Inai, Mancuso et al. 2004; Mancuso, Davis et al. 2006). Once blood vessels have breached the basement membrane and sprouted, they still need to navigate through the extracellular matrix, which is not a vacuum, but rather a fairly dense jungle constructed of interconnecting

filamentous proteins and glycoproteins such as collagen, laminin, elastin, fibronectin, and hyaluronan. The family of metallomatrix proteases consists of about 20 members, and between them all they can degrade any component of the ECM. Therefore the catalytic activity of these proteins is essential to angiogenesis.

Since Tissue Inhibitors of Metalloproteinases (TIMPs) inhibit the enzymatic action of MMPs, it has been generally assumed that TIMPs inhibit the breakdown of basement membranes and ECM by inhibiting MMPs. Following this reasoning, TIMPs would inhibit neovascularization and they would also inhibit metastasis. In fact numerous studies, which will be described later, have found this to be so. But many other studies have also found other effects of TIMPs, such as prevention of apoptosis, which could contribute to metastasis and/or angiogenesis.

There is another contradiction with all the data implicating TIMP-1 to be anti-angiogenic and anti-metastatic: if TIMP-1 inhibits cancer tumor growth and metastasis, why is its concentration in the blood found to be high in many cancers, and why has it been correlated with worsening of the disease (Table 5-1)?

In this study I show that IL-8 and TIMP-1 are made by stem/progenitor cells from early prostate tumors (PrTuSC) and attempt to discover how TIMP-1 might make an early contribution to disease progression via its effects on the vasculature.

Figure 1-1 is used with permission from PNAS: Noninvasive measurement of microvascular and interstitial oxygen profiles in a human tumor in SCID mice. I.P. Torres-Filho, M. Leunig, F. Yuan, M. Intaglietta, R.K. Jain, PNAS 91(6), 2081-2085, Figure 3 page 2084, Copyright (1994) National Academy of Sciences, U.S.A..

Figure 1-2 is used with kind permission from Springer Sciences+Business Media: Cancer and Metastasis Reviews/Vascular attack as a therapeutic strategy for cancer, 9(3), 1990, Juliana Denekamp, Figure 3.

Figure 1-3 is used with permission from Cold Spring Harbor Laboratory Press:
Molecular Genetics of Prostate Cancer, Abate-Shen, C.; Shen, M.M., 2410-2434, Figures 3,
2000.

Chapter 2: Stem Cells

2.1 Stem Cells

By definition, these are cells that can both self-renew and can also differentiate into other kinds of cells. There are two major kinds of stem cells: embryonic stem cells, which are found only in the blastocyst of the embryo, and adult stem cells, which are found in nearly every organ in mammals. Embryonic stem cells are pluripotent, i.e. they can differentiate into almost any other kind of cell. Embryonic stem cells are immortal and also tumorigenic; their growth can become uncontrollable as in cancer. They proliferate rapidly as they need to generate an entire human being in a relatively short span of time. Adult stem cells, on the other hand, are not immortal and can be found in the body in many locations, most notably the bone marrow and skin. They are not as pluripotent as embryonic stem cells, as they can usually only differentiate into the cell types that comprise their resident organ. Progenitor cells are stem cells that have already differentiated somewhat along a specific lineage. They are still capable of differentiating further but they are not as pluripotent as adult or embryonic stem cells. Adult stem cells reside in specific microenvironments called niches, which are designed to support, protect and sustain them. Adult stem cells are long-lived and relatively quiescent most of the time. When there is a need to provide new cells, the stem cells are mobilized from their niches and induced to proliferate, and their progenitors then migrate to the locations where they are needed. The adult stem cells are therefore responsible for the maintenance and upkeep of their resident organ.

Adult stem cells have been found in nearly every organ in mammals, including the bone marrow (Peichev, Naiyer et al. 2000), human skin (Moore and Lemischka 2006), the cornea of the eye (Cotsarelis, Cheng et al. 1989), mammary glands (Blanpain, Horsley et al. 2007), the esophagus (Croagh, Phillips et al. 2007), the heart (Urbanek, Cesselli et al. 2006);

the kidneys (Maeshima, Yamashita et al. 2003; Bussolati, Bruno et al. 2005), the respiratory system (Borthwick, Shahbazian et al. 2001; Hong, Reynolds et al. 2001), the pancreas (Duvillie, Attali et al. 2003), the nervous system (Uchida, Buck et al. 2000; Lee, Kessler et al. 2005), and the prostate (Kinbara, Cunha et al. 1996). When cells from the ductal tips of rat prostates were mixed with cells from urogenital neonatal rat mesenchyme, and planted under the kidney capsule in syngeneic male hosts, they grew into functional (secreting) prostate ducts (Hiroyuki, Gerald et al. 1996).

Endothelial progenitor cells (EPC) were discovered in the vascular system (Asahara, Murohara et al. 1997). These cells originate in the bone marrow, are mobilized into the circulation and are thought to travel to sites of injury or pathology where they are needed to repair the blood vessels. Their actual role in angiogenesis is controversial however. Several studies have found that EPC differentiate to perivascular but not endothelial cells (Gothert, Gustin et al. 2004; Ingram, Mead et al. 2005; Purhonen, Palm et al. 2008). It is more likely that endothelial cells already existing at the site are activated, proliferate and migrate to undertake angiogenesis. However endothelial progenitor cells, when injected or artificially mobilized into the blood stream, have been shown to have some short-term therapeutic value so they are well worth investigating (Kawamoto, Gwon et al. 2001; Rafii and Lyden 2003; Galiano, Tepper et al. 2004).

There is much hope for stem cells as a therapy in regenerative medicine. These cells could possibly be used in tissue culture to regenerate an entire organ, or injected into the pathological location to replace dysfunctional host stem cells or to aid in repair via paracrine or some other mechanism. However, stem cell research is still a field in its infancy and a great deal of work remains to be done to gain a comprehensive understanding of these cells.

2.2. Cancer Stem and Progenitor Cells

Hanahan (Hanahan and Weinberg 2000) listed 6 hallmarks of cancer:

1. Self sufficiency in growth signals (i.e. it can make its own growth factors).

2. Insensitivity to growth-inhibitory signals.
3. Extensive replicative potential.
4. Ability to avoid programmed cell death (apoptosis).
5. Sustained angiogenesis.
6. Ability to invade and produce metastases.

In order to survive and spread, cancer cells must be capable of doing all these things. And it must be remembered that the cells that comprise the tumor are not all alike, rather they are a heterogeneous collection of cells. So on a cellular level, the tumor must have cells that can do the above six things, but there can be different cells in the tumor that take on responsibility for different hallmarks. Or there can be one cell subpopulation that can differentiate into cells capable of doing some or all of these things.

Cancer stem cells have been found in many different populations and are considered to be analogous to adult stem cells. Cancer stem cells were initially found in leukemia (Lapidot, Sirard et al. 1994; Bonnet and Dick 1997). Soon thereafter they were found in a range of cancers including colorectal cancer (Brabletz, Jung et al. 2005; Dalerba, Dylla et al. 2007), head and neck cancer (Prince, Sivanandan et al. 2007), mammary carcinoma (Al-Hajj, Wicha et al. 2003), melanoma (Quintana, Shackleton et al. 2008), glioma and glioblastoma (Singh, Clarke et al. 2003; Galli, Binda et al. 2004; Singh, Hawkins et al. 2004; Bao, Wu et al. 2006), pancreatic (Li, Heidt et al. 2007), and prostate cancer (Collins, Berry et al. 2005). Characterization of all these cells and more is ongoing and involvement of cancer stem will probably vary for different kinds of cancers; but in general cancer stem cells may be adult stem cells that have acquired enough genetic mutations, epigenetic or other changes to satisfy the six hallmarks. Therefore the resident stem cell of the organ where the disease originates may have been transformed. Another possibility is a cancer stem cell may be one that has dedifferentiated in order to acquire stem cell-like properties. At any rate, the result is a single cell which, if transplanted into an animal model, can grow into an entire solid tumor, or leukemia in the case of blood cancers.

Stem cells (and cancer stem cells) are touted as highly proliferative cells, which can be confusing. Adult stem cells are normally quiescent stem cells that are coddled in a very specific protective and supportive environment known as their 'niche' (French for nest). They can be thought of as bears hibernating in a cave. They are not metabolizing much. They are usually immobilized by strong tethers to the ECM. They are usually protected from the outside environmental stresses by surrounding cells or structures (i.e. as in the bone marrow). They are long-lived. They are not commonly proliferating. When they get the signal to supply a new cell, they divide to form usually one cell like themselves (self-renewal) and one 'daughter cell' (known as asymmetric division) that is the next down in the hierarchy. They can also divide symmetrically, to form two new stem cells. Daughter cells may retain some properties of the parent stem cells, but they will probably differ from it in two major ways: 1) they are less pluripotent (they are not able to differentiate in as many ways as the parent could, i.e. their differentiation pathway is more restricted 2) they are usually highly proliferative. So stem cells can fulfill hallmark #3 by having great replicative *potential*; that is not the stem cells themselves, but their highly proliferative offspring will fulfill that replicative potential.

Metastatic cells must be able to exit their niche, get access to the bloodstream (or lymph vessel), survive in the bloodstream, leave the bloodstream and arrive at the metastatic site. They must be able to adapt to all these environments at least for the time range during which they will be present in that particular neighborhood. When the metastatic cell arrives at its new site, it does not have to be immortal to take over there and displace the resident cells; it just needs to be able to survive longer and proliferate more than the resident cells in that particular location; it must be able to differentiate into cells that satisfy the Hallmarks. Therefore the most important property for metastasis is adaptability i.e. plasticity, both forward and backward. This is why we are most interested in stem cells in cancer, the most plastic (pluripotent) of cells. It makes sense that these cells (or their direct progeny) would have this metastatic capability.

The niche is at least as critical for cancer stem cells as it is for normal adult stem cells. The cancer stem cell niche however differs from the ASC niche, mainly in that the bears are not hibernating in this case but doing some serious business. The niche is an important factor in metastasis in that metastatic cancer stem cells are more likely to settle down some place where they will be happy (Paget 1889). Once these cancer metastatic niches have been characterized, that knowledge will be a valuable base from which to formulate cancer attack plans. For example, hematopoietic stem cells that express VEGFR-1 precede cancer cells at metastatic sites (Kaplan, Riba et al. 2005). A therapy targeted to this receptor may be a way to attack the metastatic sites before the cancer even arrives there. Is vascularization an important component of cancer stem cell (CSC) niches? Ability of CSC to maintain angiogenic potential at the niche may be critical to what it does there, i.e. whether it hibernates or establishes and grows into a new tumor. There are cancer cells that have metastasized and established in new niches in small clumps that remain dormant for years; these are known as micrometastases. Micrometastases have been shown in breast and prostate cancer (Braun, Vogl et al. 2005; Weckermann, Polzer et al. 2009) and explain why cancers can remain quiescent for long periods of time, for example in breast cancer for as long as 10-20 years, and then reoccur later. One of the critical differences between whether micrometastases remain dormant or become invasive and ultimately fatal tumors is believed to be vascular supply. Therefore if the first cells that go out and initiate tumors are stem cells, and if their ability to initiate tumors rests on their ability to stimulate vascularization, it is very important to find out how cancer stem cells might be able to do this.

There is abundant evidence that putative cancer stem cells are eminently suitable for fulfilling all the qualifications enumerated by the Hallmarks of Cancer, with the exception of #5, angiogenesis. Several studies and reviews have noted the angiogenic abilities of cancer stem cells (Bao, Wu et al. 2006; Rak 2006; Eyler and Rich 2008) but not many studies have examined in detail how they are going about promoting angiogenesis.

In the prostate, the existence of cancer stem cells was hypothesized (Helmut and Klaus 1996) and eventually shown (Collins, Berry et al. 2005). Our collaborators at the Haas lab (UCSD) have isolated cells from early primary prostatectomies.

The prostate gland is a walnut-sized organ located around the urethra at the base of the bladder. It consists of a network of ducts lined with epithelial cells that secrete components of the seminal fluid. Prostate cancer consists of overproliferation of the epithelial cells. In the case of our cell sources, to stop the cancer, the entire gland was removed. Since these cells came from contained tumors as opposed to metastatic cancer, they were dubbed prostate tumor stem cells (PrTuSC). PrTuSC were isolated from tissue samples and selected on the basis of their growth on laminin and in low oxygen conditions (~5% pO₂) in a special serum-free defined medium (Haas medium #6). Clones proliferated out from a central cluster of cells (stem cell center, SCC) that expressed Oct-4, a stem cell marker. In early passages all cells in the colonies expressed stem cell markers CD44, CD133, CK5/14, integrin $\alpha 2\beta 1$, and VEGFR1. Also Bmi-1 and Sox-2 were detected in these cell populations using flow cytometry. Over time the expression of CD133 and VEGFR1 in the surrounding apron of proliferating cells decreased and their expression of CK8/18.

In the case the PrTuSC used in this study, the cells in the stem cell center (SCC), that can be labeled by Oct-4 antibodies, are probably the stem cells, and spreading out from them is found an ever-increasing proportion of progenitor 'daughter' cells. These cells share some markers with their stem cell parent but also show that they are starting to differentiate by their expression of CK8/18, a marker of differentiated epithelial luminal cells, and by their downregulation of stem cell markers CD133 and VEGFR1. The cells proliferating out from these stem cell centers are probably transit amplifying cells, a more differentiated, highly proliferative progenitor cell. The cells could be maintained for up to 8 passages *in vitro*, but in each subsequent passage the stem cell population was thought to diminish, based on lessening expression of stem cells markers. Cells from passages 1-8 grew into normal human prostate glandular structures when combined with UGM (rat urogenital sinus mesenchyme) in

a collagen matrix and implanted under the kidney capsule of male SCID mice. When these same cells (only without the UGM) were transplanted orthotopically into a SCID mouse prostate, a heterogeneous tumor formed. This demonstrates the importance of microenvironment on the fate of PrTuSC. These cells must contain some pluripotent cells, or at the very least are tumorigenic if they find themselves in the right niche. Since these cells, which appear early in prostate cancer progression, are tumorigenic, we are also very interested in their ability to promote cocommitant blood vessel formation which is essential not only for progression of the tumor but also for metastasis, per Hallmarks #5 and #6. For these studies we wanted to find out what contribution PrTuSC could possibly be making to angiogenesis in early tumors. There are several possible ways PrTuSC could influence angiogenesis: 1) by differentiating into endothelial cells, and/or 2) by releasing angiogenic proteins. In this work we looked at these two possibilities. Angiogenic proteins being made by the PrTuSC were discovered and their mechanism of angiogenesis was investigated.

Chapter 3: Pluripotency of PrTuSC in the Vascular Direction

3.1 Introduction

Since cancer stem cells are pluripotent by definition, they could possibly contribute to neovascularization by differentiating into the cells that comprise the blood vessel system. Foremost of these is the endothelial cell, which lines the blood vessels. An experiment was done to assess the plasticity of PrTuSC in the vascular direction, by determining if they could differentiate into endothelial cells *in vitro*.

3.2. Materials and Methods

Prostate tumor stem cells (PrTuSC) were obtained from the Haas lab at UCSD. These cells were isolated by the Haas lab from early primary prostate tumors (Gleason Grades 6-9) and grown on laminin in a serum-free defined medium (Haas medium) in a humidified incubator at 37°C in 10% CO₂ and ~5% oxygen. Cells from five different patients were used. Table 3-1 describes the cells, Gleason grade, and pathological description of the tissue. PrTuSC were transfected by the Haas lab to overexpress Bmi-1 and Tert. This manipulation makes the cells longer-lasting in culture. Cells were transferred to this lab, trypsinized and seeded onto fibronectin-coated 100 mm tissue culture dishes containing glass cover slips. Fibronectin (0.1% F1141-5 mg) was from Sigma. To coat the dish, 0.150 ml of stock fibronectin (pH 7.5) was added to a 100 mm tissue culture dish containing glass cover slips and 10 ml of PBS. This was incubated overnight then the fibronectin solution was removed and the medium and cells were introduced into the dish. The differentiation medium was EGM-2 (Lonza Bio-Whittaker) and control cells were PrTuSC grown in PrEGM prostate epithelial cell growth medium from Lonza (negative control), human foreskin fibroblasts (HFF) (negative control) and human umbilical vein endothelial cells (positive control) both grown in EGM-2. The medium was changed every 2-3 days. Cells were observed at this time and notes taken on their appearance.

HUVECs were obtained from Lonza and grown in the same conditions as the PrTuSC. Cells were subcultured upon attaining 90% confluence or sooner, trypsinized using 0.1% Trypsin-EDTA from Invitrogen and split at a ratio of 1:3. All cells were grown in a humidified incubator at 37°C with 5% CO₂. Passages 4-8 were used for these experiments. Human foreskin fibroblasts (HFF) were also a kind gift of the Haas lab.

At 23 day and 48 day timepoints, coverglasses containing cells were removed from culture, washed with PBS, then incubated with 2 µg/ml AcLDL-Dil (Molecular Probes, Invitrogen) for 4 hours at 37°C. Dil (1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate) is a hydrophobic lipophilic fluorescent cyanine dye used for imaging purposes. Dil has an excitation wavelength of 554 nm and an emission wavelength of 571 nm. The LDL is acetylated to stabilize it. Uptake of LDL is an indication of endothelial cell functionality commonly used to identify endothelial cells. After incubation, the cells were washed with PBS once more and viewed under the fluorescence microscope using an Omega XF108-2 filter cube. This filter cube has an excitation wavelength of 525 nm and emission wavelength of 595 nm. Cells were imaged using an Olympus E-330 digital camera mounted on an Olympus Bx51W1 microscope with an OptiQuip Model 7700 Lamphouse and an OptiQuip Model 1600 Power Supply.

3.3. Results

PrTuSC did not differentiate towards endothelial cells and indeed, did not grow very well in the EGM-2. They are normally grown in a defined serum-free medium in a low (~5%) oxygen environment, which maintains them in a stem cell-like state for up to eight passages after which they differentiate and begin to senesce. (The B+T cells used in the experiment, however, survive longer *in vitro*.) So it is possible that they would begin to differentiate immediately when grown in standard incubator oxygen levels (about 20%). But they did not differentiate towards endothelial cell lineage as they did not assume endothelial cell morphology and did not take up acLDL-Dil which is a functional indicator of endothelial cells

(Figure 3-1). As early as one week in culture on fibronectin in EGM-2, cells from all the patients began to form long cytoplasmic extensions or tubes. P62 formed the best tubes. Cells from patient p74 seemed to be the most proliferative.

3.4. Discussion

Stem and progenitor cells were isolated from primary prostate tumors and selected using a defined medium, culture on laminin and in low oxygen conditions by the Haas lab. Then the prostate tumor stem cells (PrTuSC) were transported to our lab and grown in an endothelial cell growth medium that has been shown to differentiate pluripotent cells along an endothelial lineage (De Coppi, Bartsch et al. 2007). Culture on fibronectin also promoted differentiation of endothelial progenitor cells into endothelial cells (Fan, Li et al. 2003; Errol, Salman et al. 2004). After one week, PrTuSC from most patients began to form tube-like cytoplasmic extensions (Figure 3-2), which is also seen in differentiation of endothelial cells. However, PrTuSC never did form complete tubal networks as is seen in endothelial cell capillary differentiation. They continued to show cytoplasmic extensions, but there were also a lot of spread-out and senescent-appearing cells. It may also be expected for PrTuSC to form tubes since they are duct-forming cells. At any rate, based on the lack of uptake of acetylated low density lipoprotein tagged with a fluorescent marker by PrTuSC after being grown in EGM-2 for 48 days, PrTuSC grown under these conditions did not differentiate into endothelial cells.

Gleason score is a histological rating system for prostate cancer. Gleason score less than or equal to 6 is usually considered low risk prostate cancer. Although p9 PrTuSC had the highest Gleason score of 9, the tube-formation or proliferation of these cells was not the best out of the 5 samples. Interestingly, the most vigorous cells were p74 which came from an unscored sample, which was probably BPH (benign prostate hyperplasia) or PIN (prostate intraepithelial neoplasia). The most tubal extensions were formed by p62, which had a Gleason score of six.

Vascular mimicry is an phenomenon that occurs in several cancers, among them melanoma (Maniotis, Folberg et al. 1999), breast (Shirakawa, Kobayashi et al. 2003), ovarian (Sood, Seftor et al. 2001) and prostate carcinomas (Navesh, Richard et al. 2002). In vascular mimicry, the cancer cells themselves actually align to form matrix-rich channels that carry blood. The exact subtype of cancer cells that does this has not been pinned down, but one can speculate that it may well be some kind of especially plastic cancer cell, like a progenitor or stem cell. Vascular mimicry typically only occurs in aggressive cancers, which implies that it originates with a cell that has dedifferentiated during cancer progression. Given that our PrTuSC were isolated from early primary tumors, it is not too surprising that they did not exhibit this property. Since vascular mimicry may be immune to antiangiogenic therapies, the understanding of this phenomenon is critical to treatment of cancers and bears further investigation.

Table 3-1.

PrTuSC cells used in differentiation assay

cells	Gleason score	Histology notes (from Pathology Dept, VA Hospital, UCSD)	Observation
p9B+T	9	Adenocarcinoma, high-grade poststatic intrapeithelial neoplasia and chronic inflammation	
p12B+T	7	Adenocarcinoma, conventional, not otherwise specified.. Benign prostatic hypertrophy, glandular atropy, low- and high-grade prostatic intra-epithelial neoplasia, and a focus of stromal hyperplasia	
p62B+T	6	Adenocarcinoma, high-grade prostatic intra-epithelial neoplasia; stromal hyperplasia; benign prostatic hyperplasia	Best tubes
p63B+T	6	Adenocarcinoma, high-grade prostatic intra-epithelial neoplasia; benign prostatic hyperplasia	
p74B+T	Not scored	Glandular and stromal hyperplasia	Most confluent

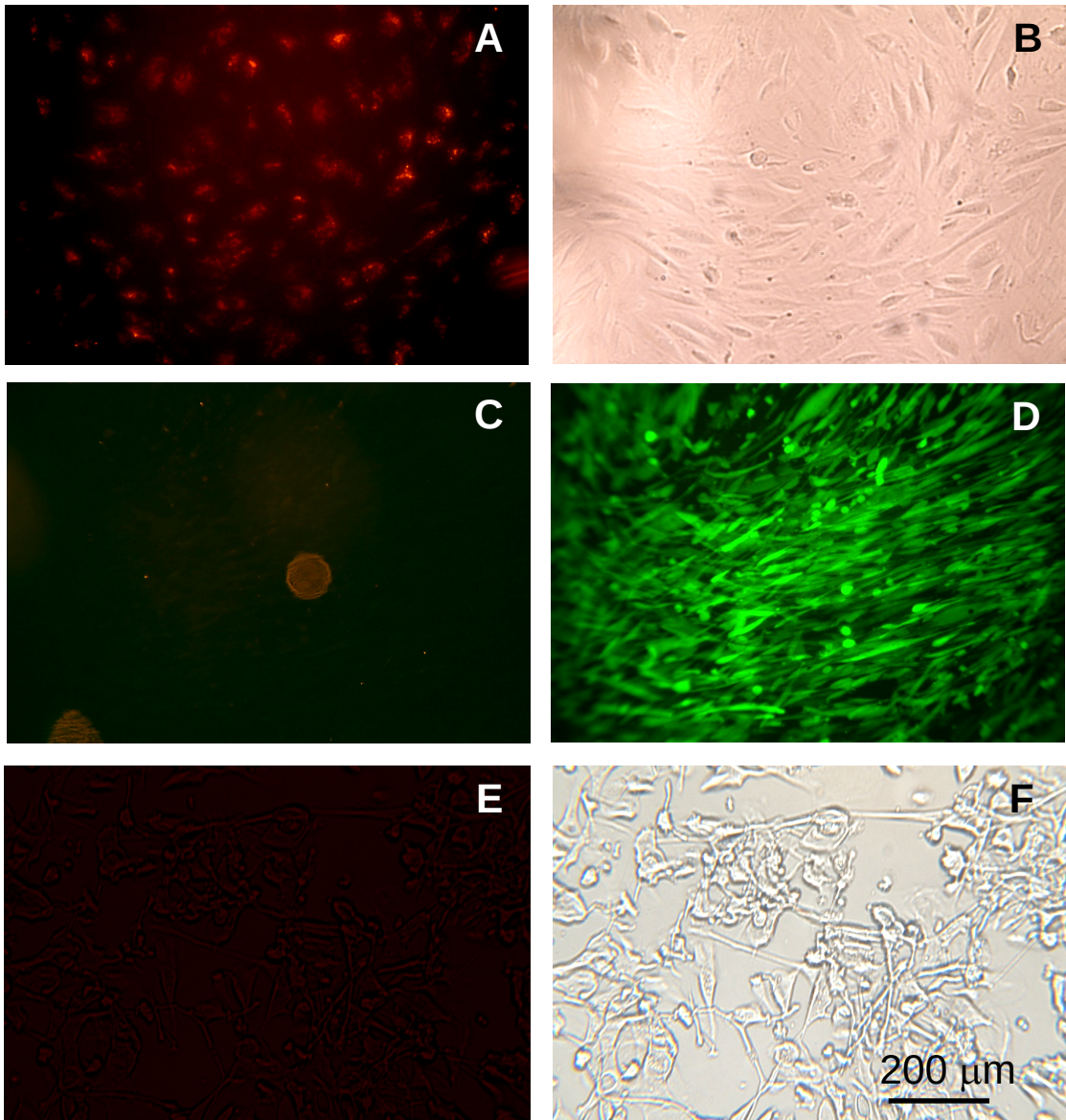


Figure 3-1.

PrTuSCs did not differentiate into endothelial cells by Day 48, based on lack of uptake of acLDL-Dil (E,F). HUVECs (A, B) were positive controls and eGFP HFF (Human Foreskin Fibroblasts, C, D) were negative controls. Pictures on left (A, C, E) taken with Dil filter, B brightfield, D GFP filter, and F phase.

We were not able to differentiate progenitor/stem cells isolated from early primary prostate tumors along an endothelial lineage in this case. However, plasticity of stem cells from prostate cancer in this direction warrants more investigation, especially in view of the occurrence of vascular mimicry in prostate cancer.

Chapter 4: Paracrine Effects of PrTuSC on Endothelial Cells *in Vitro*

4.1 Introduction

Cancer stem cells, cancer cells that can both self-renew and differentiate, were first found in blood cancer (Lapidot, Sirard et al. 1994) but since have been identified in many solid tumors (Quintana, Shackleton et al. 2008), including prostate tumors (Collins, Berry et al. 2005). In theory, a single cancer stem cell could suffice for regeneration of an entire heterogeneous tumor. This has not been done yet for prostate cancer, but an entire prostate has been regenerated from a single murine adult stem cell (Leong, Wang et al. 2008), and many other groups have regenerated prostate tumors from multiple cells. Our collaborators at the Haas lab have isolated Prostate Tumor Stem Cells (PrTuSC) from early prostate tumors, that when engrafted into the prostate of a SCID mouse, can replicate the human tumor there. Cells are required to be within 200 μm or so of a blood vessel to be able to obtain adequate nutrients and oxygen (Folkman 1971). So in order for the rapidly growing tumor to survive, it must somehow stimulate angiogenesis, the formation of new blood vessels, to keep up with its own rapid growth. If PrTuSC are tumor initiating cells, it is of vital interest to know what contribution they might make to the early formation of blood vessels in their vicinity. One way they could do this would be to secrete some protein that stimulates angiogenesis. In this study, we looked to see if prostate tumor stem cells (PrTuSC) made some proteins that are implicated in angiogenesis. We found two (Interleukin-8 and TIMP-1), and we further investigated how these proteins could act on endothelial cells in order to stimulate angiogenesis

4.2. Materials and Methods

4.2.1. Cell Culture

HUVECs (pooled human umbilical vein endothelial cells) were obtained from Lonza (Walkersville, MD) and grown in EGM-2 (Lonza Bio-Whittaker) in tissue culture flasks unless otherwise stated. EGM-2 consists of EBM-2 basal medium to which aliquots of the following are added: 5% FBS, R3-IGF-1, ascorbic acid, gentamicin sulfate, VEGF, hydrocortisone, rhFGF-B, rhEGF, and heparin. Basal medium was the as-bought EBM-2 with 0.5% wt/vol BSA (OmnPur BSA Fraction V, Heat Shock Isolation by EMD) added. Medium was changed every two to three days. Cells were subcultured upon attaining 90% confluence or sooner, and trypsinized using 0.1% Trypsin-EDTA from Invitrogen, and split at a ratio of 1:3. Cells were grown in a humidified incubator at 37°C with 5% CO₂. Passages 4-8 were used for these experiments. Human foreskin fibroblasts (HFF) were a kind gift of the Haas lab. The cells were transfected by the Haas lab to overexpress eGFP. They were grown in DMEM (Gibco 11965-092, Invitrogen Grand Island, NY) with 50 ml FBS (Omega Scientific, Tarzana, CA), 1 ml L-Glutamine 200 mM (Gibco), 5 ml Fungizone (Amphotericin B) (Gibco), 5 ml non-essential amino acids (NEaa) (Gibco) and 2 ml gentamicin (Gemini Bio-Products, West Sacramento, CA) per 500 ml bottle of basal medium. 1x PBS without calcium and magnesium was from Cellgro (Mediatech, Manassas, VA).

Exogenous growth factors used were: TIMP-1 from human neutrophils from Alexis Biochemicals, AXXORA, LLC, San Diego CA, purity >=92% by SDS-page and western blot; rhIL-8 expressed in E. coli, I-1645 Sigma-Aldrich (St. Louis MO); and rhVEGF-165 (100-44), purity >95% from Shenandoah Biotechnology Inc (Warminster, PA).

Conditioned media was obtained from the Haas lab. Upon changing media they simply saved the old media for us instead of throwing it away. Some samples were collected in 2008 from both primary and B+T PrTuSC, and media from primary PrTuSC was collected every day for a period of over two weeks in May-June 2009. The Haas lab changed half of the medium in their PrTuSC cultures every other day, and all of the medium on the next day.

Media was stored separately in 15 ml tubes (by day collected) in a -20°C non-defrosting freezer. The samples collected in 2008 were stored in a -80°C freezer. Media was thawed immediately before experiments, and tubes were rotated gently to mix it.

4.2.2. Angiogenesis Antibody Array

Conditioned media from PrTuSC was assayed for proteins involved in angiogenesis using the TransSignal Human Angiogenesis Antibody Array by Panomics #MA3610 (Fremont, CA).

Conditioned media was collected from the following cells: primary cells were from patients p62 and p74, and B+T cells from patients p9, p12 and p62. B+T cells were engineered by the Haas lab (UCSD) to overexpress Bmi-1 and telomerase. Unconditioned Haas medium was used as a control. The media conditioned by p62 B+T cells was PrEGM-2 (with growth factors added) and PrEBM-2 (basal prostate epithelial growth medium with no growth factors added). PrEB/GM media was conditioned by PrTuSC for three days. All Haas media assayed was conditioned by PrTuSC for one day.

The TransSignal Human Angiogenesis Antibody Array assay consists of a membrane prepared with an array of spots of antibodies to 19 different proteins implicated in angiogenesis: angiogenin, IL-1 α , FGF α , IFN γ , G-CSF, IL-1 β , FGF β , IL-12, HGF, IL-6, TNF α , IP-10, leptin, IL-8, TGF α , TIMP-1, TIMP-2, VEGF, and PlGF. The assay uses the same principle as a sandwich ELISA assay. Each antibody had two replicates on the membrane. When the membrane is incubated with conditioned medium, the proteins in the medium adhere to their respective antibody spots. Next the proteins adhering to the membrane are incubated with a biotinylated secondary antibody known as the detection antibody, and finally with biotin-streptavidin conjugated to HRP, an enzyme that emits a chemiluminescent signal, in order to visualize the bound protein. This assay detects protein with picogram sensitivity.

Conditioned media were thawed, centrifuged, and assayed according to Panomic's instructions. Then membranes were incubated for 5 minutes with ECL+ (Amersham, GE

Healthcare Life Sciences) and imaged using the Storm 840 Filmless autoradiography and chemifluorescence imager by Molecular Dynamics, GE Healthcare. Intensity of pixels in the dots was evaluated using ImagePro software (Molecular Dynamics) and assigned a value between 0 (the value of the negative control spots) and 100 (the value of the positive control spots on the membrane). Media samples were assayed once with 2 replicates.

4.2.3. ELISA

Human TIMP-1 and IL-8 Quantikine ELISA kits were purchased from R&D Systems (Minneapolis MN). Kits were used according to the manufacturer's instructions using conditioned media from PrTuSC. In general, TIMP-1 ELISA was repeated 2-3 times with 2 replications per experiment. IL-8 ELISA was done once with 2 replications per experiment.

4.2.4 Scratch Assay

HUVECs were grown to confluency in 30 mm tissue culture dishes. On Day 0, a straight line was drawn across the outside bottom of the tissue culture dishes with a fine Sharpie pen (Sanford Corporation, Oak Brook IL), and the cells were scratched with a 200 μ l pipet tip perpendicular to the Sharpie line. The cells were then washed twice with PBS, and two ml of the various media samples was added to each dish. Basal medium (EBM-2 with 0.5% BSA) was used as a control. The scratches were photographed using the Sharpie line as a reference, on Day 0, and again on Day 1 (24 hours later). Scratches were photographed using an Olympus E-30 digital camera mounted on an Olympus IMT-2 inverted phase microscope.

Image analysis was done using ImageJ, free software provided by the NIH. The areas of the scratches before and after 24 hours were measured, and this was divided by the length of the region of interest for each scratch to give average scratch widths before and after 24 hours. Then the change in scratch width over 24 hours was divided in two to give the distance cells migrated (from each side).

Conditioned media from two patients (p86 and p88) and basal medium containing 5, 30, 50 or 100 ng/ml of TIMP-1 were used. The results are from one experiment. There were three replicates for the medium with TIMP-1 added and two for the PrTuSC-conditioned medium experiments.

4.2.5. MTS Proliferation Assay

Proliferation of endothelial cells was quantified using the G5421 CellTiter 96TM AQueous Non-Radioactive Cell Proliferation Assay by Promega (Madison, WI) according to the manufacturer's instructions. The assay consists of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS^(a)] and an electron coupling reagent (phenazine methosulfate (PMS)). Dehydrogenase enzymes found in metabolically active cells bioreduce MTS into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan at 490 nm wavelength was measured using an E-max Plate Reader by Molecular Devices (Sunnyvale, CA). The results are directly proportional to the number of living cells in the wells.

First endothelial cell seeding density was calibrated to ensure that absorbance vs. cell density remained within the linear region during the assay. Cells were seeded in a 96 well plate at 1000, 2000, 4000, 5000, 10,000, 20,000, 100,000 and 200,000 cells/well. Two days later cells were assayed using the MTS method. The absorbance was biphasic with a change in slope at initial seeding of 5,000 cells/well. For the region of 1,000-5,000 cells/well (initial seeding) absorbance vs. cell quantity was correlated with a linear fit and R^2 of 0.9908. Since HUVECs have a doubling time of about 30 hours one can assume that two days later, absorbance will still be in the linear range for 2-day cell quantities from about 3,000 to 15,000 cells/well. The cells need to be in the exponential phase of cell growth, and dense enough to give a good signal, but not so dense that contact inhibition will set in during the course of the assay.

HUVECs were seeded into a 96 well plate at 8,000 cells/well in EGM-2 and allowed to attach. Assuming a 30 hour doubling time, this gives a projected cell quantity of about 12,000 cells/well after 24 hours, which is within the linear range. After cells attached, the media was aspirated and 200 μ l of test media was pipetted into each well. The cells were incubated for 24 hours after which 20 μ l of MTS solution was added to each well. Two to three hours later absorbance was read on the plate reader. There were three replications per sample and at least three independent experiments were done in most cases. EBM-2/0.5% BSA (basal medium) was used as a control for experiments with exogenous proteins, and Haas medium, when available, was used as a control for PrTuSC conditioned media.

4.2.6. Tube-Forming Assay

BD Matrigel Matrix from BD Biosciences (Two Oak Park, Bedford MA) was plated in 96-well plates at 55 μ l/well. Human umbilical vein endothelial cells (HUVECs) were seeded in the Matrigel-plated 96 well plates at 15000 cells/well in 100 μ l of EBM-2 (basal medium). The cells were allowed to attach for one hour, then the EBM-2 was carefully pipetted out and EBM-2/0.5% BSA containing the appropriate growth factor or PrTuSC-conditioned medium was added to each well. Cells were incubated 12-16 hours then photographed using an Olympus E-30 digital camera mounted on an Olympus IMT-2 inverted microscope with a 2x objective, which allowed capture of almost the entire well. Tube length was quantified using free NIH software ImageJ by adding the total length of tubes in pixels for each photograph. Each group was replicated two-three times and each experiment was done separately at least twice, unless otherwise indicated.

Data were analyzed using Student's T-Test in Excel and ANOVA in Minitab 16, statistical software from Minitab, Inc..

4.3. Results

4.3.1. Angiogenesis Antibody Array Results

Figure 4-1 shows two typical arrays, with the control (membrane incubated in unconditioned medium) on the left.

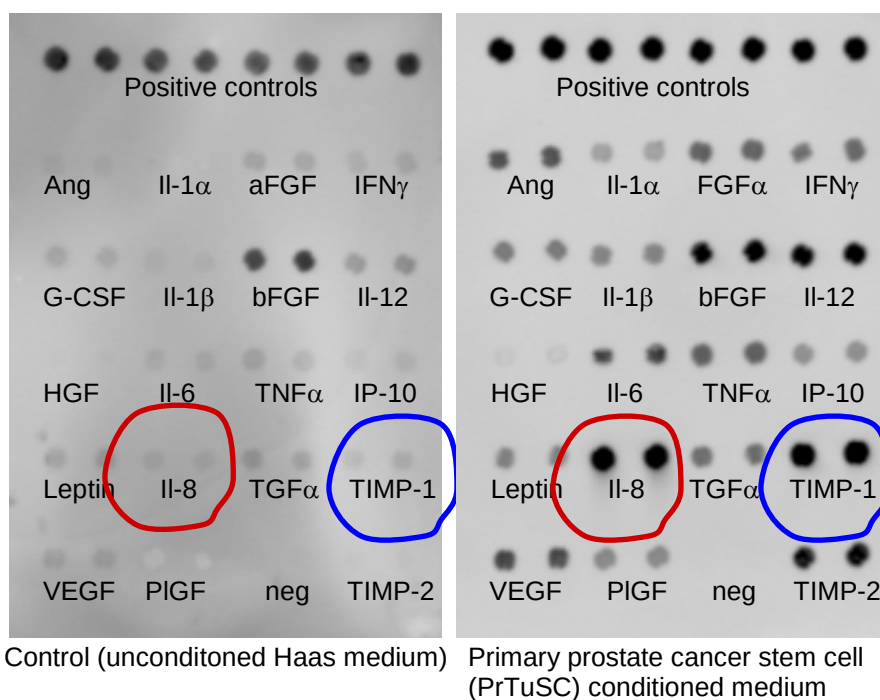


Figure 4-1.

Representative angiogenesis antibody array membranes.

Compared to the control (Haas medium that did not have cells grown in it), PrTuSC expressed Il-8 and TIMP-1 the most strongly. They also expressed VEGF, Angiogenin, Il-6, TIMP-2 and Il-12 in relatively lesser quantities (Figure 4-1). Note that Haas medium already had bFGF added, so this was not a conclusive assay for bFGF. The results shown are only for the primary cells assayed in Haas medium, but they are similar to results for TIMP-1 and Il-8 for the B+T cells. Figure 4-2 shows image analysis relative quantification of the antibody arrays using ImageProPlus software.

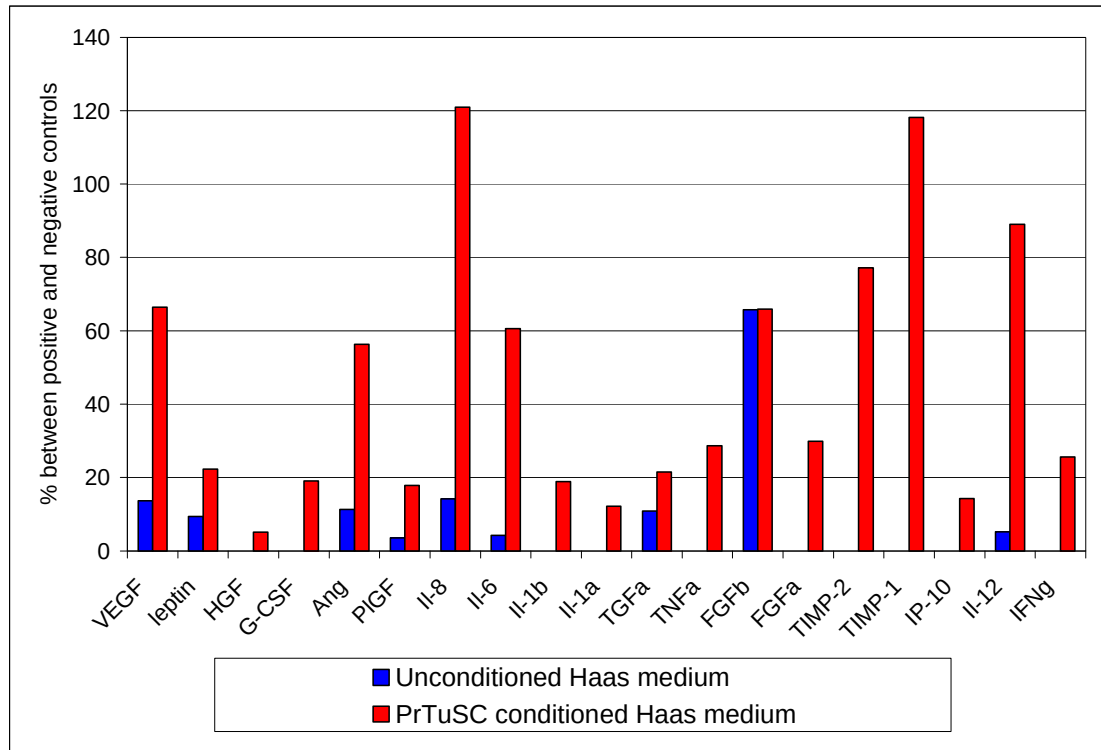


Figure 4-2.

Angiogenesis antibody array results

4.3.2. ELISA Results

The amount of TIMP-1 and IL-8 in conditioned media from PrTuSC was quantified using ELISA (Figure 4-3). ELISA found that all PrTuSC assayed secreted TIMP-1 and IL-8 into their cell culture media. B+T-transfected PrTuSC secreted about 1/3 as much TIMP-1 as the primary cells. There was quite a bit of variation between the patients, with p86, p74 and p76 secreting the highest quantities of TIMP-1. Primary cells contained a mean of 74.7 +/- 48.5 ng/ml TIMP-1 in their conditioned media. Haas medium contained negligible amounts of TIMP-1 and IL-8. Haas medium conditioned by fibroblasts was used as a positive control since fibroblasts are well known to secrete TIMP-1.

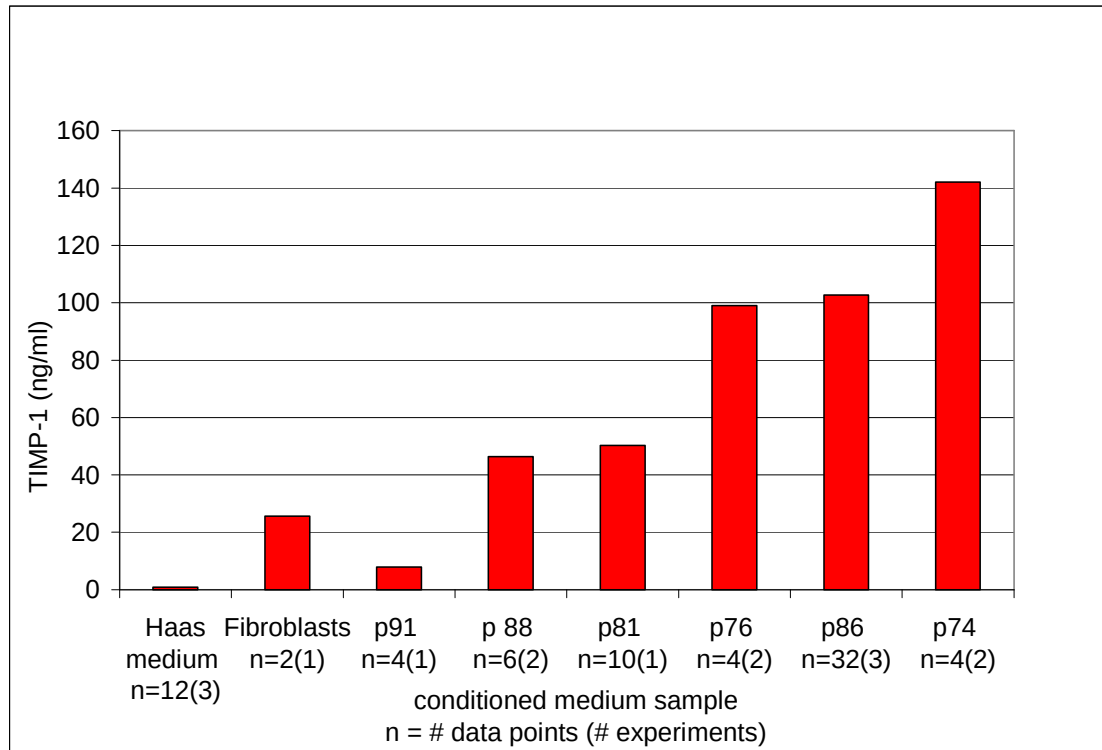


Figure 4-3.

TIMP-1 ELISA of medium conditioned by PrTuSC

All primary PrTuSC assayed also secreted IL-8, but the concentrations were lower than TIMP-1. The mean for all conditioned medium samples assayed was 6.4 +/- 5.0 ng/ml. The same three highest TIMP-1 samples were also the highest IL-8-producing samples (p74, p76 and p86) (Figure 4-4).

4.3.3. Scratch Assay Results

Migration of HUVECs was highest at 100 ng/ml TIMP-1. However none of the TIMP-1 values were significantly higher than basal medium (EBM-2/BSA) (Figure 4-5).

Media conditioned by cells from two patients, p86 and p88, was used. Interestingly, the media from p86, the patient with the highest Gleason score, most proliferation promoting and among the highest TIMP-1 concentrations produced, promoted the most migration of the

PrTuSC conditioned media samples. However none of these values differed significantly from basal medium (EBM-2/BSA).

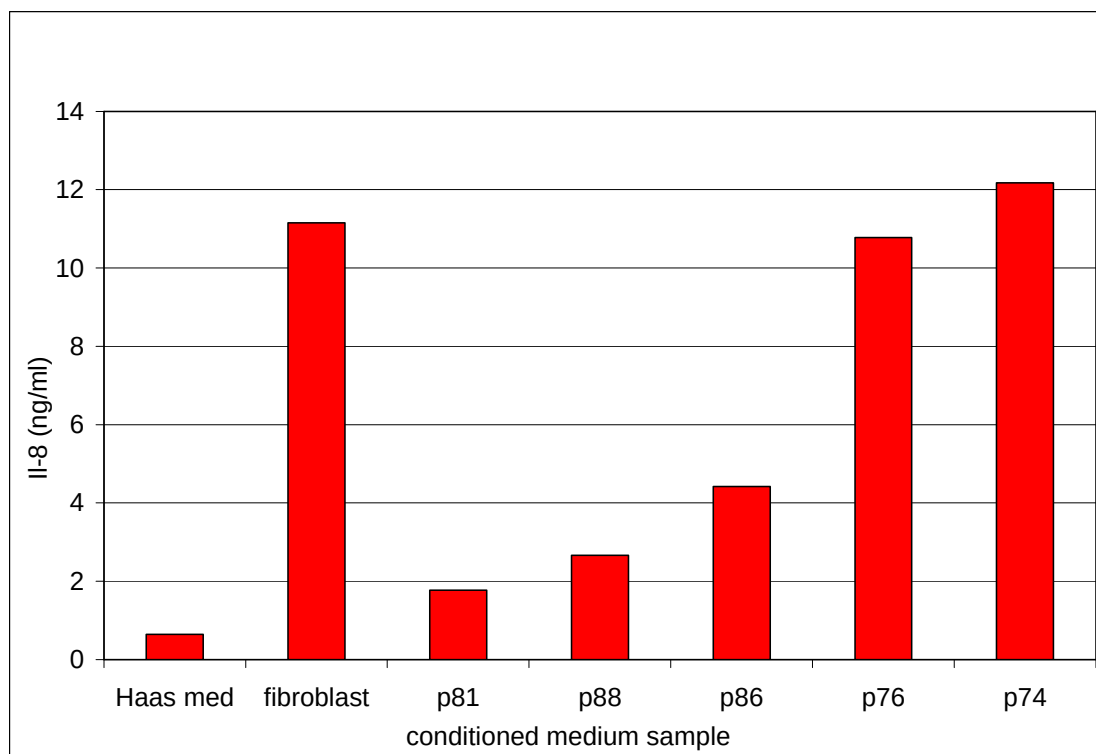


Figure 4-4.

IL-8 ELISA of medium conditioned by PrTuSC. Number of data points = 2 (1 experiment) for all bars except for p81 n=10(1) and p86 n=26(1)

4.3.4. MTS Proliferation Assay

Concentrations of TIMP-1 of 200, 400 and 1000 ng/ml in EBM-2/0.5% BSA basal medium significantly increased proliferation of HUVECs in a concentration-dependent manner. Concentrations below this also increased proliferation but not significantly.

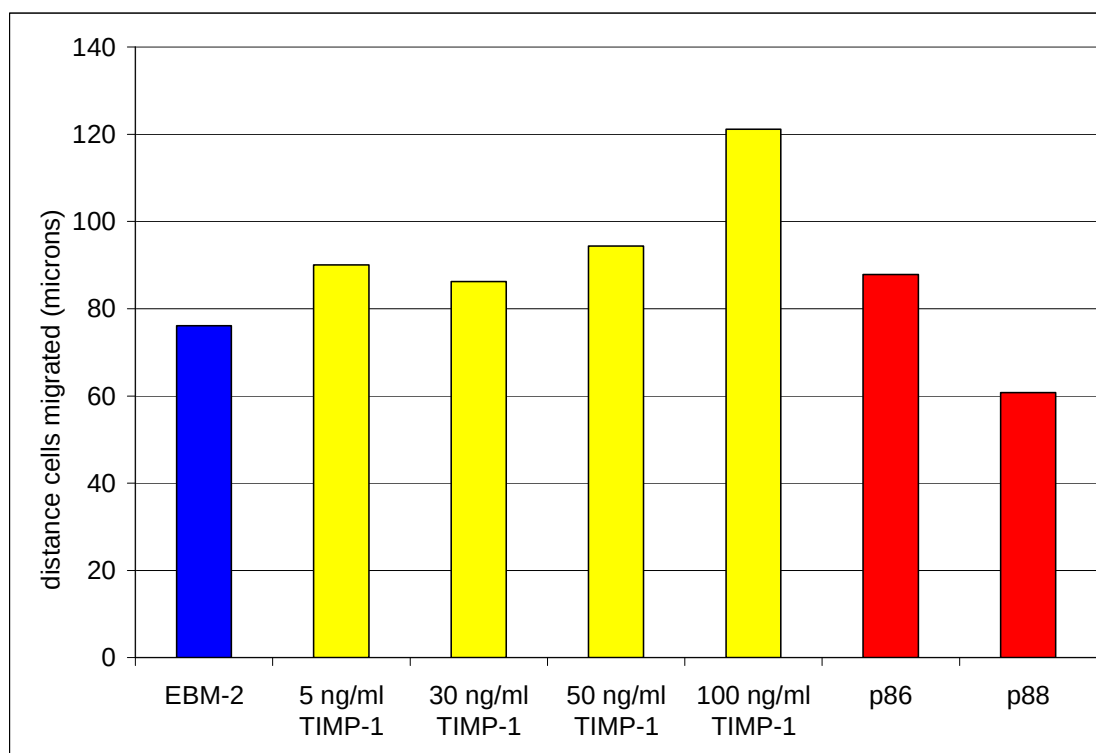


Figure 4-5.

Scratch Assay results; HUVECs grown in basal medium alone (blue), basal medium with exogenous TIMP-1 (yellow), and two PrTuSC conditioned medium samples (p86 and p88, red). n = 3(1) for EBM-2 and TIMP-1 samples; n = 10(1) for p86 and 8(1) for p88.

4.3.4. MTS Proliferation Assay

Concentrations of TIMP-1 of 200, 400 and 1000 ng/ml in EBM-2/0.5% BSA basal medium significantly increased proliferation of HUVECs in a concentration-dependent manner. Concentrations below this also increased HUVEC growth but not significantly.

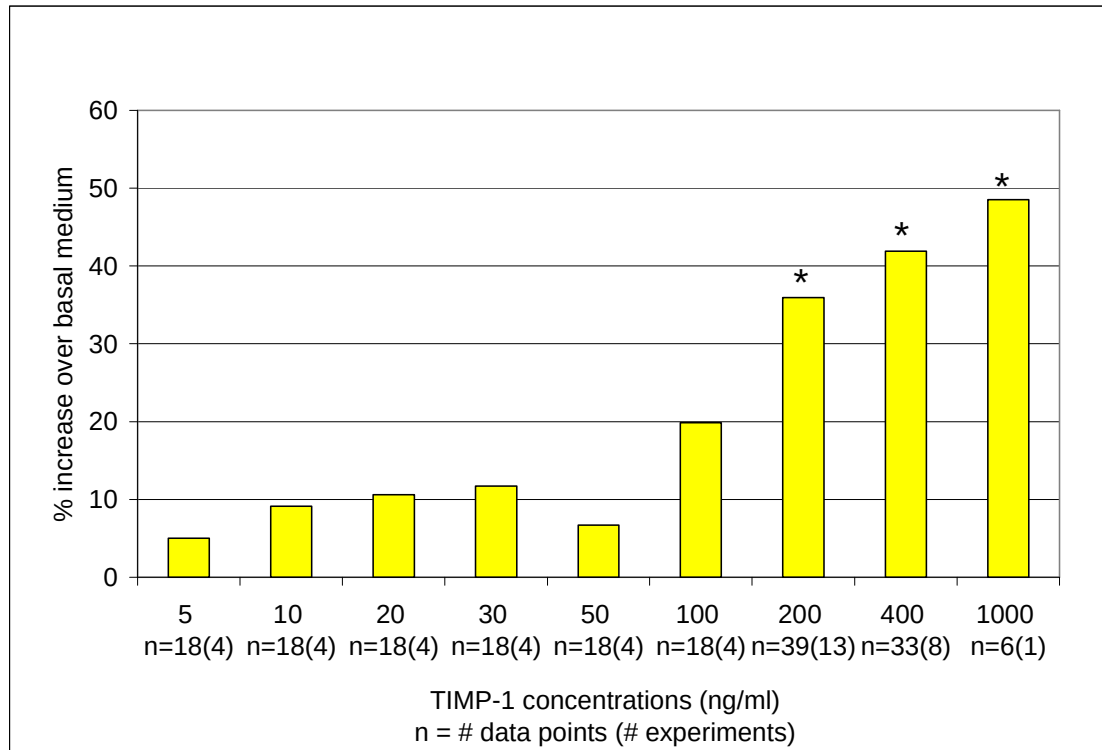


Figure 4-6.

Proliferation of HUVECs in TIMP-1, *p < 0.05.

Addition of exogenous IL-8 and VEGF, which are a well-known angiogenic proteins, to EBM-2/BSA (basal medium) also increased the proliferation of HUVECs (Figure 4-7) as expected. EGM-2 is a commercially available medium that optimizes endothelial cell growth, so this was used as a positive control. It contains growth factors and 5% serum (Figure 4-7).

Media conditioned by PrTuSC from four patients increased proliferation over Haas medium, which is the unconditioned medium or basal medium in this case, to different degrees (Figure 4-8). Only medium from p86 increased proliferation significantly.

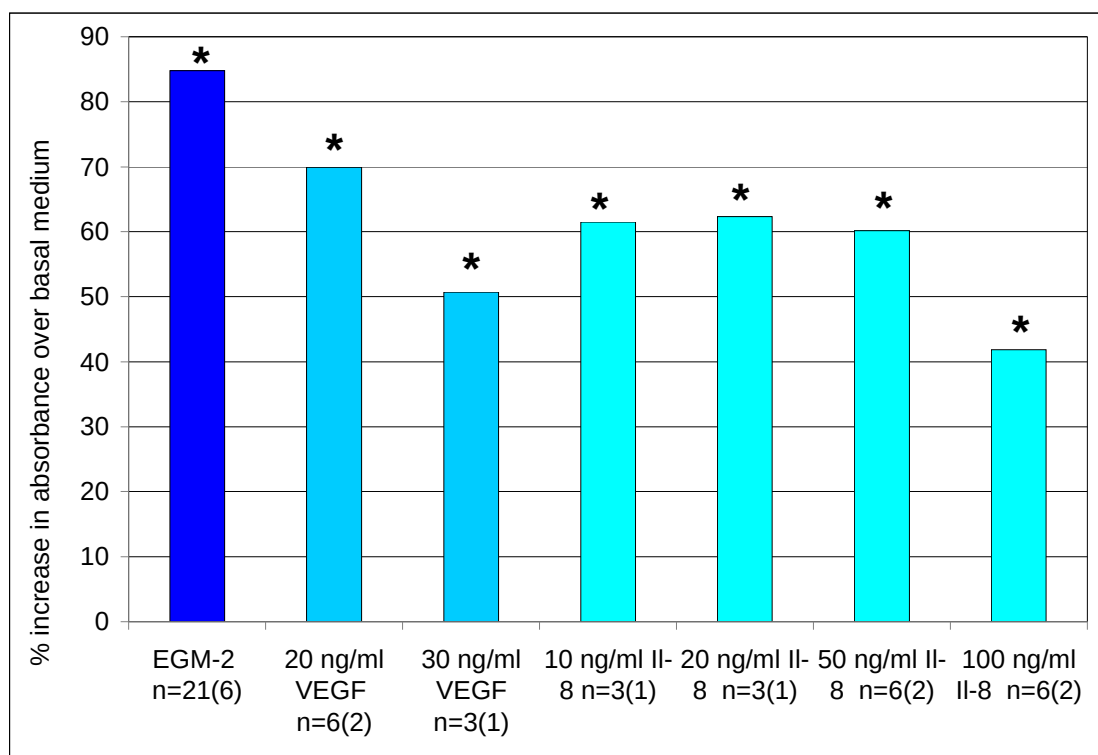


Figure 4-7.

Proliferation of HUVECs in VEGF, IL-8 and EGM-2 medium, positive controls. * $p < 0.05$.

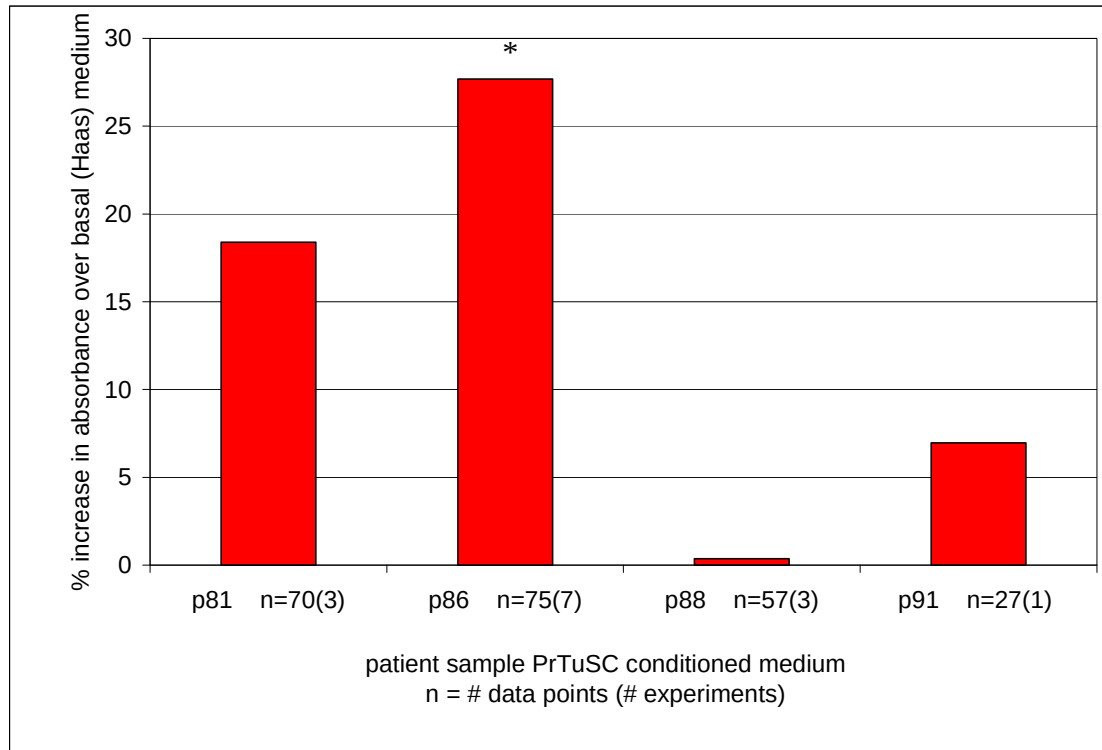


Figure 4-8.

Proliferation of HUVECs in PrTuSC conditioned medium from four patients: p81, p88, p86, and p91. * $p < 0.05$.

4.3.5. Tube Forming Assay Results

All concentrations of TIMP-1 increased tube formation of HUVECs to some degree but none increased it significantly. 400 ng/ml TIMP-1 significantly increased tube formation in preliminary studies, so it was chosen as the concentration to use in all subsequent experiments. Unfortunately, when all the results of preliminary and subsequent experiments were analyzed together this concentration did not result in a significant increase in tube formation.

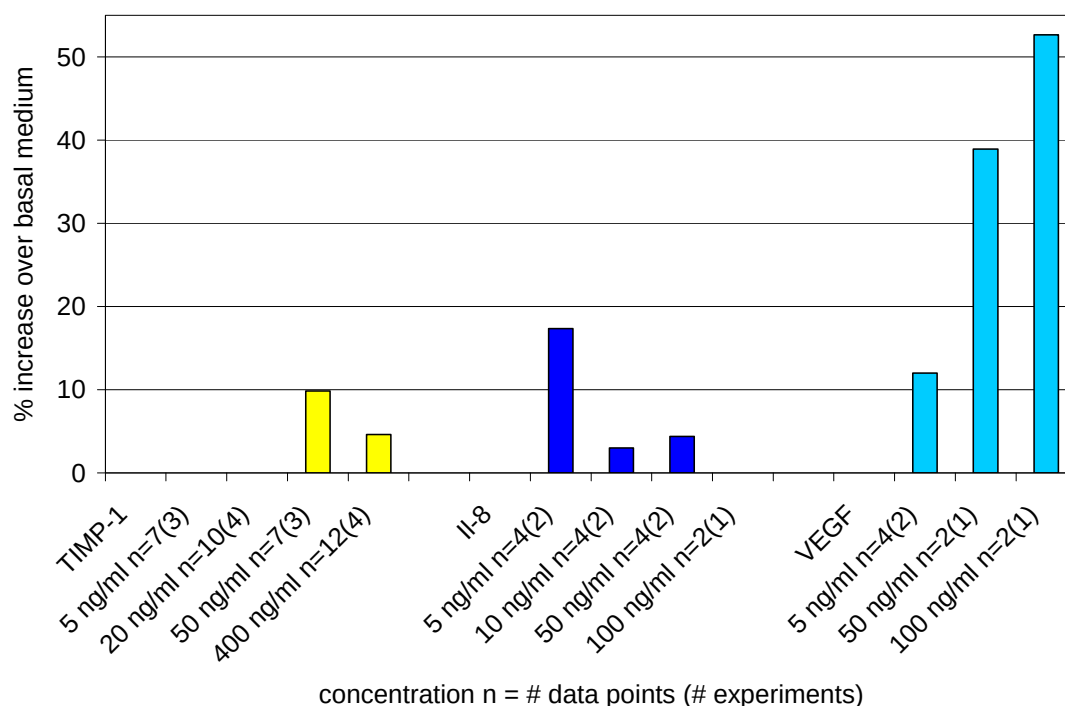


Figure 4-9.

Tubes formed by HUVECs in exogenous proteins TIMP-1, IL-8, and VEGF. VEGF is a positive control and IL-8, which is also known to increase EC proliferation, is another positive control.

Huvecs grown in Matrigel in PrTuSC conditioned medium also did not show a significant increase in tube formation, probably due to extreme variation in the assay. All PrTuSC conditioned media, however, stimulated tube-forming better than unconditioned Haas medium. Medium from patient p81 grew the most tubes (Figure 4-10).

Table 4-1 gives a synopsis of Chapter 4 results, PrTuSC samples used, their Gleason grade and histological description.

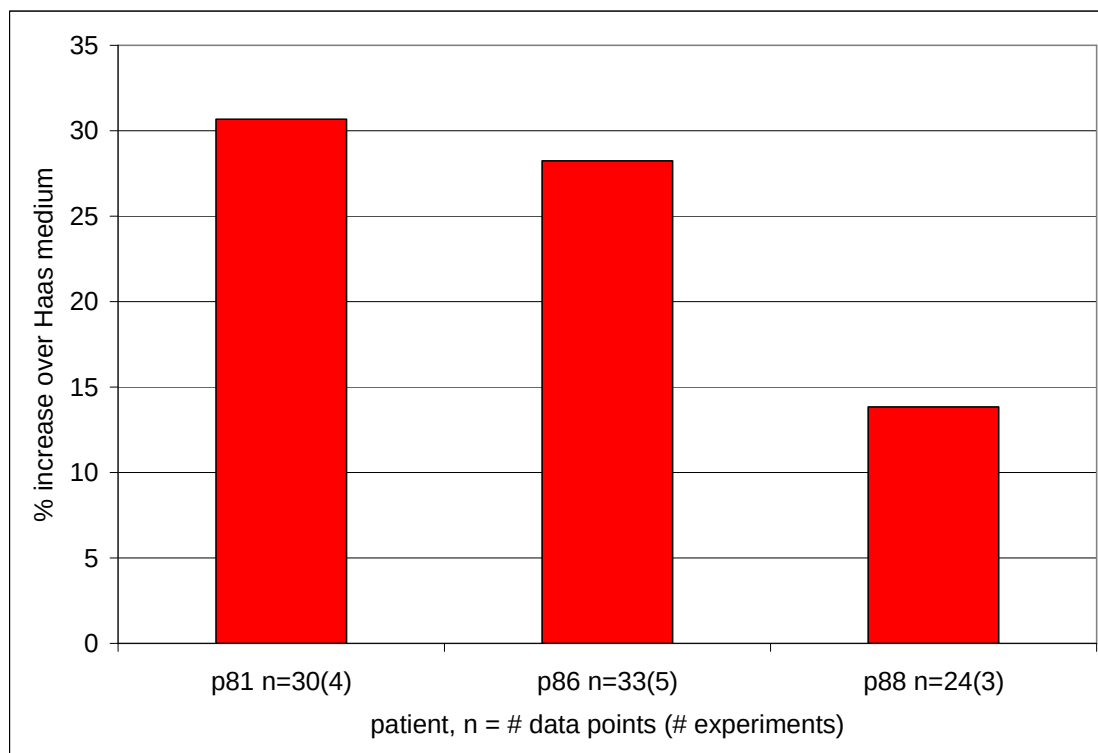


Figure 4-10.

Tubes formed by HUVECs in PrTuSC conditioned media

Table 4-1.

Synopsis of Chapter 4 PrTuSC results.

Patient #	ELISA	ELISA	Scratch Assay	Proliferation Assay	Tube-forming Assay	Cancer Pt 1=Yes, 0=No	Gleason score	Histology notes
	TIMP-1 (ng/ml)	Il-8 (ng/ml)	distance cells migrated (μ m)	% increase in absorbance over basal medium	% increase in length of tubes formed over Haas medium			
p74	142.0	12.2	--	--	--	0	--	glandular and stromal hyperplasia
p76	99.0	10.8	--	--	--	1	6	Adenocarcinoma, small acinar type, high-grade and low-grade prostatic intraepithelial neoplasia, glandular hyperplasia
p81	50.2	1.8	--	--	30.7	1	7	Adenocarcinoma small acinar type
p86	102.7	4.4	87.9	28.4	28.2	1	8	Adenocarcinoma
p88	46.4	2.7	60.8	0.0	13.9	1	7	Adenocarcinoma
p91	8.0	--	--	7.0	--	1	7	Adenocarcinoma

4.4. Discussion

4.4.1. TIMP-1 and Il-8

Our collaborators have isolated stem/progenitor (PrTuSC) cells from early prostate tumors. In looking for possible angiogenic effects of these cells, we discovered by angiogenesis antibody array that they secrete relatively high quantities of TIMP-1 and Il-8 *in vitro*. These results were verified and quantified by ELISA of PrTuSC-conditioned medium for Il-8 and TIMP-1.

For the past several decades Interleukin-8 (Il-8) has been well known and studied as a metastatic and angiogenic protein. Il-8 (CXCR-8) belongs to the superfamily of CXC cytokines, with its gene located on chromosome 4q. It has 72 amino acids, including an ELR+ (Glu-Leu-Arg positive) motif (Araki, Omori et al. 2007). Il-8 has two high affinity cell surface receptors, CXCR1 and CXCR2 (Holmes, Lee et al. 1991). CXCR1 and –2 are G protein coupled serpentine receptors. CXCR1 has greater affinity than –2 for Il-8. Historically, Il-8's main function was chemotaxis, as it was initially known as the neutrophil chemoattractant (Matsushima and Oppenheim 1989). Il-8 has a wide range of proinflammatory effects. In addition, it has various roles in immune surveillance, growth, differentiation, and angiogenesis. It is produced by many cells including lymphocytes, monocytes, fibroblasts, hepatocytes and keratinocytes. Il-8 targets include endothelial cells, liver cells, keratinocytes, macrophages, mast cells, and neutrophils. Endothelial cells make Il-8 and store it in their Weibel-Palade bodies. Il-8 is constitutively expressed by HUVEC's and human dermal microvascular endothelial cells (HDMVECs), which also express CXCR1/2 mRNA and proteins (Li, Dubey et al. 2003).

Il-8 was found to be an autocrine growth factor for many cancer cells, including melanoma cells, human liver and pancreatic cancer cells (Miyamoto, Shimizu et al. 1998). Il-8 is constitutively produced by most metastatic and solid tumors of breast, melanoma, colon, and prostate, primary pancreatic carcinoma and primary glioblastomas (Youngs, Ali et al. 1997; Fasciani 2000; Fujimoto, Sakaguchi et al. 2000; Xie 2001; Huang 2002; Brat, Bellail et al. 2005). It is produced by human ovarian cancer cells (Yoneda, Kuniyasu et al. 1998; Xu and Fidler 2000), prostate cancer cells (Ferrer, Miller et al. 1998), and gastric cancer cells (Kitadai, Haruma et al. 1998; Kitadai, Haruma et al. 2000). Il-8 has been correlated with metastatic potential in human cancers. Endogenous Il-8 also modulated MMP-2, MMP-9, E-cadherin, and VEGF (which are involved in tumorigenicity and metastasis) production in melanoma tumor cells (Luca, Huang et al. 1997; Kim, Uehara et al. 2001) and correlates with

levels of constitutive NF- κ B activity (Huang, DeGuzman et al. 2000). IL-8 was found to induce motility in melanoma and breast carcinoma cells (Youngs, Ali et al. 1997; Xie 2001).

IL-8 is a known promoter of prostate cancer (Izawa and Dinney 2001). In prostate cancer, levels of both IL-8 and its receptors are elevated, resulting in an increase of both autocrine and paracrine signaling, which promote cell proliferation and angiogenesis. IL-8 also augments prostate cancer invasiveness through the regulation of MMP synthesis and secretion. High levels of IL-8 made prostate tumors secrete more MMP-2 and MMP-9, while VEGF mRNA and E-cadherin levels decreased (Murphy, McGurk et al. 2005). Also, gene expression of MMP-9 and IL-8 correlated with metastasis (Greene, Kitadai et al. 1997). Higher levels of osteopontin and IL-8 found in staining of human prostate cancer tissue samples were good indicators of negative prognosis (Caruso, Carmack et al. 2008). IL-8 was expressed by the PC-3 line of human prostate cancer cells and correlated with angiogenesis, tumorigenicity, and metastasis (Kim, Uehara et al. 2001). One study using three prostate cancer cell lines of varying metastatic potential found that all three constitutively produced both IL-8 receptors, but LNCaP and LAPC-4 did not make much IL-8. However the more metastatic line PC-3 made a lot of IL-8 (Araki, Omori et al. 2007). The highly metastatic prostate cancer cell line PC-3M-LN4 overexpressed bFGF, IL-8 and MMP-9 mRNA. The power of IL-8 was shown by an experiment where PC-3M-LN4 cells were transfected to underexpress IL-8, while the weakly metastatic prostate cancer cell line PC-3P was transfected to overexpress IL-8. The two cell lines seemed to trade phenotypes upon this manipulation, with the PC-3P line becoming very metastatic and angiogenic *in vivo*, and the other line becoming less so (Inoue, Slaton et al. 2000).

Androgen and estrogen suppress IL-8 transcription. If androgen levels go down, IL-8 goes up. Increases in serum IL-8 levels can indicate androgen-deprivation resistance and have been correlated to tumorigenicity and metastasis in prostate cancer. IL-8 was found to be undetectable in androgen-positive prostate cancer cells (LNCaP and LAPC-4) but was highly expressed in androgen-independent metastatic cells (PC-3). IL-8-induced cell proliferation mediated through the CXCR1 receptor was independent of AR (the androgen receptor). Also,

exposure of both cells lines to exogenous Il-8 decreased their androgen dependence. Both Il-8 treated and extra-secreting cells made less Prostate Specific Antigen (PSA) and androgen receptors (AR). Il-8 overexpressing cells also made two to three times as much phosphorylated-EGFR, src, Akt, and NF-kB. They were more chemoresistant. Il-8 overexpressing cells had three to five times the mobility, invasion, and MMP-9 (but not MMP-2) and VEGF. They made rapidly growing tumors with higher microvascular density (MVD) and abnormal tumor vasculature. This study found that Il-8 is a molecular determinant of androgen independent prostate cancer growth and progression. However it is unknown if Il-8 is a cause or a consequence of androgen independence and metastatic tumor progression (Araki, Omori et al. 2007). Il-8 was found to be strongly expressed by neuroendocrine tumor cells in androgen-independent prostate cancer. These cells did not express the CXCR1 receptor, but this receptor was upregulated instead on non-neuroendocrine cells in malignant prostate cancers, suggesting an autocrine mechanism to replace some of the growth-stimulating duties of the missing androgen (Huang, Yao et al. 2005). All these data imply that the expression of high levels of Il-8 by PrTuSCs may play a role in progression of androgen-deprivation resistant prostate cancer.

Il-8 causes VEGF and bFGF levels to increase and directly modulates endothelial cell proliferation and migration (Fasciani 2000; Fujimoto, Sakaguchi et al. 2000). Il-8 is constitutively expressed by HUVECs and Human Dermal Microvascular Endothelial Cells (HDMVECs), which also express CXCR1/2 mRNA and proteins. In vitro, human Il-8 caused endothelial cell proliferation and capillary tube organization. Incubation of endothelial cells with Il-8 inhibited endothelial cell apoptosis and increased expression of antiapoptotic gene ratios such as Bcl-x_L:Bcl-x_s and Bcl-2:Bax. Incubation of endothelial cells with 10 and 100 ng/ml Il-8 increased MMP-2/9 protein and mRNA expression (Li, Dubey et al. 2003). Il-8- but not VEGF-mediated angiogenesis was inhibited in a streptozotocin-induced diabetic rat corneal angiogenesis model (Bek, McMillen et al. 2002). Human recombinant Il-8 administered intraperitoneally (IP) was able to induce de novo angiogenesis in a rat mesenteric window

angiogenesis assay. IL-8 also accelerated ongoing angiogenesis in the rat surgical subcutaneous sponge assay (Hu, Hori et al. 1993).

The finding of high levels of IL-8 expressed by PrTuSC gives them something in common with angiogenic, androgen-deprivation-resistant, metastatic forms of prostate cancer even though these cells were isolated from primary tumors. In addition to promoting angiogenesis, IL-8 secreted by PrTuSC could be contributing to development of androgen-deprivation resistance seen in advanced prostate cancer.

TIMP-1 is historically most well known as an inhibitor of metalloproteinases (thus its name) and this simple fact has led investigators to view its main purpose to be inhibition of matrix metalloproteinases. However it also does a great deal of other things, the details of which shall follow, some as a side effect of MMP inhibition but many totally independent of that.

TIMP-1, before it even carried that name, was originally discovered as a growth factor. Erythroid-potentiating activity protein (EPA) was first found as a product of T-lymphocytes that stimulated erythroid precursors and certain cancer cells (Golde, Bersch et al. 1980). Later on EPA was purified (Westbrook, Gasson et al. 1984) and sequenced (Gasson, Golde et al. 1985). A few months later, TIMP-1 was sequenced and found to be identical to EPA (Docherty, Lyons et al. 1985).

TIMP-1 is a 28 kDa (calculated molecular weight 20.685 kDa) glycoprotein in its most commonly found glycosylated form, a member of a family along with TIMPs -2, -3 and -4. All four TIMPs have similar sequence homology and secondary structure. This secondary structure consists of an N-terminal and a C-terminal domain, both of which have six conserved cysteine residues that form three disulfide loops (Figure 4-1). The N-terminal domain inhibits MMPs. The N-terminal domain has an overall structure of a wedge-shaped OB- (oligosaccharide/oligonucleotide-binding fold) which is found in DNA or oligosaccharide-binding proteins. The OB fold consists mainly of beta-sheet structures separated by small flexible loop regions (Westbrook, Gasson et al. 1984). The wedge fits into the catalytic site of

MMPs to inhibit them. The TIMP-1 core protein has 207 amino acids, the first 23 of which comprise a signal sequence that is cleaved upon secretion. TIMP-1 has two conserved *N*-linked glycosylation sites at N30 and N78 (Hayakawa 1994a). TIMP-1 clearance depends on sugar chains linked to these sites. CHO boxes on the diagram indicate the sugar binding sites. Different carbohydrates including sialic acids can bind to TIMP-1 in these two locations, giving it a heterogeneous charge. The C-terminal domain also seems to have many functions that will be discussed shortly.

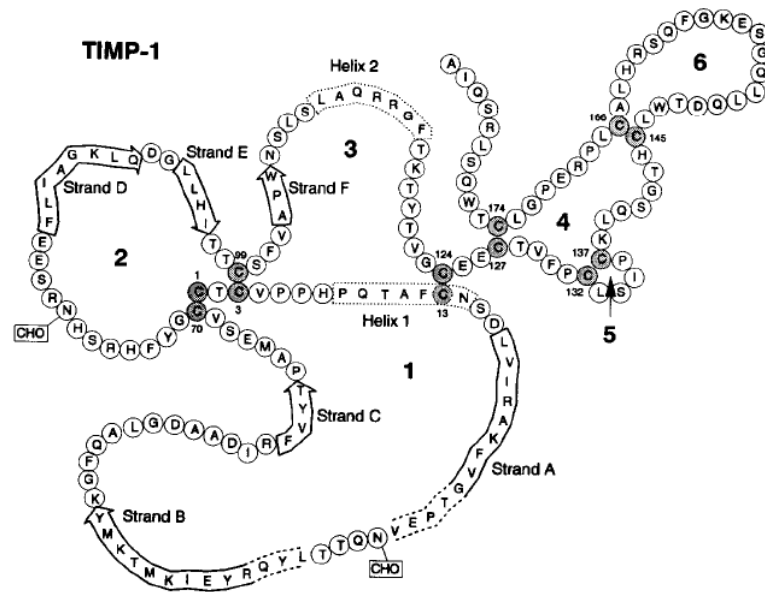


Figure 4-11.

TIMP-1 diagram.

Arrows indicate strands, dotted outlines are the two alpha helices, and CHO labels the two glycosylation sites. (Murphy, Willenbrock et al. 1995).

The gene encoding TIMP-1 is located at the short arm of the X chromosome (Xp11.3-p11.23) nested in the synapsin gene (Docherty, Lyons et al. 1985). TIMP-1 has a 162 bp promoter gene that lacks a classic TATA box but has binding sites for signal transducer and activator of transcription (STAT), Pea3/Ets, AP-1 (Fos/Jun), and AP-2 transcription factors (Edwards, Beaudry et al. 1996; Trim, Samra et al. 2000).

Matrix metalloproteinases (MMPs) are a family of at least 20 zinc-binding proteolytic enzymes that degrade structural proteins of the extracellular matrix (ECM). All MMPs have a

catalytic site in their N-terminal domain. An important role of MMPs is to break up the ECM to allow cells to migrate through it, but MMPs can also cleave other non-ECM molecules including growth factor precursors, binding proteins, cytokines, and cells surface receptors (Sternlicht and Werb 2001). MMPs have five defining features: 1) an active Zn^{2+} site that binds the conserved histidine domain sequence HEXXHXXGXXH for their catalytic mechanism, 2) an 80 amino acid long prodomain containing the consensus sequence PRCXXPD, 3) they are synthesized as an inactive zymogen with aforementioned prodomain attached; 4) they can degrade or cleave at least one ECM protein; and 5) their proteolytic activity is inhibited by TIMPs (Rayment and Upton 2009). Gelatinases A and B (MMP-2 and -9) degrade type IV collagen, which is found in basement membranes. MMP-9 can degrade collagen types I through V, and can also break down the core proteins of proteoglycans and elastin, which are ECM components (Libby and Schonbeck 2001). It is primarily produced by inflammatory cells, such as neutrophils and macrophages, but keratinocytes and many other cells also secrete it (Rayment and Upton 2009).

TIMP-1 non-covalently binds and inhibits MMPs one to one, especially MMP-9, via its N-terminal domain. The rate of ECM turnover is regulated by the activated MMP: free TIMP ratio. As a rule of thumb, all four TIMPs can bind and inhibit almost all MMPs with nanomolar affinity or less, but affinities do vary. TIMP-1 also preferentially forms a 1:1 complex with activated MMP-1, MMP-2 and MMP-3 and forms a complex with proMMP-9 that blocks MMP-9 activation by stromelysins (MMPs -3, -10 and -11). (Goldberg, Strongin et al. 1992). There are thought to be two steps in the inhibition of MMPs by TIMPs. The C terminal domain of TIMP-1 associates with the hemopexin domain of MMPs in the first step of binding, in such a way that the N-inhibiting end of TIMP-1 is properly aligned with the active site of the MMP. This is a relatively fast and reversible step. The second step is slower and results in tighter binding and involves the catalytic site of the MMP (Olson, Gervasi et al. 1997).

Cleavage of ECM proteins by MMPs can have other effects besides loosening up the ECM to allow cell passage. Extracellular matrix (ECM) degrading enzymes can release growth

factors from ECM, and can activate or inactivate growth factors and their cell surface receptors. MMPs can cleave elastin and collagen (two important ECM components) to make endostatin and angiostatin respectively, which are both potent anti-angiogenic factors (Cornelius, Nehring et al. 1998; Wen, Moses et al. 1999).

Since TIMP-1 was secondarily discovered and has been characterized mainly as an MMP inhibitor, investigators want to know if the TIMP-1 effect they are studying is a side-effect of MMP inhibition, or if it occurs independently of MMP inhibition. This can be done by using a synthetic MMP inhibitor such as GM6001. If the synthetic inhibitor of MMPs produces the same effect as TIMP-1 did, it is likely that the effect observed is a side effect of MMP inhibition by TIMP-1. One group found that TIMP-1 and GM6001 both caused proliferation of MDA-MB-435 breast carcinoma cells. Therefore the effect was judged to be dependent on MMP inhibition by TIMP-1 (Porter, Shen et al. 2004). Researchers have engineered TIMP-1 molecules with a mutation or alkylation in their MMP binding sites, or have removed the binding site, so that these modified TIMP-1s will not inhibit MMPs. If the altered molecule also reproduces the effect, then it is probable that this effect occurs independently of inhibition of MMPs by TIMP-1. For example, a non-MMP-inhibiting TIMP-1 mutation still promoted growth of erythroid precursor cells (Chesler, Golde et al. 1995; Chesler, Golde et al. 1995), and TIMP-1 with alkylated sulfhydryl groups could not inhibit MMP's but it could stimulate growth in Raji cells (Hayakawa, Yamashita et al. 1992). Many effects of TIMP-1 have been found to be independent of MMP inhibition including proliferation and inhibition of apoptosis (Stetler-Stevenson 2008).

Therefore TIMP-1 seems to be a multifunctional protein that can act directly (probably by activating a cell signaling pathway, most likely initiated through cell surface receptors) or indirectly through modulation of protease activity.

A vast array of cells secrete TIMP-1 *in vitro*. These include fibroblasts, keratinocytes (Bertaux, Hornebeck et al. 1991), haematopoietic cells (Marquez-Curtis, Dobrowsky et al. 2001), polymorphonuclear leukocytes (Triebel, Blaser et al. 1995), megakaryocytes and

platelets (Cooper, Eisen et al. 1985), monocytes and macrophages (Opdenakker, Masure et al. 1991), epithelial cells, endothelial cells, osteoblasts, chondrocytes, smooth muscle cells, plus many tumor cells (Hayakawa 1994a). Normally, TIMP-1 is not expressed constitutively. However human rheumatoid synovial fibroblasts seem to express it constitutively (Hanemaaijer, Koolwijk et al. 1993), so constitutive expression can be seen in disease states. MCF7 breast carcinoma cell line but NOT non-neoplastic breast epithelial cells were found to secrete TIMP-1 (Yoshiji, Kuriyama et al. 2002). TIMP-1 was made by astrocytes during reactive gliosis (Wilczynska, Gopalan et al. 2006). TIMP-1 is thought to contribute to fibrosis by its inhibition of MMPs and prevention of apoptosis. TIMP-1 is upregulated in fibrotic conditions of the lung (Ruiz, Ordonez et al. 2003; Hajj, Lesimple et al. 2007), liver (Roeb, Behrmann et al. 2000; Murphy, Waung et al. 2004; Roderfeld, Geier et al. 2006), and kidney. TIMP-1 upregulation is believed to slow down recovery from liver fibrosis (Iredale, Benyon et al. 1998). TIMP-1 has been found in tissue explants including aorta, cartilage, embryonic bone, tendon, dental pulp, gingival, synovium, and uterus (Hayakawa 1994a). TIMP-1 is found in nearly every body fluid (Hayakawa 1994a). TIMP-1 is commonly found in reproductive organs (Fedorcsak, Polec et al.; Boujrad 1995; Robinson, Sznajder et al. 2001).

TIMP-1 and IL-8 were the most strongly expressed proteins on the angiogenesis array. ELISA further quantified these two proteins. We found that conditioned media from PrTuSC contains an average of 74.7 ± 48.5 ng/ml TIMP-1 and 6.4 ± 5.0 ng/ml. It should be noted that other proteins such as VEGF, TIMP-2, and IL-12 were also expressed by PrTuSC and they may also have important effects on angiogenesis, but they were not specifically addressed in this study.

Cells transfected to overexpress Bmi-1 and TERT (PrTuSC B+T) expressed about 1/3 as much TIMP-1 as primary cells did. Therefore even though B+T cells are more durable in cell culture and in *in vivo* experiments, they may not be a very good tool to study TIMP-1 effects.

4.4.2 Proliferation induced by TIMP-1 and IL-8

In order to grow new blood vessels from existing, it is fairly obvious that new endothelial cells will be required. Therefore endothelial cell proliferation is a prerequisite part of angiogenesis. It is thought to be one of the earliest steps in angiogenesis. Possibly TIMP-1 could be contributing to angiogenesis by stimulating proliferation of endothelial cells. Past study on this topic has not been extremely consistent. TIMP-1 stimulation of proliferation seems to depend on cell type.

In one study, 100 ng/ml TIMP-1 stimulated endothelial cell proliferation (Hayakawa, Yamashita et al. 1992). Another study found that while TIMP-2 inhibited EC proliferation in a concentration-dependent manner, TIMP-1 did not (Murphy, Unsworth et al. 1993). TIMP-1 (at concentrations of 33-100 ng/ml) increased proliferation of scleroderma fibroblasts (Kikuchi, Kadono et al. 1997). TIMP-1 also increased proliferation of cells in a mixed neuroglial culture and also in primary cultured astrocytes (Hernandez-Guillamon, Delgado et al. 2009); keratinocytes (Bertaux, Hornebeck et al. 1991) and many other cell types (Hayakawa, Yamashita et al. 1992).

Smooth muscle cells associate with arteries by surrounding and sharing a common basement membrane with the endothelial cells. Therefore TIMP-1 could promote stabilization of new arteries by promoting proliferation of smooth muscle cells. Concentrations of 100 and 500 ng/ml but not 20 ng/ml TIMP-1 increased proliferation of SMC significantly. This effect did not depend on MMP inhibition (Akahane, Akahane et al. 2004). Another study, however, found that TIMP-1 did not affect SMC proliferation (Baker, Zaltsman et al. 1998). TIMP-1 effect on SMC was not examined in our study.

Results have also been mixed for epithelial cell proliferation. Fata et al found TIMP-1 decreased epithelial cell proliferation (Fata, Leco et al. 1999), while 50 and 100 ng/ml TIMP-1 stimulated proliferation in rabbit corneal epithelial cells *in vitro* (Saika, Kawashima et al. 1998).

TIMP-1 also has growth factor-like activity for a variety of neoplastic cell types *in vitro* including MCF7 (a breast carcinoma cell line) (Hayakawa, Yamashita et al. 1992; Yoshiji,

Harris et al. 1998). Another study found that neither overexpression of TIMP-1 in vitro or exogenous rTIMP-1 (100 ng/ml) could increase proliferation of MCF7; only in vivo did TIMP-1 over-expressing MCF7 increase tumor size, which is interesting because most studies found that TIMP-1 decreases tumor size (Yoshiji, Harris et al. 1998). TIMP-1 overexpressing BEL-7402 hepatocellular carcinoma cell line reduced proliferation by 40% and migration by 70% (Guo, Shen et al. 2007). Porter et al found that TIMP-1 caused proliferation of MDA-MB-435 breast carcinoma cells in an MMP-dependent fashion (Porter, Shen et al. 2004). Luparello et al developed two human clonal breast carcinoma cell lines, BC-3A and BC-61, BC-61 being the more tumorigenic of the two. Both lines secreted TIMP-1 but only BC-61 responded to doses of 40, 100 or 1000 ng/ml TIMP-1 with proliferation (Luparello, Avanzato et al. 1999). TIMP-1 concentrations of 10-1000 ng/ml increased proliferation of Burkitt lymphoma cell line Raji cells over serum-free basal medium, but not as much as medium with 10% FCS. Serum is known to contain TIMP-1 (Hayakawa 1994a).

In this research, exogenous TIMP-1 promoted endothelial cell proliferation significantly at concentrations of 200, 400 and 1000 ng/ml (Figure 4-8). At a concentration of 1000 ng/ml TIMP-1 was able to increase proliferation of HUVECs by nearly 50% above that of cells grown in basal medium. These concentrations are in line with the literature reported above. To assay proliferation of HUVECs in PrTuSC conditioned media, we collected media from a mixed population of cells, which consisted of a stem cell center surrounded by a corolla of transit amplifying cells. Since their proportion increased as time in cell culture progressed, we were collecting and assaying mainly proteins being made by the transit amplifying cells, with perhaps a small contribution by the stem cells themselves. However, since the transit amplifying cells are the rapidly proliferating cells, then these are the cells that blood vessel growth has to keep up with, and the effect of these cells on blood vessel growth is of vital interest. P86 PrTuSC-conditioned medium stimulated the most proliferation of all the patient samples, with an increase of almost 30% over basal medium. These data were compared with proliferation increases due to stimulation by two known angiogenic proteins, IL-8 and VEGF. 20

ng/ml VEGF increased proliferation by 70% over basal medium, and 20 ng/ml IL-8 increased it by about 63%. EGM-2 is a commercial endothelial cell growth medium that has been optimized for cell proliferation. It stimulated HUVECs to grow ~85% more than basal medium. Therefore while the stimulation of HUVEC proliferation by TIMP-1 did not exceed these positive controls, it was not far below them either (Figures 4-8 and 4-9). GM6001, an MMP inhibitor, abrogated TIMP-1-induced proliferation of HUVECs (data not shown). Therefore this effect is not a side-effect of TIMP-1 inhibition of MMP, but apparently MMPs must be present and active for the proliferative effect of TIMP-1 to occur.

4.4.3. TIMP-1 effect on endothelial cell migration

It is critical in angiogenesis for endothelial cells to be able to migrate and to communicate with each other and with the extracellular microenvironment, and also to rearrange their structure in order to migrate and reassemble into tubes. Possible roles of TIMP-1 in these functions will be discussed below.

In physiological conditions, TIMP-1 is basically thought to inhibit cell migration since it inhibits MMPs, and MMPs are necessary to loosen cell-cell junctions, basement membrane and the extracellular matrix so endothelial cells can get through. For example, when the TIMP-1 gene was disrupted in murine embryonic stem cells partially differentiated along a mesenchymal lineage, increased cell invasiveness of basement membrane resulted. Addition of exogenous TIMP-1 (10 ug/ml) reversed this effect (Alexander and Werb 1992). Migration of human granulocytes through Collagen IV and basement membrane was significantly inhibited by 20, 100 and 500 ng/ml exogenous TIMP-1 (Chromek, Tullus et al. 2004).

HUVECs are endothelial cells from a large vein, while angiogenesis occurs in the microvasculature, probably mainly by sprouting from venules (Denekamp 1990). Endothelial cells isolated from these two different locations are known to have different phenotypes (Langenkamp and Molema 2009). However HUVECs are often used and, in most cases, are a valid model for angiogenesis *in vitro*. In the literature, experiments examining the effect of

TIMP-1 on both micro-and macro-vascular endothelial cells obtained the same results for both EC types. For example, when both HUVECs and human fat microvascular endothelial cells (HFECs) were used in tandem, TIMP-1 (concentrations ~ 200, 600 and 2000 ng/ml) inhibited tube formation of both HUVECs and HFECs in Matrigel similarly (Schnaper, Grant et al. 1993). The authors believed this inhibition was due to inhibition by TIMP-1 of effects of MMP-9 on Collagen IV in the Matrigel. Adipocyte-conditioned medium and FGF stimulated and TIMP-1 inhibited migration of both HUVECs and BCE (bovine capillary endothelial cells) similarly in a Boyden chamber migration assay. Concentrations greater than 10 ng/ml TIMP-1 inhibited both HUVEC and BCE migration. Both endothelial cell types behaved the same in tube-forming assays as well (Grant, Lelkes et al. 1991). A study comparing TIMP-1 secretion by FSE (human foreskin microvascular endothelial cells) and HUVECs found that upon stimulation, FSE secreted substantially more TIMP-1 than HUVECs (Jackson and Nguyen 1997). Since we studied cell response to TIMP-1 stimulation, not TIMP-1 expression, in endothelial cells, it was almost better to use cells whose TIMP-1 secretion was minimal so that stimulation quantities could be known and controlled. Therefore only HUVECs were used in these experiments. In addition, they are also less expensive and easier to grow than microvascular endothelial cells.

In the human testis, tight junctions between Sertoli cells and the ECM must be broken to allow spermatocytes to migrate through the blood-testis barrier. Dynamically regulated secretion of various proteins by Sertoli cells allowed this to happen. $\text{TNF}\alpha$ was found to upregulate production of Collagen IV, MMP-9, and TIMP-1. It was thought that first the MMP-9 was upregulated to perturb the ECM and tight junctions to allow the spermatocytes to migrate through, followed by upregulation of Collagen IV, to rebuild the ECM, and of TIMP-1 to inhibit the MMP-9 while the ECM was being reconstructed (Siu, Lee et al. 2003). Therefore it is simplistic to say that TIMP-1 is anti-migratory, since in a natural process the ECM and cell-cell junctions need to be reconstructed after migration and TIMP-1 may be important for this. Since migration of endothelial cells is essential to angiogenesis, the role of TIMP-1

immediately after migration is also probably essential to angiogenesis although its overall role in most experiments to date has been anti-migratory, perhaps due to the fact that many of the experiments did not consider dynamics or extremely localized effects.

In the scratch assay, which assays cell migration, media conditioned by cells from two patients, p86 and p88, was used. Interestingly, the medium from p86, the patient with the highest Gleason score, most proliferation promoting and among the highest TIMP-1 concentrations (Table 4-1), promoted the most migration. However this result was still not significant. 100 ng/ml TIMP-1 caused HUVECs to migrate at least 1/3 farther than basal medium, but again results were not significant.

4.4.4. TIMP-1 and angiogenesis

Endothelial cells are not growing in a vacuum, they are bordered first by the basement membrane (which is mainly type IV collagen, laminin and fibronectin) and beyond that by an extracellular matrix composed of collagen fibrils, elastin, microfibrillar proteins, proteoglycans and more. In order for blood vessels to sprout, there must be a break in the basement membrane. Next the cells need a passage through the extracellular matrix so they can migrate out, proliferate, and reassemble into new blood vessels. Matrix metalloproteinases break up the basement membrane and ECM to give EC this required access. Therefore TIMPs are believed to primarily inhibit angiogenesis by inhibiting MMPs.

Liu et al found that fibroblasts potentiate blood vessel formation partially through secreted TIMP-1. Fibroblast-conditioned medium and fibroblast/HUVEC cocultures encouraged tube formation in a 3D angiogenesis assay. Fibroblasts (and myofibroblasts (Still, Robson et al. 2000)), but NOT endothelial cells, secreted TIMP-1 in this study. Exogenous TIMP-1 also increased vessel assembly. The fibroblast-angiogenesis-enhancing activity of TIMP-1 was found to be MMP dependent, as chemical inhibition of MMP-2/9 also increased angiogenesis. Addition of exogenous MMP-9 blocked the enhancing effect of TIMP-1. Therefore the mechanism was found to be dependent on the inhibition of MMP-9 by TIMP-1.

Endothelial cells cultured in a Matrigel tube forming assay in 30 ng/ml VEGF with 200 ng/ml TIMP-1 or 400 ng/ml TIMP-1 increased angiogenesis by 24% and 46% respectively. The authors found some variability in the TIMP-1 lots, but overall the effect was dose-dependent. Exogenous TIMP-1 obtained two-fold less results than conditioned medium from the fibroblasts however. The authors believe that this effect might involve TIMP-1 inhibition of formation of tumstatin, an anti-angiogenic collagen IV fragment produced by cleavage by MMP-9 (Liu, Chen et al. 2008).

In addition to production of tumstatin from collagen IV, MMPs can also cleave elastin and collagen (two important ECM components) to make endostatin and angiostatin, two anti-angiogenic proteins, respectively (Cornelius, Nehring et al. 1998; Wen, Moses et al. 1999). In doing so, MMPs can be anti-angiogenic. It was thought that TIMP-1 might contribute to angiogenesis by preventing MMP-cleavage of ECM to produce anti-angiogenic proteins. An interesting study using renal and colon carcinoma cells, however, found that if this cleavage effect is activated, cells increase cell-specific growth factor production to override angiogenesis inhibition by ECM-product proteins (Fernando, Koch et al. 2008).

The angiogenic response to conditioned media from adipocytes (known to be angiogenic cells) and bFGF was blocked by TIMP-1, both *in vivo* in the rat corneal assay, and *in vitro* using HUVECs and bovine capillary endothelial cells. Both types of endothelial cells responded similarly to TIMP-1 concentrations. They think TIMP-1 inhibited angiogenesis in part by inhibiting migration. Migration *in vitro* was inhibited at concentrations of TIMP-1 greater than 10 ng/ml (Johnson, Kim et al. 1994).

In another study, PVA sponges containing a blocking antibody to TIMP-1 were implanted in mice, followed by injection of more blocking antibody into the sponge every two to three days. This doubled the amount of angiogenesis in the sponge without increasing proliferation of cells that migrated into the sponge. *In vitro* TIMP-1 inhibition also increased migration; EC *in vitro* changed to a migratory phenotype when TIMP-1 was blocked. Endothelial cells treated with TIMP-1 blocking antibody exhibited an invasive morphology.

Basically the authors believe that TIMP-1 inhibits angiogenesis by blocking migration but it does not affect proliferation either way (Reed, Koike et al. 2003).

TIMP-1 was found to increase the concentration of soluble VEGFR-1 in HUVECs *in vitro*. sVEGFR-1 critically regulates human ovarian angiogenesis by competitively binding VEGF. TIMP-1 caused HUVECs to make more sVEGFR-1 *in vitro*. SiRNA depletion of TIMP-1 resulted in lowering of sVEGFR-1 levels. VEGFR-1 (not soluble) is a protein that is expressed by cells in the premetastatic niche. In this experiment, however, levels of the soluble version of VEGFR-1 were increased by TIMP-1 and competitive binding of VEGF resulted in a net decrease in angiogenesis (Bruegmann, Gruemmer et al. 2009).

Commercially available glycosylated rTIMP-1 inhibited capillary tube formation *in vitro* (Yoshiji, Harris et al. 1998).

In view of all these results, it is not too surprising that TIMP-1 did not significantly promote tube formation of HUVECs in our experiments. The tube assay is one of the best functional *in vitro* angiogenesis assays. The results of the tube assay, when all gathered together, however, were not significant. Differences in the averages could be observed, with 50 ng/ml TIMP-1 resulting in almost 10% greater tube formation, but due to huge variations in the data, significance could not be achieved. The same applied to tube formation as stimulated by PrTuSC conditioned media: tube formation of HUVECs in conditioned media increased, but not significantly.

TIMP-1 and Il-8 were expressed by prostate tumor stem/progenitor cells from unmetastasized tumors. Il-8 is a well-known angiogenic protein. TIMP-1 was found to stimulate proliferation of endothelial cells. Only media conditioned by PrTuSC from patient p86 increased HUVEC proliferation significantly. Media conditioned by PrTuSC increased migration and tube formation, albeit not significantly. Therefore these proteins produced by PrTuSC could be contributing to angiogenesis, but probably not by promoting endothelial cell migration or assembly into tubes.

Chapter 4, in part, is currently being prepared for submission for publication of the material. Frimodig, Jane; Gough, David. The dissertation author was the primary investigator and author of this material.

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Chapter 5: TIMP-1 Levels in the Literature

Although TIMP-1 has been found by many studies to be anti-cancer, its blood and tissue levels seem to increase with cancer progression and have been correlated with negative prognosis in many cancers (Table 5-1).

Elevated TIMP-1 expression has generally been correlated with poor prognosis of breast cancer. High levels of TIMP-1 in tissue extracts from primary breast cancer were significantly associated with poor prognosis (McCarthy, Maguire et al. 1999; Schroh, Christensen et al. 2003; Schroh, Meijer-van Gelder et al. 2006). In breast cancer tissue, TIMP-1 was expressed in ECM, cancer cells, and by fibroblasts at the invasive front (Egeblad and Werb 2002). TIMP-1 mRNA levels correlated with the occurrence of distant metastases in breast cancer (Ree, Florenes et al. 1997). A study of invasive breast carcinomas found that TIMP-1 mRNA levels in tumor tissue correlate with poor outcome (Nakopoulou, Giannopoulou et al. 2002). Plasma TIMP-1 was correlated with poor prognosis especially for node-negative breast cancer (Wurtz, Moller et al. 2008). In a different study, high concentrations of TIMP-1 mRNA were correlated with positive outcome, while high concentrations of TIMP-1 protein are generally correlated with poor prognosis. Therefore mechanisms of post-transcriptional TIMP-1 processing bear closer examination (Sieuwerts, Usher et al. 2007).

In gastric cancer, TIMP-1 was expressed by cancer cells and also by inflammatory cells in cancer infiltrates. The latter correlated strongly with negative prognosis (Mroczko, Lukaszewicz-Zajac et al. 2009).

Both in-situ hybridization of colorectal cancer tissue samples for TIMP-1 mRNA and antibody protein staining detected TIMP-1 positive fibroblast-like cells at the invasive front of the tumors. TIMP-1 was not found in benign or malignant epithelial cells (in contrast to breast cancer), nor in vascular or smooth muscle cells (Holten-Andersen, Hansen et al. 2005). This

contrasts with the findings of elevated TIMP-1 levels in serum and plasma of colorectal cancer patients (Table 5-1).

MMPs in the blood are preferential inactivated by the protein α 2-macroglobulin (Woessner and Nagase 2000). TIMP-1, as its name implies, is the resident tissue inhibitor for MMPs. However, serum and plasma levels of TIMP-1 have been found to be elevated in many disease states (Table 5-1). Blood levels of TIMP-1 may not be directly related to levels of TIMP in tissue, and it is difficult to determine the origins of TIMP-1 in the blood.

In most cancers, serum or plasma TIMP-1 levels were higher in cancer patients than in normal healthy controls (Table 5-1). Two exceptions are TCC, transitional cell bladder cancer (Staack, Badendieck et al. 2006), and the Balacescu prostate cancer study, which is described below.

Expression of TIMP-1 has been studied in tissue and body fluids of prostate cancer patients. Most studies have found increased levels in prostate cancers, but a few have not, for example a study that used the detection of eight angiogenic molecules in serum to see if this could be an aid to prostate cancer diagnosis. Their result showed that there are significant differences in serum concentration of TIMP-1 between prostate cancer, benign prostatic hyperplasia, chronic prostatitis, and the control group for the angiogenic molecules KGF, angiopoietin-2, PDGF-BB, and TIMP-1(n=40). TIMP-1 was LOWER in prostate cancer, BPH and chronic prostatitis. This study used FAST Quant^R Human Angiogenesis Antibody Array (Whatman) similar to the Panomics array used in our study, only theirs included standards so they were able to quantify proteins directly (Balacescu, Neagoe et al. 2008). Brehmer et al immunostained tissue from radical prostatectomies. They found that normal prostate epithelium expressed TIMP-1 strongly while cancer cells did not (Brehmer, Biesterfeld et al. 2003). These studies serve to emphasize the need for more thorough investigation of TIMP-1's expression and role in prostate cancer progression.

Breast carcinoma is a parallel cancer to prostate: they are both cancers of the ductal epithelial cells of reproductive organs, so both are subject to hormone regulation. The many

breast cancer studies that show a strong correlation of TIMP-1 blood levels with poor prognosis indicate that TIMP-1 may also be a useful diagnostic in prostate cancer. In fact, an older study of TIMP-1 in tissue and plasma (Jung, Lein et al. 1998) and some recent studies (Leitzel, Kantoff et al. 2007; Oh, Leitzel et al. 2008; Vargas, Jacobus et al. 2008) support the prognostic strength of TIMP-1 blood levels.

TIMP-1 levels can also be increased in other pathologies. Increased TIMP-1 in serum or plasma has been found in women with migraine with aura (Martins-Oliveira 2009), during bone healing (Weiss 2005), and in diabetics (Maxwell, Timms et al. 2001).

TIMP-1 has potential as a prognostic blood protein in a wide range of diseases, but it is apparent from Table 5-1 that rigorous standardization will be required first. For example, serum levels are usually higher than plasma, probably because platelets contain TIMP-1 in their granules. Whether citrate or EDTA is used to prevent coagulation and a plethora of other factors can also affect results. Centrally standardized protocols will be necessary in order to use TIMP-1 as a viable prognostic.

Chapter 5, in part, is currently being prepared for submission for publication of the material. Frimodig, Jane; Gough, David. The dissertation author was the primary investigator and author of this material.

Table 5-1. TIMP-1 Blood Levels							
	LEVEL (ng/ml) otherwise noted	SPECIES/ GENDER	CONDITION	n	ASSAYED	ASSAY	REFERENCE
NORMAL	65-240	human	normal	20	plasma	ELISA Systems, Abbott Diagnostics	(Dresse, Nagel et al. 2008)
	218 +/- 81.3	human	normal	212	plasma	ELISA R&D	Viachopoulos et al. 2007)
BLADDER CANCER	423 (121-1776)	human	TCC transitional cell carcinoma of the bladder				(Staack, Badendieck et al. 2006)
	184 (57.8- 1084)		normal	79	plasma	ELISA Amersham	
	300 (207-1029)		non-metastasized metastasized	57 11			
BREAST CANCER	<454	women	normal postmenopausal	522	plasma	ELISA Oncogene Science/Siemens	(Lipton, Leitzel et al. 2008)
	0-2388		breast cancer	49			
	163.8 +/- 39.2	women	normal	15	serum	ELISA	(Wu, Wu et al. 2008)
	163.2 +/- 29.3		benign breast disease	18		Biosource International	
	251.5 +/- 55.1		breast cancer	60			
	96.7 (45.5- 498.1) 515.6)	women	primary breast cancer	519	plasma serum	ELISA in house	(Wurtz, Moller et al. 2008)

Table 5-1. TIMP-1 Blood Levels, Cont.							
	LEVEL (ng/ml) mean unless otherwise noted	SPECIES/ GENDER	CONDITION	n	ASSAYED	ASSAY	REFERENCE
COLORECTAL DISEASE	296.5 +/- 20.6	human	normal	50	serum	ELISA R&D	Kapsoritaki et al. 2008)
	414.9 +/- 17.6		ulcerative colitis	53			
	446.1 +/- 22.8		Crohn's disease	52			
	368.5		women (all conditions together)				
	399.8		men				
COLORECTAL CANCER	88.9 (51.0-156)	human	normal	108	plasma	ELISA in house	(Holten-Andersen, Christensen et al. 2002)
	86.5 (51.2-190)		women normal	69			
	141 (53.7-789)		colorectal cancer	588			
	126 (64.1-640)		rectal	250			
	159 (53.7-789)		colon	338			
	41 (19-82)		ulcerative colitis	24			
	32 (18-85)		Crohn's disease	26			
	95 (45.5-289)		primary breast cancer	322			

Figure 5-1. TIMP-1 Blood Levels, Cont.							
	LEVEL (ng/ml) mean unless otherwise noted	SPECIES/GENDER	CONDITION	n	ASSAYED	ASSAY	REFERENCE
PROSTATE CANCER	740	men	normal	35	plasma	ELISA	(Jung 2007)
	900		BPH	29		Amersham	
	1200		PCa no metastasis	29			
	3000 +/- 3000		PCa with metastasis	18			
	152.7 +/- 34	men	normal	66	plasma	ELISA	(Leitzel, Kantoff et al. 2007)
	20.6-1391	DU145 PCa cell line (androgen-independent)	prostate cancer (hormone refractory)	60	conditioned medium		
	46.2		normal	6	serum	FAST Quant™ Array	(Balacescu, Neagoe et al. 2008)
	22.9		prostate cancer	16		Whatman	
	23		BPH	11			
	22.9		chronic prostatitis	6			
METABOLIC	223 (99-334)	human	normal	35	plasma	ELISA	(Maxwell, Timms et al. 2001)
	518 (112-2020)		Type I diabetes	43		Amersham	
	143.1 +/- 20.1	human	normal	28	plasma	R&D	(GLOWINSKA- Olszewska and Urban 2007)
	161.2 +/- 32		obese adolescents about 14-15	45		ELISA	

Figure 5-1. TIMP-1 Blood Levels, Cont.							
	LEVEL (ng/ml) otherwise noted	SPECIES/ GENDER	CONDITION	n	ASSAYED	ASSAY	REFERENCE
CARDIOVASCULAR DISEASE	>100	upper quartile	male undergoing coronary angiography	389	plasma	ELISA EMD Biosciences	(Cavusoglu, Ruwende et al. 2006)
	66.5-100	interquartile					
	<66.5	lower quartile					
	731 +/- 133 (447-1410)	human	subjects from the Framington heart study	1069	plasma	ELISA Amersham	Evans et al. 2004)
OTHER	890	human	normal	52	serum	ELISA Amersham	Giral et al. 2003)
	1100		hyperlipidemic	52			
	990		hyperlipidemic w/out atherosclerotic lesions				
	1500		hyperlipidemic with atherosclerotic lesions				
MIGRAINE							Speciali et al. 2009)
	300	women	normal	40	plasma	ELISA R&D	
	300		migraine without aura	40			
FSH-WOMEN	500		migraine with aura	40			
	49	women	FSH-stimulated women	78	follicular fluid	ELISA RayBioTech	(Bruegmann, Gruemmer et al. 2009)
	57		granulosa cells		serum		
	10		HUVECs		medium		
	8				medium		

Chapter 6: Molecular Mechanisms of TIMP-1 Effects on Endothelial Cells

6.1 Introduction

TIMP-1 has been found to promote proliferation in many cell types (Hayakawa, Yamashita et al. 1992). We have previously shown that proliferation is induced by TIMP-1 in endothelial cells *in vitro*. The next question to answer is, how is TIMP-1 doing this, i.e., which molecular pathway to cell proliferation does TIMP-1 activate? Is there a receptor that transduces the TIMP-1 signal to some intracellular pathway?

In order to determine the pathway used by TIMP-1 to induce endothelial cell proliferation, HUVECs were grown with exogenous TIMP-1 and various small molecule inhibitors of the potential pathways. PrTuSC conditioned media was also investigated to see which pathways it activated. Cell surface proteins on HUVECs stimulated by TIMP-1 were fluorescently labeled in an effort to identify a TIMP-1 receptor.

6.2. Materials and Methods

6.2.1. MTS Proliferation Assay with Inhibitors

HUVECs were cultured to ~70-90% confluence in EGM-2 (Lonza Bio-Whittaker). The EGM-2 was aspirated, cells were washed once with PBS, and the EBM-2 (basal medium) and inhibitors were added to the tissue culture dishes. Inhibitors were incubated for one hour and then the TIMP-1 or PrTuSC conditioned medium were added. In the case of PrTuSC conditioned media, after one hour the EBM-2 containing inhibitor was aspirated and replaced with the conditioned medium, to which was added fresh inhibitor to equal the previous

concentration. Cells were incubated for 24 hours after which cell proliferation was assayed using MTS.

The following small molecule inhibitors were used: 1-2 $\mu\text{g/ml}$ (about 5 μM) GM6001 inSolution by EMD Biosciences Inc, San Diego CA, a general MMP inhibitor; 10 μM LY294002, PI3K/Akt inhibitor 9901 by Cell Signaling Technology, Danvers MA; 100 nM TGX-221, 7-methyl-2-(4-morpholinyl)-9-[1-(phenylamino)ethyl]-4H-pyrido(1,2-*b*) pyrimidin-4-one, inhibitor of the PI3K p110 β subunit 10007349 by Cayman Chemical, Ann Arbor MI; 100 nM PI-103, inhibitor of the PI3K p110 α subunit (#10009209 Cayman Chemical, Ann Arbor, MI); 10 μM Genistein, a tyrosine kinase inhibitor (5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-one), (Tocris Bioscience #1110, Ellisville, MO); 10 μM SB203580 [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole], a p38 MAPK inhibitor (Enzo Life Science, Alexis Biochemicals); and 50 μM U0126 [1,4-Diamino-2,3-dicyano-1,4-*bis* (2-aminopheylthio)-butadiene], a specific MEK inhibitor (Alexis Biochemicals, AXXORA, LLC, San Diego, CA).

All inhibitors except TGX-221 were initially dissolved in DMSO, therefore a sample equal to the end DMSO dilution (1%) of the worst case (strongest DMSO concentration) inhibitor was used for a control. Generally DMSO is toxic to cells at concentrations greater than 2-3%.

6.2.2. Tube-forming Assay with Inhibitors

Growth Factor-Reduced Matrigel (Benton-Dickson Biosciences, Two Oak Park, Bedford MA) was pipeted into 96-well plates at 55 $\mu\text{l/well}$. Human umbilical vein endothelial cells (HUVECs) were seeded in 96 well plates at 15000 cells/well in 50 μl of EBM-2 (basal medium). Inhibitor was added to the appropriate wells. After one hour of incubation with inhibitors, 100 μl of either PrTuSC conditioned media or EBM-2/0.5% BSA with or without the growth factors of interest were added to each well. This added media also contained inhibitors in the appropriate wells to keep inhibitor concentration consistent. Cells were incubated in a

humidified incubator at 37°C with 5% CO₂ for 12-16 hours then photographed using an Olympus E-30 digital camera mounted on an Olympus IMT-2 inverted microscope with a 2x objective, which allowed capture of almost the entire well. Tube length was quantified using free NIH software ImageJ by adding the total length of tubes in pixels for each photograph. Each group was replicated three times and each experiment was done separately at least twice, unless otherwise indicated.

HUVECs from passages 4-8 were used. They were passaged at least twice after thawing before use in the assay. Primary endothelial cells must be passaged at least twice after freezing, before use in tube assays. This is because differentiation of capillary endothelial cultures is more efficacious in later passage cells (Folkman and Haudenschild 1980).

6.2.3. pAkt ELISA

The PathScan® Phospho-Akt(Ser473) Sandwich ELISA kit 7160 from Cell Signaling Technology (Danvers, MA) was used to monitor the amount of activated Akt protein in HUVECs with and without TIMP-1 stimulation. Kits were used according to the manufacturer's instructions.

HUVECs were grown to ~95% confluence in 10 cm dishes and incubated in a humidified incubator at 37°C and 5% CO₂ for one hour with the following samples: 5 ml EBM-2/0.5% BSA alone or with 20 ng/ml IL-8, 30 ng/ml TIMP-1 or 200 ng/ml TIMP-1; or 5 ml conditioned media from p81 6/6 and p86 6/4 PrTuSC. After one hour the protein was harvested, processed, and run through the assay. The experiment was done once with three replicates of each sample for the TIMP-1 groups, and two replicates of each for the conditioned medium samples.

Data from the previous experiments was analyzed using Student's T-test in Excel and ANOVA using Minitab 16, Minitab, Inc..

6.2.4. Immunofluorescent microscopy

Cells were grown to 50-70% confluence on 12 x 12 mm glass coverslips mounted on silicone (70462-12 by Electron Microscopy Sciences, Hatfield PA). Cells were washed with PBS and transferred to a 6-well plate for one hour with or without 200 ng/ml TIMP-1, after which they were washed with PBS then were fixed with 4% formaldehyde for 10 minutes at room temperature. Cells were washed again with PBS three times for five minutes each, and non-specific sites were blocked by incubation for 1 hour in 2% (v/v) goat serum (G9023 Sigma) with 1% (w/v) BSA (OmnPur BSA Fraction V Heat Shock Isolation by EMD) in PBS. Next slides were transferred to humidity chambers, and the primary antibodies (diluted as follows in blocking solution) were carefully dropped onto the slides, and incubated overnight in a humidity chamber in the refrigerator (2-8°C):

1. mouse anti-human TIMP-1 Ab-2 (102D1) monoclonal antibody, (MS-608-P0 NeoMarkers, Fremont CA) 1:100.
2. rabbit antihuman CD44 clone EPR1013Y monoclonal antibody (04-112 Millipore, Temecula CA) 1:50.
3. rabbit anti-human CD63 monoclonal antibody (HPA010088 Sigma Life Science Prestige antibodies, St. Louis MO) 1:50.
4. rabbit anti-human Matrix Metalloproteinase-9 propeptide region affinity isolated antibody (M0306 Sigma Aldrich, St. Louis MO) 1:100.

The TIMP-1 antibody was mixed with each one of the other antibodies, so three different antibody cocktails were used. Afterwards the slides were transferred back to fresh 6-well plates, washed three times with PBS, placed in the humidity chamber and incubated for one hour at room temperature in the dark with a cocktail of two secondary antibodies diluted in blocking solution as follows:

1. Alexa Fluor 488 goat anti-rabbit igG (H+L) 1:250.
2. Alexa Fluor 555 goat anti-mouse igG (H+L) 1:250.

Both by Molecular Probes, Invitrogen, Eugene OR. After incubation cells were washed four times with PBS and once with distilled water. The silicone backing was removed from the slides, and they were carefully laid onto a drop of hard-set Vectashield with Dapi (H-1500 by

Vector Laboratories, Burlingame CA) on 25 x 75 x 1 mm slides (Colorfrost microslides 48312-705 by VWR Scientific, Westchester PA).

To check for nonspecific staining, secondary antibodies were also incubated with cells which had not been incubated with primary antibodies.

Positive controls were the following pre-made unstained tissue slides: Post Control s1 MS-1118-PCS Melanoma NM-387 c for CD63, Post Control 2 Ms-608-805 Pancreas NM-463F for TIMP-1, and for CD44 Post Control s1 MS-668-PCS Breast Cancer NM-1075, all from NeoMarkers (Lab Vision, Thermo Fisher Scientific, Fremont CA).

Slides were viewed using an Olympus laser scanning confocal microscope (Fluoview 1000 Confocal System) and Olympus Fluoview 1000 software with Olympus objective FM1-U2B530 40x Oil Uplan Fluorite, NA = 1.30. Excitation wavelengths used were 473 and 559 nm.

Image analysis was done with free NIH software ImageJ. Free NIH plug-in JACoP (Just Another Colocalization Program) (Bolte and Cordelieres 2006) was used to analyze the overlap of TIMP-1 and possible receptor signals. This plug-in is actually an assemblage of many colocalization programs that can all be run at once and then compared.

6.3. Results

6.3.1 Proliferation Assay with Inhibitors

HUVECs were incubated with basal medium (EBM-2/BSA) with exogenous protein and inhibitors added. The addition of 200 ng/ml of TIMP-1 to basal medium increased HUVEC proliferation significantly, as was shown in Chapter 4 ($p < 0.05$). There was no significant difference between the growth of HUVECs in basal medium and that of HUVECs in basal medium with any of the inhibitors added. However, addition of GM6001, LY294002, PI-103 and U1026 inhibited TIMP-1-induced increase in proliferation significantly. TGX-221 also inhibited TIMP-1 increase in proliferation but not as strongly ($p \sim 0.07$) (Figure 6-1).

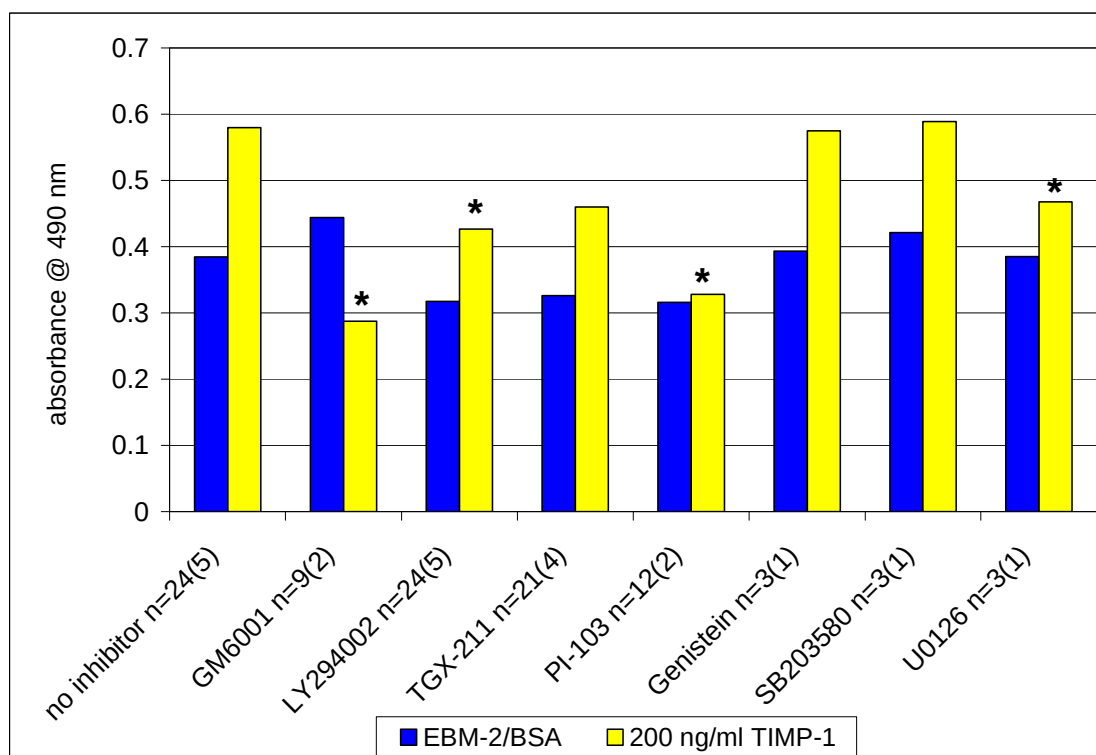


Figure 6-1.

Proliferation of HUVECs in basal medium with and without TIMP-1 and inhibitors. Significances between 200 ng/ml with and without inhibitors * $p < 0.05$. n = # data points (# of experiments).

Growing cells in conditioned medium from patient p86 increased proliferation over basal medium. Only U0126 inhibited this CM-induced growth increase. Since GM6001 is an MMP inhibitor, this means that CM-induced growth does not depend on inhibition of MMPs. For patient p81, proliferation did not increase as much as for p86 and no inhibitors were able to inhibit this proliferation significantly. U0126 was not tested with p81 PrTuSC conditioned medium (Figure 6-2).

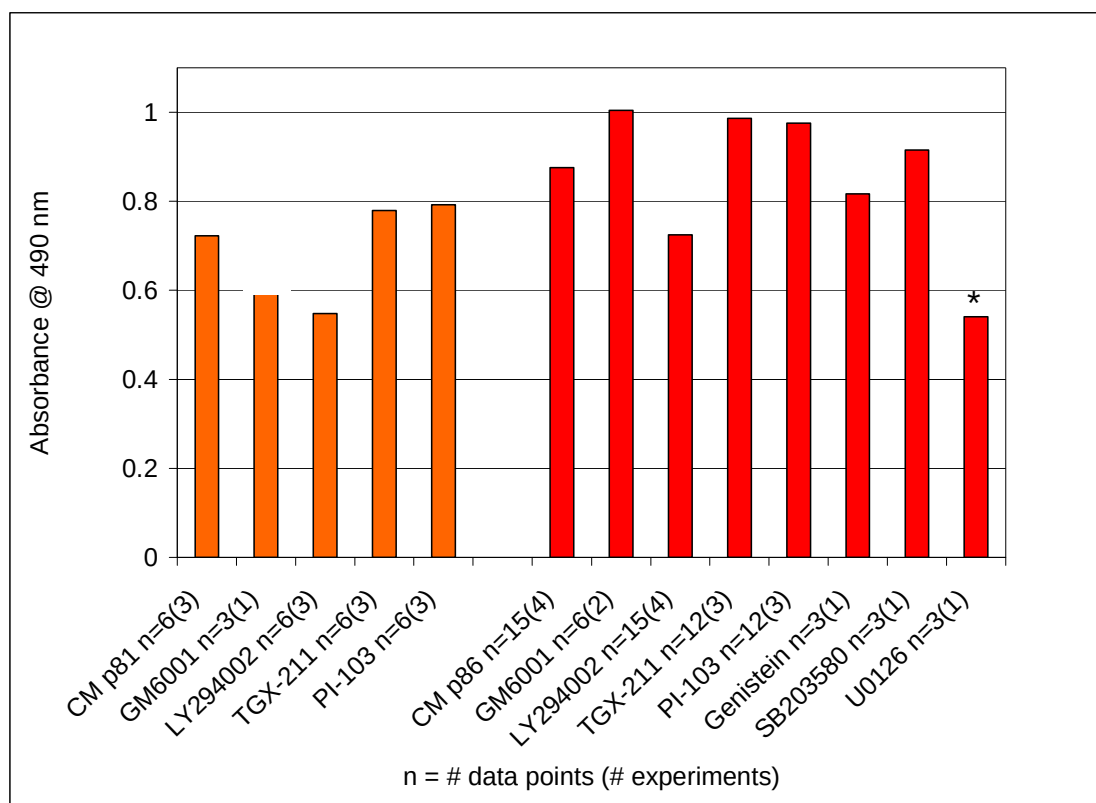


Figure 6-2.

Proliferation of HUVECs grown in PrTuSC conditioned medium with inhibitors, * $p < 0.05$. Data from p81 is on the left and p86 is on the right. The first column on the left of each group is the proliferation of that CM with no inhibitor added.

6.3.2. Tube Forming Assay with Inhibitors

Exogenous TIMP-1 did not increase the quantity of tubes formed by HUVECs grown in Matrigel significantly. There was some inhibition of tube forming in the basal medium by TGX-221 and PI-103. These inhibit the PI3K p110 α and p110 β subunits respectively. This inhibition was rescued by 400 ng/ml TIMP-1, but not significantly. LY294002 also inhibited tube forming of HUVECs in both basal medium and basal medium with 400 ng/ml TIMP-1 added, but again, not significantly (Figure 6-3).

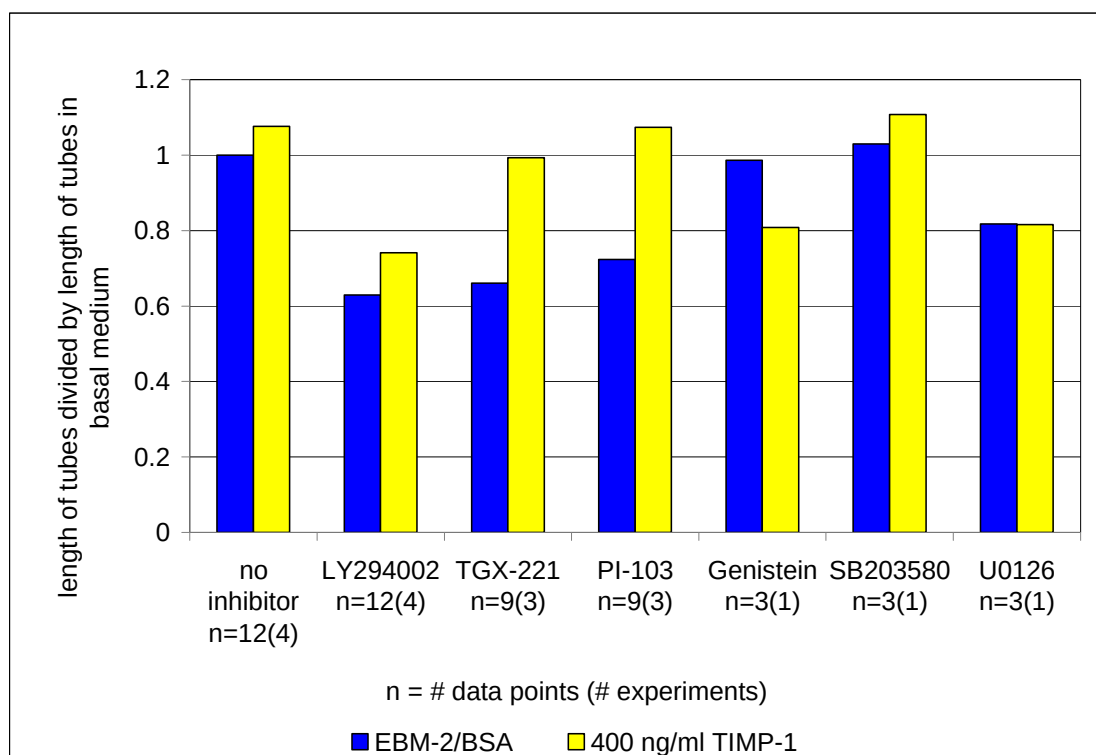


Figure 6-3.

Tube assay with TIMP-1 and inhibitors.

Medium conditioned by PrTuSCs from patient p86 and p88 caused slightly less tubes to form than basal medium did. The tube forming ability of p86 PrTuSC conditioned medium was inhibited significantly ($p < 0.05$) by GM6001 (Figure 6-4).

Figure 6-5 shows a tube-forming experiment where cells were accidentally left in the incubator for two days longer. Generally they are thrown away after photographing at the 16 hour timepoint. In this case the cells were just left alone with no media changes. Almost all of the cells in the TIMP-1 (except for two wells) continued to proliferate and formed clusters.

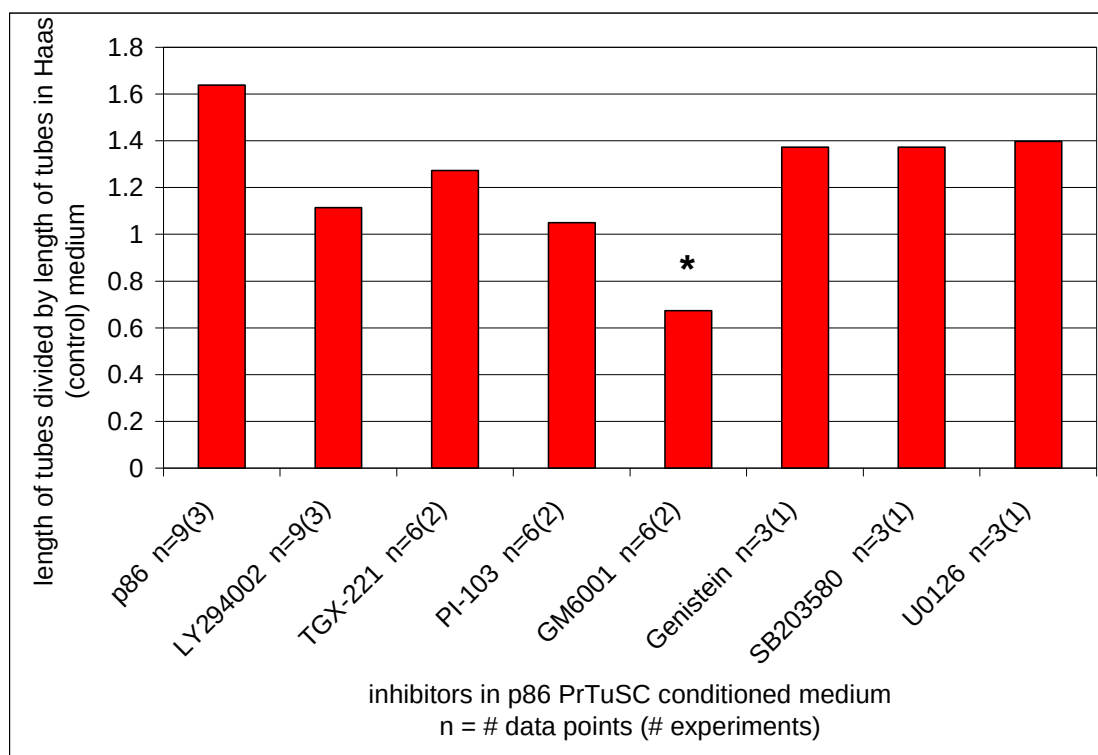


Figure 6-4.

Tube assay with PrTuSC conditioned medium from p86 and inhibitors. * $p < 0.05$.

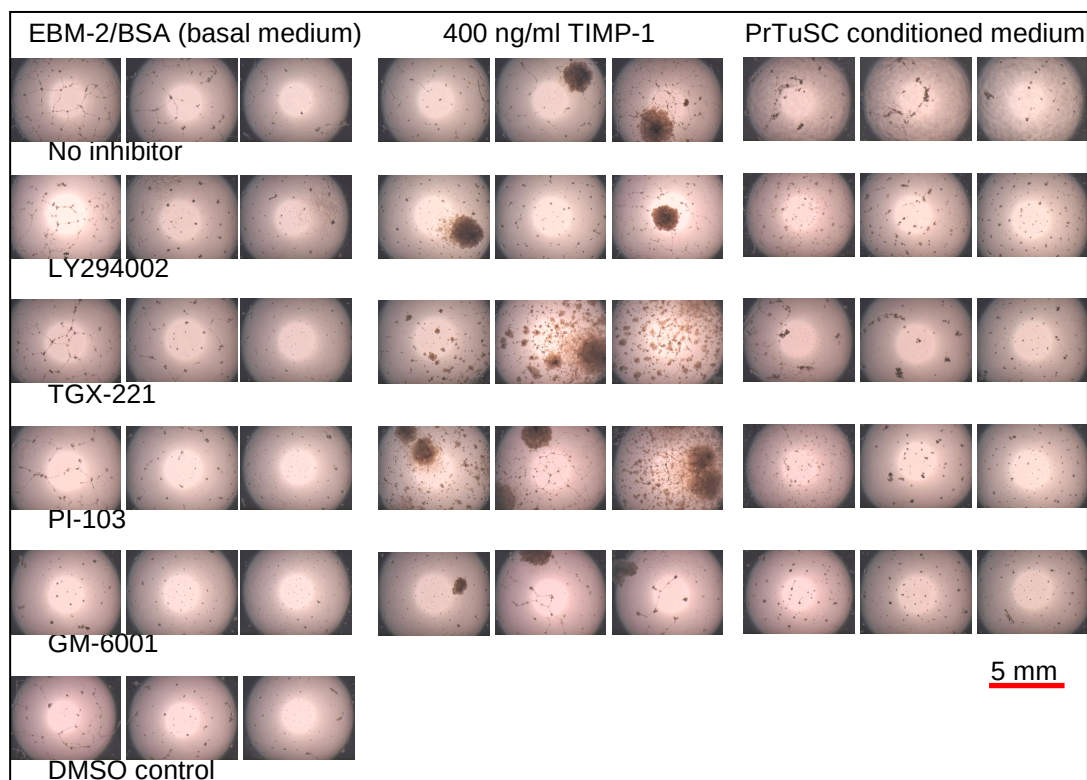


Figure 6-5.

Tube assay with cell clusters.

6.3.3. pAkt ELISA

HUVECs were incubated with II-8, TIMP-1 and PrTuSC conditioned media to determine if the pAkt pathway was activated by any of these. This is an ELISA assay that detects phosphorylation of Akt-1 on serine 473 which means that the protein is activated. TIMP-1 significantly activated Akt as did conditioned medium from p81 and p86 ($p > 0.05$). 20 ng/ml II-8 did not significantly activate Akt (Figure 6-6).

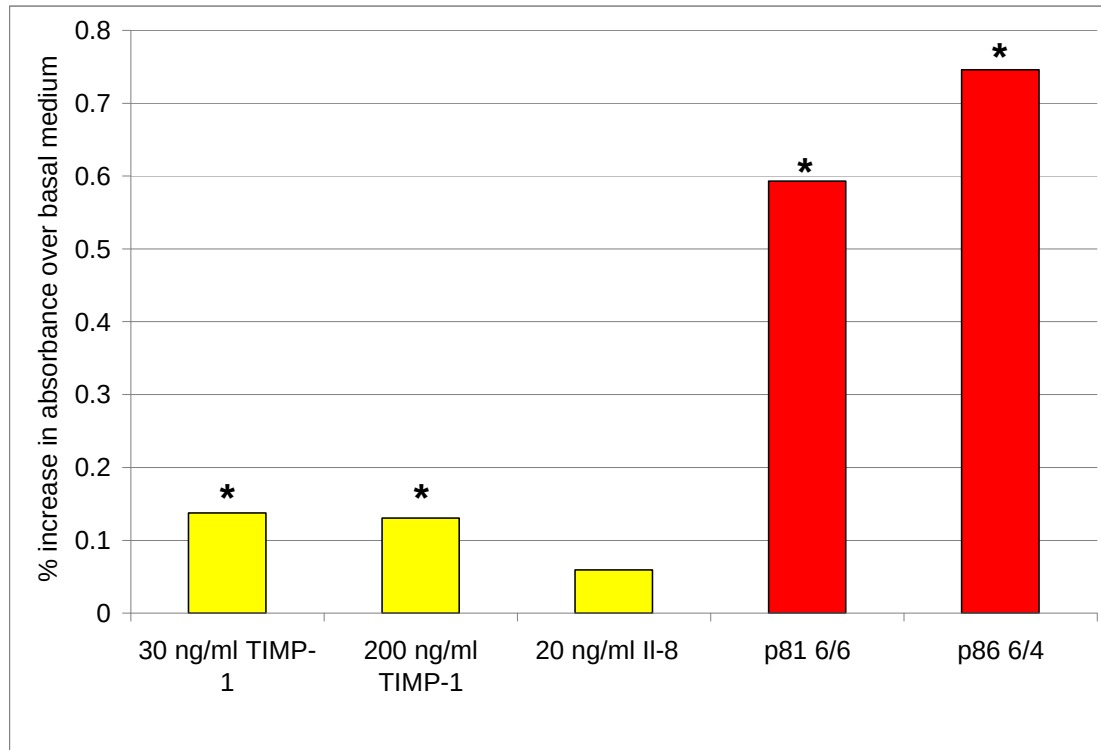


Figure 6-6.

ELISA detection of phosphorylated Akt-1. *p < 0.05. n = 3(1) for protein, 2(1) for PrTuSC conditioned medium.

6.3.4. Immunofluorescent microscopy

HUVECs were stimulated with TIMP-1 and then fluorescently labeled for the putative receptor components and for TIMP-1. Stimulated and unstimulated cells showed punctuate membrane staining for CD44 and CD63. The staining was strongest for proMMP-9, and the TIMP-1 stimulated sample appeared to have stronger proMMP-9 staining, indicating that TIMP-1 may have upregulated proMMP-9 expression. Small amounts of TIMP-1 staining were visible, again in the cell membrane region, in all slides, including the unstimulated ones. This is possible as endothelial cells have been known to express TIMP-1. Image analysis quantification, however, revealed slightly increased colocalization in the TIMP-1-stimulated samples, indicating that more TIMP-1 bound to the cell surface than on the unstimulated cells, especially on the proMMP-9 sample (Figure 6-7). The controls without primary monoclonal

antibody showed no or very slight staining, indicating that the secondary antibody was not responsible for any non-specific staining. The positive controls, which were prepared slides of tissue known to express CD44 (breast cancer), pancreas (TIMP-1) and melanoma (CD63), stained beautifully with their appropriate antibodies (not shown). Colocalization analysis using Mander's coefficient revealed that proMMP-9 and TIMP-1 had the highest percentage of overlap, followed by CD63 and TIMP-1. CD44 and TIMP-1 colocalized as well, but to a smaller degree. Figure 6-7 is a graph of Mander's Coefficients for the samples. Figure 6-8 shows the immunostained HUVECs. Table 6-1 shows Mander's Coefficients A and B for the different samples.

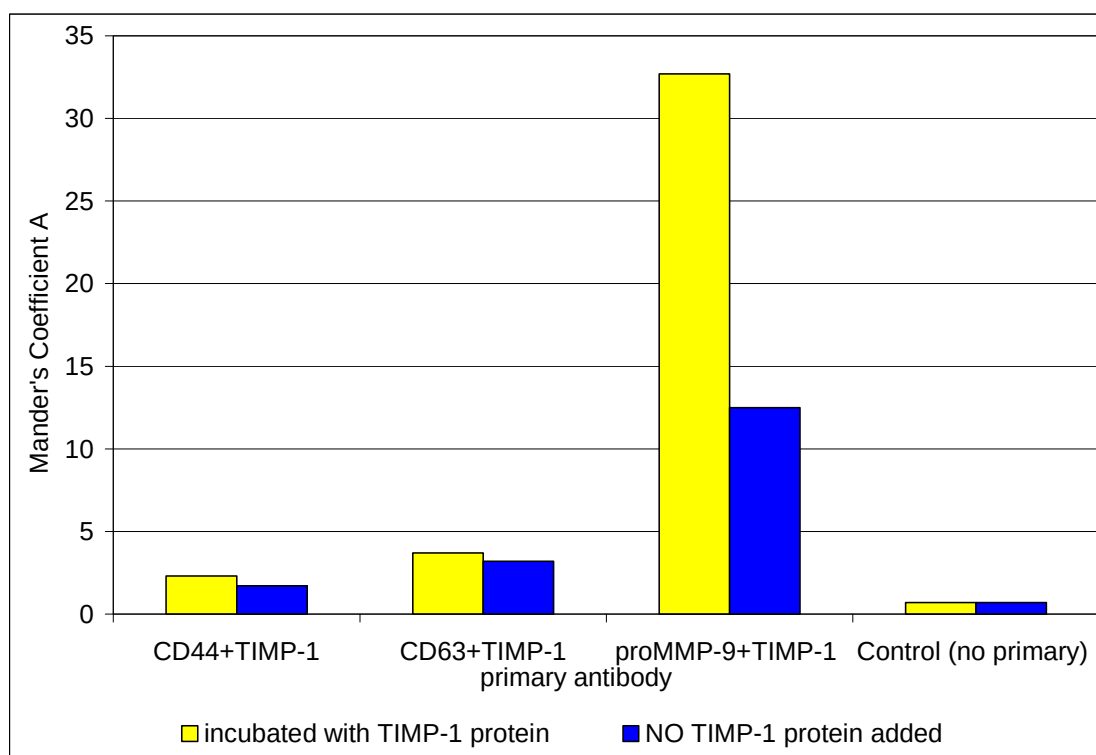


Figure 6-7.

Colocalization: Mander's Coefficient A

Mander's Coefficient A is the % of red signal (TIMP-1) that overlaps with the green (putative receptor) signal. n = 1(1).

Table 6-1.

Colocalization of putative receptors and TIMP-1: Mander's Coefficient

Receptor	With TIMP-1		Without TIMP-1	
	Mander's Coefficient A	Mander's Coefficient B	Mander's Coefficient A	Mander's Coefficient B
	% of TIMP-1 overlapping receptor	% of Receptor overlapping TIMP-1	% of TIMP-1 overlapping receptor	% of Receptor overlapping TIMP-1
CD63	4.1	1.4	2.6	1.4
CD44	2.3	3.7	1.7	3.2
proMMP-9	32.7	1.5	12.5	1.0

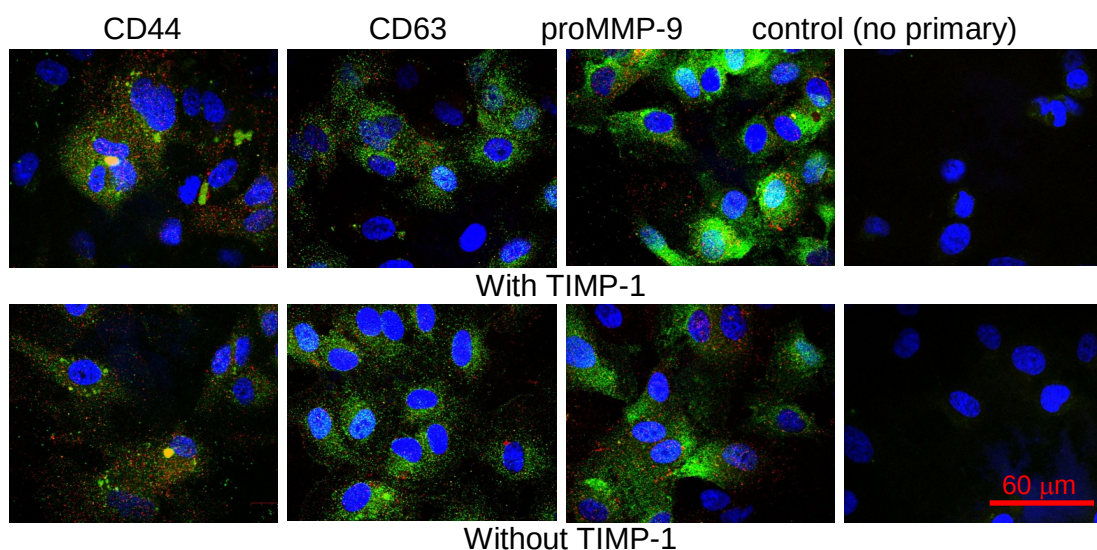


Figure 6-8.

Confocal images of HUVECs fluorescently stained for TIMP-1, CD44, CD63 and proMMP-9. Red stain indicates TIMP-1 and green stain indicates putative receptor. Yellow signal indicates TIMP-1/receptor overlap. DAPI was used to stain the cell nuclei blue.

6.4. Discussion

6.4.1. Pathways

The mechanism of TIMP-1 induced proliferation of endothelial cells is not known, but the literature points to several pathways.

Akt (PKB), a serine-threonine protein kinase, is an important node in multiple signaling pathways active in angiogenesis and cancer (Figure 6-9). *Akt* is commonly regarded as an oncogene. It is involved in anti-apoptotic, pro-angiogenic, and pro-mitotic pathways (Crowell, Steele et al. 2007). Moreover, Akt activation has been found to contribute to chemotherapy and/or radiation resistance (Clark, West et al. 2002). There is evidence that traditional chemotherapy actually selects for cancer stem cells, due to their multidrug resistance (MDR) mechanisms, one of which is Akt pathway activation (Li, Lewis et al. 2008). Akt can be activated by binding of ligands/growth factors (such as PDGF, EGF, and IGF-1) to their surface receptors.

PI3K is a major activator of Akt. PI3K (Phosphoinositide 3-kinases) are a conserved family of enzymes that have both protein and lipid kinase functions. They all phosphorylate the D3 hydroxyl group of membrane phosphatidylinositols (PtdIns) activated by RTKs (receptor tyrosine kinase) and G-protein-coupled receptors (GPCR) or Ras. Class I PI3Ks are heterodimers composed of two main subunits, the p85 regulatory subunit and the p110 catalytic subunit. The p110 subunit has four isoforms, α , β , δ and γ . p110 α and p110 β are ubiquitous and abundantly expressed in the vascular system; the other two isoforms are expressed mainly by leukocytes (Morello, Perino et al. 2009).

When the inactive p85 subunit is recruited to the membrane (usually stimulated by some surface receptor activation), PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂) at the 3' position on its inositol ring, which converts PIP₂ to PIP₃ (PIP₃ (PtdIns(3,4,5)P₃)). PIP₃ can then recruit other downstream molecules to the membrane by binding to their pleckstrin-homology domains. Akt-1 is one of these downstream proteins.

Once at the membrane, Akt is phosphorylated in order by its two kinases: the general name of the first kinase is PDK2 (phosphoinositide dependent kinase 2). A common PDK2 is mTORC2 (mammalian target of rapamycin complex 2) which phosphorylates Akt at Ser-473. Other proteins that can serve as PDK2s include ILK (integrin-linked kinase), DNA-PK (DNA-dependent protein kinase) and MAPKAPK2 (Mitogen-Activated Protein Kinase Activated Kinase-2). Secondly, PDK1 (phosphoinositide dependent kinase 1, which is also recruited to the membrane by PIP₃ binding to its pleckstrin homology domain) phosphorylates Akt at Thr-308. Akt is then activated and can go on to effect its many substrates (Figure 6-9) (Paez and Sellers 2003; Xia, Meng et al. 2006; Crowell, Steele et al. 2007).

The PI3K/Akt pathway is known to promote proliferation and survival (Figure 6-9). Many studies have shown that TIMP-1 can prevent apoptosis via PI3K/Akt. In a study using UT-7 human erythroleukaemia cell line and murine myeloid cell line 32D, TIMP-1 prevented apoptosis (but did not cause proliferation) via PI 3-kinase activation. pAkt prevents apoptosis by phosphorylating Bad at Ser136, causing Bad to be sequestered by 14-3-3 proteins in the cytoplasm, so it cannot heterodimerize with and inactivate Bcl-XL (an anti-apoptotic protein). JAK2, a tyrosine kinase that may be activated by the putative TIMP-1 receptor, was implicated in TIMP-1 signaling for apoptosis prevention. The JAK/signal transduction and activators of transcription pathway (STAT) also controls Bcl-XL and its receptor. JAK is also the tyrosine kinase that activates Ras (which can be upstream of the MEK/ERK MAPK pathway) (Lambert, Boudot et al. 2003). TIMP-1 inhibited apoptosis in breast epithelial cells via activation of FAK, PI3K and ERK pathways. This protection was independent of MMP inhibition. (Liu, Bernardo et al. 2003).

In endothelial cells, Akt pathways regulate angiogenesis (Chen, Somanath et al. 2005). PI3K p110 α was found to be critical to VEGFA-dependent migration of endothelial cells by RhoA activation. In this case it did not seem to be involved in EC viability and survival (Morello, Perino et al. 2009). Although TIMP-1 has shown survival benefits for many cells, endothelial cells have not been specifically addressed nor did we address apoptosis in this

study. However we did show that TIMP-1 can induce proliferation of endothelial cells and also, the continued proliferation and formation of cell clumps in the tube-forming assay (Figure 6-5) implies that survival mechanisms may be in play although we did not show that explicitly.

Activation of Akt can increase TIMP-1 expression. Activation of Akt pathway was found to increase TIMP-1 expression by an extravillous trophoblast cell line (Qiu 2004) and by thyroid carcinoma cells (Soula-Rothhut, Coissard et al. 2005). TIMP-1 expressed by embryonic stem cells inhibited apoptosis in H9c2 cells through the PI3K/Akt but NOT the ERK/MAPK pathway (Singla, Singla et al. 2008). On the other hand, TIMP-1 overexpressing MCF7 breast carcinoma cells caused an increase in angiogenesis *in vivo* as opposed to *in vitro* migration, tube-forming and proliferation assays, where they did NOT cause any increase. This study looked but did not find activation of p-Akt, ERK or Fak pathways (Cardelli and Bigelow 2007).

To discern if PI3K/Akt pathways were used by TIMP-1 in endothelial cell proliferation and tube-forming, we used small molecule inhibitors LY294002, PI-103 and TGX-221. LY294002 is a PI3K inhibitor that was originally developed from quercetin in search of a more specific PI3K inhibitor. Quercetin is a naturally occurring bioflavonoid which has been shown to have anti-oxidant and anti-inflammatory properties and low toxicity (Formica and Regelson 1995). LY294002 inhibits the PI3K/Akt pathway by acting on the ATP-binding site of PI3K (Poh and Pervaiz 2005). PI-103 specifically inhibits the p110 α PI3K subunit and TGX-221 inhibits the PI3K p110 β subunit. The p110 α subunit is usually activated by receptor tyrosine kinases (RTKs), while the p110 β is more subject to activation by a ligand binding to a GCPR (G-coupled protein receptor) (Zhao and Roberts 2006). Therefore use of these specific inhibitors might also indicate what kind of receptor TIMP-1 is using. We found that TIMP-1-induced proliferation was inhibited by LY294002 and PI-103 ($p < 0.05$). Therefore the PI3K/Akt pathway was being activated by TIMP-1, most likely through the p110 α subunit of PI3K. Therefore it is possible that activation is occurring through stimulation or perhaps costimulation of a tyrosine kinase receptor such as VEGFR2. In the tube forming assay, there

was no significant inhibition, but LY294002 inhibition did seem to be rescued somewhat by TIMP-1. Stimulation of inhibition of HUVECs by PrTuSC conditioned medium was not significantly inhibited by any of the compounds tested except for GM6001.

There are six known mitogen activated protein kinase (MAPK) (as of Kolch 2000) signaling modules. They exist in all eukaryotes and control important cellular processes such as proliferation, differentiation, survival and apoptosis. Some kind of surface receptor is stimulated, which then signals to three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK) which then activates a MAPK. The best known MAPK module is the Ras/Raf/MEK/ERK pathway. Ras is the G-protein, Raf is the MAPKKK, MEK (MAPK/ERK) kinase is the MAPKK and finally ERK is the MAPK. (Figure 6-10). Activated ERK goes into the nucleus to activate Elk which turns on genes which can control proliferation, differentiation, survival and apoptosis. MEK, the MAPKK which is inhibited by U0126, is activated by phosphorylation of two serine residues (S22 and S218) on its activation loop. MEK is a rare dual-specificity kinase, i.e. it can phosphorylate both threonine and tyrosine residues. MEK in turn phosphorylates T183 threonine and Y185 (tyrosine first) residues in the Thr-Glu-Tyr motif of the activation loop of ERK1/2 (Zheng and Guan 1994). ERK is a busy serine/threonine kinase, having more than 50 substrates (Kolch 2000). Activated ERK phosphorylates Elk-1 leading to transcription activation of cFos and cJUN genes, which results in AP-1 activation (Cano and Mahadevan 1995). U0126 inhibition of MEK results in functional antagonism of AP-1 transcriptional activity. (TIMP-1 does have an AP-1 binding site on its promoter (Edwards, Rocheleau et al. 1992)). U0126 selectively and noncompetitively inhibits MEK (Favata, Horiuchi et al. 1998).

This MAPK pathway is known to be important in proliferation (Meadows, Bryant et al. 2001) survival (Gupta, Kshirsagar et al. 1999), and migration (Eliceiri, Klemke et al. 1998) of ECs during angiogenesis. Also, Mek1 knockout mice are embryonic lethal, dying from a placental defect due to impaired angiogenesis (Giroux, Tremblay et al. 1999). The MAPK

pathway induced EC survival and sprouting by inhibition of Rho (Mavria, Vercoulen et al. 2006).

Shear stress activated the MEK/ERK MAPK pathway in microvascular endothelial cells both *in vitro* and *in vivo*, which caused upregulation of mRNA for transcription factor Ets-1, which in turn caused upregulation of TIMP-1 (Milkiewicz, Uchida et al. 2008).

In one study, both TIMP-1 and TIMP-2 increased Ras-GTP levels, but they utilized different pathways to do so. TIMP-1 activated the MAPK pathway and TIMP-2 used PKA Ras/PI3K complex formation. This fork upstream of Ras indicates that TIMP-1 and TIMP-2 might have different receptors. (Wang, Yamashita et al. 2002).

In human osteosarcoma cell line MG-63, TIMP-1 activated Ras through a tyrosine kinase MAPK pathway, causing an increase in Ras-GTP in a dose-dependent manner. Ras-GTP then complexed with the Raf-1 effector molecule. TIMP-1 also stimulated phosphorylation of Raf-1. Ten minutes of stimulation with 20 ng/ml TIMP-1 but not 100 or 200, and only somewhat 1, caused Ras-GTP (activated Ras) to increase. 20 ng/ml TIMP-1 did not change cAMP levels. Tyrosine kinase inhibitor herbimycin A abrogated this effect (Wang, Yamashita et al. 2002). In another study using MG-63 (human osteosarcoma) cells, TIMP-1 stimulated proliferation by activating protein tyrosine kinases and extracellular signal-related protein kinase -2 (ERK-2) (Yamashita, Suzuki et al. 1996).

MEK also upregulates TIMP-1 in smooth muscle cells. Induced cerebral ischemia in a rat model cause upregulation of both MMP-9 and TIMP-1 in smooth muscle cells associated with the cerebral arteries, and U0126 abrogated this effect (Maddahi 2009).

The MEK pathway is not only activated by TIMP-1, it also upregulates other growth factors. Bancroft et al found that angiogenic proteins IL-8 and VEGF were upregulated in human head and neck squamous cell carcinoma via activation of the MEK-MAPK and IKK-NF- κ B pathways (Bancroft, Chen et al. 2001). IL-8 promoters contain recognition sites for (NF)- κ B and Activator Protein AP-1, which is interesting as this is a transcription factor activated by the MEK MAPK pathway. The fact that a small molecule inhibitor of CXCR1/2 (the IL-8 receptors)

decreased ERK1/2 phosphorylation in melanoma cells is consistent with IL-8 activation of this pathway (Singh, Sadanandam et al. 2009). So if both TIMP-1 and IL-8 activate this pathway, and activation of this pathway could be also be upregulating expression of IL-8 and VEGF in endothelial cells.

We found significant inhibition of HUVEC proliferation by U0126, the specific MEK inhibitor, in HUVECs stimulated with 200 ng/ml TIMP-1. There was only one experiment done with this inhibitor; more experiments are needed to solidify this result.

In addition to PI3K/Akt inhibitors, we used Genistein and SB203580, a p38 inhibitor. P38 is part of another MAPK pathway. We saw no effects with this inhibitor, nor with Genistein. Genistein is a plant-based isoflavonoid the precursors of which are found in soy products (Setchell and Adlercreutz 1988). It inhibits by competitively binding the ATP catalytic domain of tyrosine kinases. It inhibits the tyrosine kinase activities of EGFR (Akiyama, Ishida et al. 1987) and PDGFR (Hill, Dean et al. 1990). The VEGF receptors are RTKs, and Genistein ablated VEGF mRNA production, but at a higher concentration (360 μ M) than was used in our study (which was 100 μ M) (Mukhopadhyay, Tsiokas et al. 1995). Therefore it is still possible that TIMP-1 activates PI3K/MAPK and or MEK/ERK MAPK pathways through stimulation or costimulation of an RTK.

Innumerable studies have found that if you shut down one biological pathway, another one steps up to take its place. While TIMP-1 is probably not absolutely essential for survival (TIMP-1 knock-out mice do survive, (Brew and Nagase 2010)) and is not a critical angiogenic regulator like VEGF or FGF-2, it is probably part of a back-up or supplemental system. TIMP-1 may give some kind of survival advantage to cancer, since so many studies have correlated its blood levels with cancer progression (Table 5-1). MEK/ERK MAPK and PI3K/Akt pathways are in some ways parallel pathways that have similar downstream responses, i.e. inhibition of apoptosis, proliferation, etc. (Figure 6-9). Recent work in a cancer model has found that if one of these pathways is inhibited, the other will take over (Wee, Jagani et al. 2009). When basal cell breast cancer was subjected to MEK inhibition, it increased PI3K activity (Hoeflich,

O'Brien et al. 2009). Ren et al found that if murine or zebrafish arterial growth is inhibited by Akt-1 suppression, it can be restored by ERK signaling (Ren, Deng et al. 2010) (Figure 6-10). Other studies are also finding that these are two parallel pathways that can take over for each other if required, in order to arrive at the same results such as inhibition of apoptosis or stimulation of growth (Hoefflich, O'Brien et al. 2009).

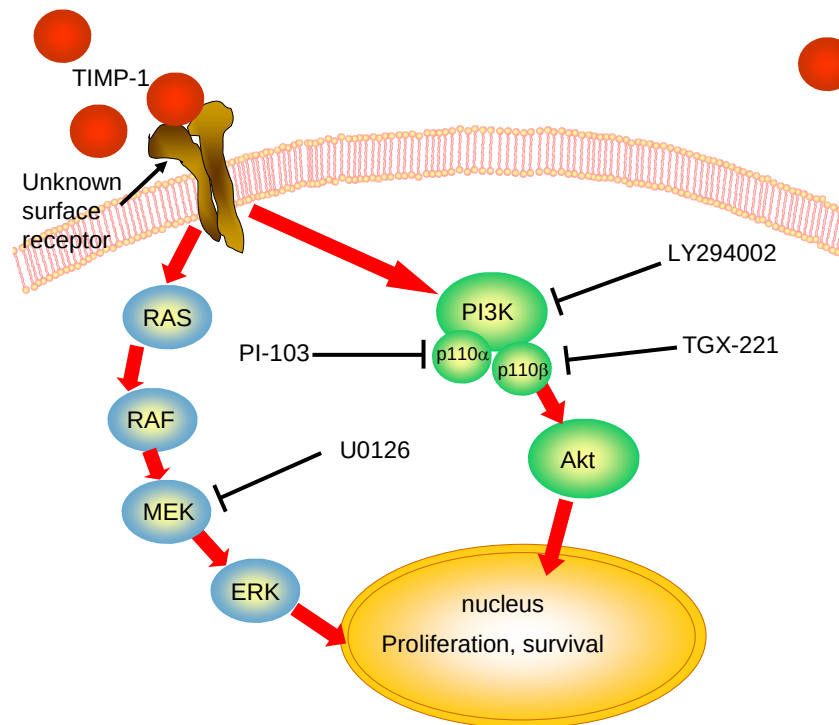


Figure 6-9.

PI3K/Akt vs. MEK/ERK MAPK pathways.

Cell surface receptors such as receptor tyrosine kinase (RTKs) activate PI3K and MEK/ERK MAPK signaling pathways resulting in survival and proliferation. If one pathway is inhibited, the other can compensate. The figure shows where the various inhibitors used affect these pathways. The two pathways could also be activated by different receptors.

As shown in Figure 6-9, binding of a ligand to a cell surface receptor can activate PI3K/Akt and MEK/ERK pathways. Stimulation of TIMP-1 induced proliferation of HUVECs was inhibited by PI3K inhibitors LY294002 and PI-103, as well as by MEK inhibitor U0126.

In work by Soula-Rothhut et al, EGF stimulation in thyroid carcinoma cells activated both PI3K/Akt and MEK/ERK MAPK pathways resulting in increased expression of TIMP-1

and thrombospondin (Tsp-1), which ultimately resulted in increased migration/invasion of these cells. This may be one explanation for the high levels of TIMP-1 seen in cancers, where EGF is often upregulated. TIMP-1 may amplify this response by further stimulation of the PI3K/Akt and MEK/ERK pathways. Inactivating mutations of PTEN, which is a natural suppressor of PI3K/Akt, occur in as many as 60% of advanced prostate cancer (Soula-Rothhut, Coissard et al. 2005). Loss of PTEN removes a brake from the PI3K/Akt pathway, which promotes proliferation and migration and inhibits apoptosis, and might cause emphasis to switch from MAPK pathways to PI3K/Akt ones.

Interestingly, EGF also induced expression of TIMP-1 via PI3K/Akt and ERK MAPK pathways in a extravillous trophoblast cell line. In this case, activation of both pathways was required for TIMP-1 induction (Qiu 2004). So it is possible that these pathways work both ways with TIMP-1; they can be stimulated by TIMP-1 and they can be used to induce TIMP-1. However these effects are probably cell-type dependent, and this study did not investigate TIMP-1 induction in endothelial cells.

The proliferation and antiapoptotic effects of TIMP-1 have sometimes been shown to be enacted through its inhibition of MMPs. We did not find this to be the case. It may be that this is also dependent on cell type. One group found that TIMP-1 and GM6001, an MMP inhibitor, both caused proliferation of MDA-MB-435 breast carcinoma cells. Therefore the effect was judged to be dependent on MMP inhibition by TIMP-1. ERK and p38 inhibitors also inhibited this effect, demonstrating activation of those pathways by TIMP-1 inhibition of MMPs. LY294002, a PI3K/Akt inhibitor, decreased the proliferation somewhat less. TIMP-1 and GM6001 both activated ERK1/2 and p38 pathways (Porter, Shen et al. 2004).

In our study, GM6001 did not repeat but often abrogated the effects of TIMP-1 or PrTuSC conditioned medium (data not shown). Therefore, the TIMP-1-induced proliferation was not a side effect of MMP inhibition, but rather the presence of active MMP was required for the TIMP-1-induced effect to take place. Inhibition of TIMP-1 induced HUVEC proliferation by LY294002, a general PI3K/Akt inhibitor, and by PI-103, a specific PI3K p110 α subunit

inhibitor, was seen. Both parallel activation by and expression of VEGF and IL-8 are also seen with these pathways, raising the possibility of synergistic effects with TIMP-1 in angiogenesis.

6.4.2. TIMP-1 Receptor

TIMP-1 is a soluble ligand that may transmit a signal into the cell by binding to a molecule on the cell's surface. It binds to and inhibits matrix metalloproteinases (MMPs), which are soluble proteases, in solution but at lower concentrations of TIMP-1 (less than 500 ng/ml), inhibition of MMP decreases while certain biological activities (such as cell proliferation and survival) that are known to be stimulated by TIMP-1 are still seen. The surface receptor for TIMP-1 on endothelial cells, if it exists, is not known. Recent publications have reported the discovery of a CD63/ β 1 integrin complex on breast epithelial cells that binds TIMP-1 (Jung, Liu et al. 2006) and a CD44/proMMP-9/TIMP-1 ternary complex that forms on the surface of erythroleukemic cells (Lambert, Bridoux et al. 2009), both of which induce prevention of apoptosis. In order to find a surface receptor on endothelial cells for TIMP-1, we stimulated HUVECs with TIMP-1 and then fluorescently stained them using antibodies to some of these components.

TIMP-1 was found early on by several groups to use classical cell receptors but they were not specifically identified. A specific TIMP-1 receptor with a K_d of 8.7 nM was found on human keratinocytes (Bertaux, Hornebeck et al. 1991). Avalos et al found two classes of binding sites for TIMP-1 on human erythroleukemic cell line K562: one low affinity (K_d = 62 nM) with about 60000 receptors per cell. About 1% of the total sites they found were high affinity (K_d of 100 pM). The high affinity receptors were observed in some experiments but not all. The authors believe that the binding mode is probably a covalent direct linkage, and that this is the receptor that mediates TIMP-1's cell proliferation effect, and that the effect is autocrine. The low affinity receptor had a molecular weight of 32 kDa (Avalos, Kaufman et al. 1988).

An 80 kDa transmembrane protein that bound TIMP-1 was found in human breast carcinoma BC-61 cell line (Luparello, Avanzato et al. 1999). This putative receptor had a K_d of 70 pM and interacted both *in vitro* and *in vivo* with TIMP-1.

A pertussis toxin-sensitive (PTX) G protein, perhaps a G protein coupled receptor, was found to be involved in rendering breast carcinoma T-47D cells resistant to apoptosis following TIMP-1 binding. The authors believe that an integrin may complex with the GPCR and TIMP-1. TIMP-1 induced phosphorylation of Akt and ERKs, and also of C-Src. PI3K and Akt were thought to be downstream from the G protein. In this case they did not find any increase of phosphorylation of FAK or JAK2. (Lee, Yoo et al. 2003).

TIMP-1 went into the nuclei of proliferating human gingival fibroblasts in the S-phase. (Zhao, Li et al. 1998). TIMP-1 also bound to the surface of breast carcinoma cell line MCF7 and after 72 hours localized to the nuclei. TIMP-1 did not bind to the surface of HBL-100 (normal breast epithelial) cells, indicating the utilization of a differentially expressed surface receptor (Ritter, Garfield et al. 1999). Cell cycle-associated nuclear accumulation of TIMP-1 may mean that TIMP-1, which does possess a DNA-binding-like structure, can influence transcriptional activation.

CD63 is a member of the tetraspanin family, a group of proteins with four transmembrane domains. Tetraspanins organize multimolecular membrane complexes and thereby regulate cell migration, fusion and signaling. In the cell membrane they organize into TEMs (tetraspanin-enriched microdomains) that are believed to be involved in signal transduction affecting cell adhesion, motility and survival (Berdichevski, Tolia et al. 1997; Berdichevski 2001; Hemler 2005). CD63 has a molecular weight of 40-60 kDa. It is an intracellular lysosomal/endosomal/granule protein that is found in Weibel-Palade bodies of vascular endothelium. It is translocated to the surface of activated platelets and endothelium (de Haas and Azorsa). CD63 has been known to regulate PI3K, MEK/ERK MAPK, FAK, Sarc and Akt signaling (Berdichevski, Tolia et al. 1997).

A complex of $\beta 1$ integrin, TIMP-1 and CD63 inhibited apoptosis in breast epithelial MCF10A cells (Jung, Liu et al. 2006). CD63 had previously been shown to associate with $\beta 1$ integrins. $\beta 1$ integrins heterodimerize with an α integrin and CD63 can associate with the $\beta 1$ integrin whether its dimerized with $\alpha 3$, $\alpha 4$, $\alpha 5$ or $\alpha 6$ (Yunta and Lazo 2003). Keratinocytes, which also possess binding sites for TIMP-1, also express CD63 on their surface which associates there with $\alpha 3\beta 1$ integrin (Okochi, Kato et al. 1997). Normally, attachment of epithelial cells to their basement membrane activates signals which prevent apoptosis. Jung et al found that the binding of TIMP-1 in this complex constitutively activated FAK and ERK signaling which inhibited apoptosis whether cells had contact with basement membrane or not.

CD44 is a cell surface protein most well known as a binding site for hyaluronan. A complex of CD44, TIMP-1 and proMMP-9 inhibited apoptosis in K562, an erythroleukemic cell line (Lambert, Bridoux et al. 2009). TIMP-1 is known to complex with only one MMP zymogen, proMMP-9 (Figure 6-10). They are thought to complex via their C domains (the hemopexin domain on the C terminal of proMMP-9) (Figure 6-10). MMP inhibitors did not replicate the survival effect, so TIMP-1 induced survival is not a side effect of MMP inhibition. However the MMP inhibitors did block the TIMP-1/proMMP-9 interaction and the survival effect, therefore they think that the TIMP-1/proMMP-9 interaction site is close to the MMP-9 catalytic site. The authors hypothesize that first, proMMP-9 anchors to the CD44 on the cell surface, during which the MMP active site is demasked. Therefore if MMP inhibitors arrive first, they competitively occupy the MMP active site and the TIMP-1 is not able to bind to the complex, and the effect is abrogated. Contrary to previous knowledge, preliminary studies reveal that the interaction site with proMMP-9 might be in the N-terminal domain of TIMP-1. MMP-9 is well known to associate with CD44, in fact MMP-9 associates with CD44 at the leading edges of migrating cancer cells where it is well situated to break down impeding ECM and facilitate migration (Yu and Stamenkovic 1999). It is possible that binding of TIMP-1 to the CD44/proMMP-9 complex might stimulate cell proliferation, but one study found that TIMPs

complexed with their proMMPs did not promote cell growth. (Hayakawa, Yamashita et al. 1994b). Another study found that 10% of the isolated MMP-9 was in its latent 95-kDa form (proMMP-9) complexed with TIMP-1 (Triebl, Blaser et al. 1995).

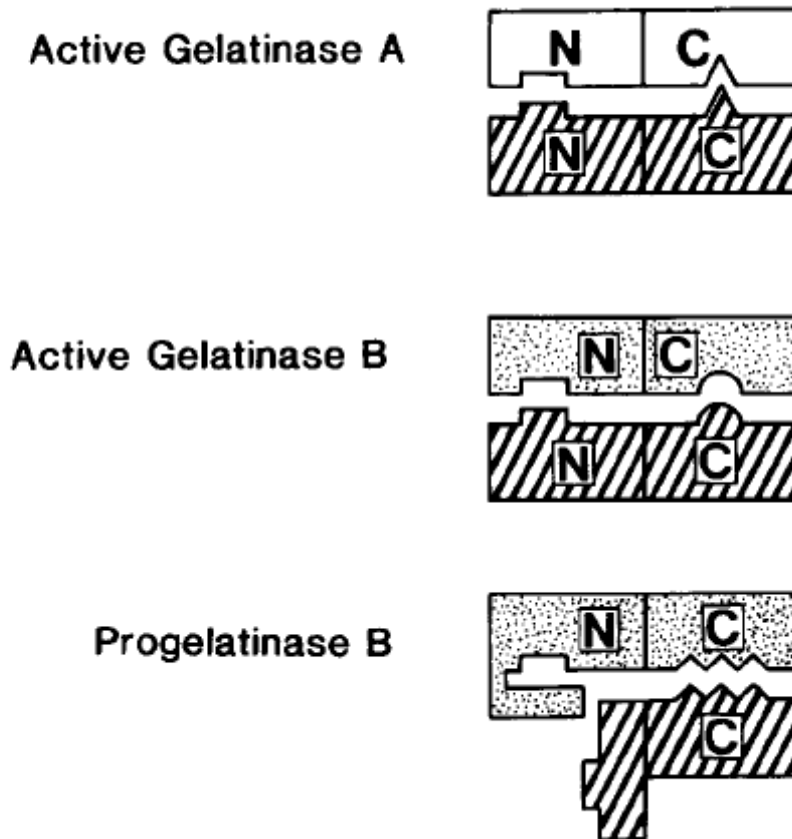


Figure 6-10.

Binding of TIMP-1 (cross-hatched) to MMP-2 (Active Gelatinase A), MMP-9 (Active Gelatinase B), and proMMP-9 (Progelatinase B). TIMP-1 does not bind to proMMP-2 (Murphy, Willenbrock et al. 1995). TIMP-1 was thought to bind to proMMP-9 via the hemopexin domain on proMMP-9's C-terminal domain

CD44 was found to be an activation marker for endothelial cells (Trochon, Mabilat et al. 1996; Griffioen, Coenen et al. 1997). CD44 expression was increased on endothelial cells isolated from human breast carcinoma tumors (Grange, Bussolati et al. 2006). Tumor associated endothelial cells expressed it, while normally quiescent HUVECs did not. HUVECs begin to express CD44 *in vitro*, since subconfluent cells *in vitro* are actively proliferating. VEGF and bFGF were able to increase CD44 expression of HUVECs *in vitro* (Griffioen,

Coenen et al. 1997). Hyaluronan is well-known to be the activating ligand of CD44 for angiogenesis, but it must be the right size hyaluronan (4-25 disaccharide units) (Trochon, Mabilat et al. 1996). It is possible that TIMP-1 in complex with proMMP-9 further activates endothelial cells. This might explain why, if TIMP-1 induces cell proliferation, such high circulating levels of TIMP-1 as found in cancer do not cause system-wide angiogenesis; possibly there is an upregulated receptor on endothelial cells at the tumor or wound site where endothelial cells are actively proliferating.

The standard (also known as the hematopoietic form, CD44H or CD44s) is a 85-95 kDa molecule. (Jalkanen, Bargatze et al. 1987). In the Lambert study, the CD44 glycoform was CD44H. However, this form was also found to be expressed by quiescent endothelial cells (Price, Coombe et al. 1996).

None of the previously identified TIMP-1 receptors were involved in proliferation, only in cell survival via prevention of apoptosis. The identification of a TIMP-1 receptor that instigates a proliferation cascade has yet to occur, as does the identification of a TIMP-1 receptor on endothelial cells. We have shown through immunocytochemistry that TIMP-1 does colocalize with CD44, CD63 and proMMP-9 separately on the surface of HUVECs. ProMMP-9 had the strongest association with TIMP-1, followed by CD63, with CD44 close behind. Therefore the strongest possibility is that TIMP-1 may activate proliferation in HUVECs by forming a ternary complex with CD44 and proMMP-9 on the cell surface, which in turn may activate PI3K/Akt and/or MEK/ERK MAPK pathways. This does not exclude the possibility of formation of a CD63/ β 1 integrin complex. Further work is necessary to verify the existence of these complexes on the surface of endothelial cells and to tie them to their functionality.

6.4.3. HUVEC cluster formation in tube assay

We have already shown that 200 and 400 ng/ml of TIMP-1 significantly increased HUVEC proliferation in 2D cell culture. When grown in Matrigel (a 3D culture) and basal medium containing 400 ng/ml TIMP-1, HUVECs formed tubes in the first 16 hours similar to

the EBM-2 controls without TIMP-1. Two days later, however, the HUVECs in EBM-2 and in PrTuSC conditioned medium were still in tube formation but the HUVECs in the 400 ng/ml TIMP-1 had continued to proliferate and formed large cell clusters (Figure 6-5). Two days after that the cells continued to proliferate but some necrotic areas were apparent, probably due to the fact that the medium was not changed for five days. The only TIMP-1 they received was the initial 400 ng/ml dose on day 0 of the assay yet proliferative effects continued to be seen. This effect was seen in all the wells containing TIMP-1, regardless of inhibitor added, save two. Since none of the inhibitors prevented this phenomenon, it is possible that:

1. The effect of the inhibitors is short-lived (they will not inhibit after 24 hours, and then the pathways were able to activate again).
2. The effect of one-time delivery of 400 ng/ml TIMP-1 is long-lived, as the proliferation effect was still seen after five days without any apparent inhibition.
3. TIMP-1 was activating a pathway that was not being inhibited.

Normally, endothelial cells do not proliferate during *in vitro* tube formation. They basically just go about the process of migrating into tube formation and differentiating into a quiescent phenotype as is seen in normal vasculature. Both microvascular endothelial cells and HUVECs (cells from small and large vessels) cultured on Matrigel do not proliferate, while cells cultured on laminin or tissue culture plastic do (Kubota, Kleinman et al. 1988). One study found that pAkt is reduced 3-fold and pERK1/2 is reduced 4-fold early (during the first hour) in tube formation (Grove, Prabhu et al. 2002). So these proliferation pathways are inactivated early in tube formation in order to suppress cell proliferation. My research indicates that pAkt (activated Akt) is increased by TIMP-1 in 2D cell culture, and that TIMP-1 stimulated HUVEC proliferation through the PI3K/Akt and MEK/ERK MAPK (somewhat) pathways in a 2D cell culture. Even though pAkt levels were not assayed in the 3D Matrigel cell culture, it is possible that TIMP-1 was stimulating activation there as well. Perhaps being placed into the Matrigel environment allowed the normal signaling (most likely ECM-integrin related signaling) that suppresses these proliferation pathways to initially occur. But eventually the TIMP-1 remaining in the medium was enough to perturb the cells so they did not remain quiescent; perhaps the

PI3K/Akt and MEK/ERK MAPK pathways were stimulated enough to overcome the initial ECM microenvironmental controls, so the HUVECs could continue to proliferate and form clusters. TIMP-1 was probably maintaining activation of those pathways so proliferation did not cease and the prominent effect was proliferation, not tube formation.

In a second tube assay (not shown), HUVEC proliferation after two and five days in 400 ng/ml TIMP-1 was inhibited by tyrosine kinase inhibitor Genistein and MEK inhibitor U0126. Cell clumps still grew in the LY294002 PI3K/Akt inhibitor wells, indicating that either this pathway was only inhibited for a short time or that proliferation was not occurring through this pathway, but probably through the MEK/ERK MAPK pathway instead.

TIMP-1 is known to promote cell adhesion (Schultz, Silberman et al. 1988; Roeb, Winograd et al. 1999). This may be why the cells aggregated instead of spreading out more as they proliferated. VE-cadherin in adherens junctions on ECs is required for cell-cell adhesion, but its presence inhibits proliferation signaling (Lampugnani, Zanetti et al. 2003). Interestingly, VE-cadherin expression is lower in tumor associated endothelial cells (TEC) (Xiong, Sun et al. 2009). In our HUVECs in Matrigel with 400 ng/ml TIMP-1 after three days, this contact inhibition effect was not taking place; somehow this inhibitory effect was being overridden.

TIMP-1 may have a role in the proliferative function immediately preceding tube formation in neovascularization, so that there is an adequate supply of new cells available for assembly of the new blood vessels. It probably also has a role in restabilizing ECM and basement membrane and cell adhesion after MMP and tube-forming activity is done by inhibiting MMPs. Therefore TIMP-1 could have two very important roles in angiogenesis that are overlooked in assays that emphasize tube formation.

6.4.4. TIMP-1 and VEGF

VEGF is perhaps the best known and studied angiogenic protein. Through review of the literature a trend is apparent: that TIMP-1 and VEGF levels increase cocommitantly in some cancers. This is a possible explanation for the correlation of high blood TIMP-1 levels

with a negative prognosis. It is perhaps not a cause but may be an indicator or a result of other angiogenic effects. Below are some details.

VEGF is well known to be overexpressed in prostate cancer. Interestingly, VEGF reduction is believed to be one of the first steps in the involution of the prostate that occurs upon castration, or androgen-deprivation, leading to ablation of blood vessels which in turn reduces the prostate down to a few cells (which they think are the prostate stem cells) that can regenerate the prostate upon resupply of androgen. Involution and restoration of the prostate in this manner can be repeated over and over many times, and seems to be dependent upon blood vessel supply (Gray, Huss et al. 2004). PrTuSC, as has been noted, are like many other stem cells that can survive in reduced oxygen conditions. So probably, upon reduction of the blood vessel supply, the stem cells are the ones that are able to survive in order to restore the organ later when androgen is resupplied and the blood vessels reform. In an experiment with rats, VEGF levels dropped 80% within one week of castration. This effect was reversed within another week with an eight-fold increase when animals were given androgens (Joseph, Nelson et al. 1997). In a model of prostate cancer with castration, VEGF decreased in 24 hours, a drop in neovascularization was seen within three days and tumor regression was apparent by day eight (Hrouda, Nicol et al. 2003). Therefore VEGF levels (and their induction of angiogenesis) are a critical factor in androgen response of the prostate, and possible synergy with TIMP-1 might make TIMP-1 important in androgen response as well.

In a rat mammary carcinoma cell line overexpressing TIMP-1, the resulting clones were ranged by the amount of TIMP-1 they expressed (Figure 6-11). The highest expressing clone made 4.6 times larger subcutaneous tumors than the control in two weeks. In Figure 6-11, the mammary epithelial cell lines are ranked from most to least aggressive. The more aggressive cells express both more TIMP-1 and more VEGF. Increased TIMP-1 secretion resulted in increased VEGF levels and increased vascularization of subcutaneous tumors. MCF7 transfected with TIMP-1 also increased VEGF expression *in vivo* but not *in vitro*, and exogenous TIMP-1 did not cause VEGF to increase *in vitro* so the effect of TIMP-1 in this case

might be indirect. The authors were not sure how TIMP-1 causes VEGF levels to increase (Yoshiji, Harris et al. 1998).

In ovarian cancer, siRNA downregulation of the p110 α subunit of PI3K resulted in downregulation of HIF-1 α which in turn reduced expression of VEGF, resulting in reduced tumorigenesis and angiogenesis. In addition they saw decreased Akt signaling (Xia, Meng et al. 2006). Perhaps TIMP-1 could reverse this trend and reactivate VEGF expression by increasing Akt signaling through the p110 α subunit of PI3K. In a recent study, TIMP-1 and VEGF serum levels were tracked before, during and after chemotherapy in ovarian cancer patients. At all times, TIMP-1 correlated positively with VEGF, and both were prognostic for poor outcome (Mahner, Woelber et al. 2010).

Table 6-2.

TIMP-1 and VEGF production in human breast epithelial cell lines *in vitro* (Yoshiji, Harris et al. 1998). The cell lines are arranged from most to least aggressive.

Cell type	Secretion into media ¹	
	TIMP-1 (ng/ml)	VEGF (pg/ml)
Carcinoma		
MDA-MB 231	338.0	3,096
MDA-MB 415	326.5	5,621
MDA-MB 435	183.0	3,210
MDA-MB 468	144.0	2,217
BT-483	91.0	3,108
MDA-MB 453	73.9	1,852
BT-20	36.8	1,884
T-47D	25.5	1,206
Non-neoplastic		
HBL-100	15.14	416
HS-578BSt	4.57	201

¹Concentrations of TIMP-1 and VEGF were measured by ELISA.

It's possible that VEGF and TIMP-1 might synergistically stimulate angiogenesis. One study found that fibroblasts potentiate blood vessel formation partially through secreted TIMP-1. Fibroblast-conditioned medium and fibroblast/HUVEC cocultures encouraged tube formation in a 3D angiogenesis assay. Fibroblasts (and myofibroblasts (Still, Robson et al.

2000)) secrete TIMP-1. In this study, TIMP-1 was secreted by fibroblasts but NOT by endothelial cells. Addition of exogenous TIMP-1 to medium containing VEGF also increased vessel assembly. Endothelial cells cultured in 30 ng/ml VEGF with 200 ng/ml TIMP-1 increased angiogenesis 24%, and addition of 400 ng/ml TIMP-1 increased angiogenesis 46%. So the addition of TIMP-1 enhanced VEGF-dependent tube formation in a dose-dependent manner. Since chemical inhibition of MMP-2/9 also increased angiogenesis, and addition of exogenous MMP-9 blocked the enhancing effect of TIMP-1, the mechanism was found to be dependent on the inhibition of MMP-9 by TIMP-1. The authors believe this effect might involve TIMP-1 inhibition of formation of tumstatin, an anti-angiogenic collagen IV fragment produced by cleavage by MMP-9 (Liu, Chen et al. 2008). However, another study found that TIMP-1 also weakly inhibited VEGF-induced proliferation of HUVECs (Bruegmann, Gruemmer et al. 2009).

VEGF is not only an angiogenic paracrine, but also an autocrine growth factor in prostate tumor cells which have demonstrated increased expression of VEGF, which in turn stimulates tumor cell motility and tumor angiogenesis. The fact that TIMP-1 has been found to use the same pathways, increase in parallel and synergize with VEGF means that TIMP-1 might have some important role in angiogenesis, and perhaps an autocrine effect on tumor cells as well.

6.4.5. Possible autocrine effects of PrTuSC

In this study, only angiogenesis was addressed, but it should be kept in mind that prostate epithelial and vascular endothelial cells are both sheet-forming (*in vitro*) cells that line tubes or ducts *in vivo*. Due to and also in addition to morphological and functional similarities between the two cell types, many of the paracrine effects of the proteins being expressed by PrTuSC on endothelial cells may also be having autocrine effects on the PrTuSC themselves, especially since cancer cells are known for their self sufficiency (one of the Hallmarks of Cancer). In support of this possibility, the same two pathways (PI3K/Akt and MEK/ERK

MAPK) that were activated in HUVECs by TIMP-1 were found to be activated during progression of androgen-independent prostate cancer (Bok and Small 2002). In another study, PC3 prostate cancer cell lines were selected for CD133+/CD44+ cells and grown in spheres, as this was found to increase tumorigenicity upon implantation in SCID mice. These cells were dependent on the PI3K/Akt pathway for survival. PTEN deletion (which would take the brakes off PI3K signaling) seemed to increase the stem/progenitor cell subpopulation (Dubrovskaya, Kim et al. 2009). The fact that PrTuSC express at least two proteins (TIMP-1 and IL-8) that activate these pathways further defines them as stem/progenitor cells in agreement with the above study, and also identifies a possible autocrine growth/survival mechanism for the cells.

TIMP-1 may have an autocrine effect on cancer cell migration, but most studies of cancer cells have shown that TIMP-1 inhibits cell migration. One study however showed slight stimulation of migration by TIMP-1. HepG2 (human hepatoma) cells were stably transfected with TIMP-1 and TIMP-1 antagonist (MMP-9_H401A) which is a catalytically inactive MMP. In other words the protein MMP-9_H401A is not an active enzyme but it can still bind and neutralize TIMP-1. In Boyden chamber migration assays, cells overexpressing TIMP-1 migrated slightly more (115%) and cells overexpressing TIMP-1 inhibitor MMP-9_H401A migrated slightly less (62%). GM6001, an MMP inhibitor, reduced migration in all cases. Therefore increased migration was not a side effect of TIMP-1 inhibition of MMPs, but MMPs were required to be present for the migration effect to take place. Actually, the researchers think TIMP-1 upregulation of MMPs was the cause of the small increase in migration. (Roeb, Bosserhoff et al. 2005). In an earlier experiment similar to the above one, less migration was achieved using TIMP-1 overexpressing human hepatoma cells (HepG2). It was noted that TIMP-1 increased expression of MMP-2 and MMP-9, but inhibited migration, and seemed to increase cell-cell adhesion (Roeb, Winograd et al. 1999). In vitro, TIMP-1 seemed to increase adhesion of B16-F10 murine melanoma tumor cells to an amniotic membrane that they were supposed to invade through. TIMP-1 injections into C57Bl/6 mice inoculated with B16-F10 cells significantly inhibited the number of metastatic lung tumors formed but had no significant

effect on their size. Also subcutaneous tumors showed no difference in size from controls. The authors believe that rTIMP does not affect tumor growth but it does affect the invasion step (Schultz, Silberman et al. 1988).

In opposition to the above work is the recent dissertation of Rosemarie Chirco, which found that TIMP-1 promotes EMT (epithelial-mesenchymal transition, change into a migratory phenotype) of breast cancer cells (Chirco 2008).

Inhibition of apoptosis by TIMP-1 may help cancer cells survive various therapies. TIMP-1 gene deficient mouse lung fibrosarcoma cells were more sensitive to chemotherapy. The authors speculated that TIMP-1 might protect cells from chemotherapy by preventing apoptosis. (Davidsen, Wurtz et al. 2006). Researchers found that tissue levels of TIMP-1 of primary breast cancer tumors were prognostic of resistance to chemotherapy in patients with metastatic breast cancer (Schrohl, Meijer-van Gelder et al. 2006). Plasma TIMP-1 was assayed from 90 patients with metastatic colorectal cancer. Patients with low plasma TIMP-1 were more likely to respond better to chemotherapy (Sorensen, Bystrom et al. 2007).

On the other hand, TIMP-1 could sometimes play a role in promoting efficacy of drugs. Cisplatin inhibited invasion of human cervical carcinoma (HeLa) through Matrigel. Cisplatin also caused TIMP-1 to increase in this situation. This effect was accompanied by activity increases in p38 and p42/44 MAPK pathways and could be ablated by specific inhibitors of these pathways. SiRNA knock-down of TIMP-1 completely reversed the suppression of invasion by cisplatin. TIMP-1 also inhibited migration of C33A (another human cervical carcinoma cell line) and A549 (human lung carcinoma cell line) through Matrigel. This anti-migratory effect through upregulation of TIMP-1 could be another way cisplatin fights cancer progression (Ramer, Eichele et al. 2007).

The possible receptor component CD44 is expressed in prostate cancer, especially in tumorigenic, metastatic cells (Liu 1994; Lokeshwar, Lokeshwar et al. 1995). In fact, CD44 is a marker for prostate cancer stem/progenitor cells in general (Hurt, Kawasaki et al. 2008), including PrTuSC (Finones 2009).

Our previous study (Chapter 4) showed that PrTuSC secrete relatively high quantities of TIMP-1 and IL-8, and that TIMP-1 is able to stimulate proliferation of HUVECs. In the present study, small molecule inhibitors of various pathways were tested to see which ones were able to abrogate the growth effects of TIMP-1 and PrTuSC conditioned media. Activation of the PI3K/Akt and MEK/ERK MAPK pathways was found. *In vitro*, HUVECs were found to express components of TIMP-1 receptor complexes discovered by others. TIMP-1 was found to colocalize somewhat with proMMP-9, CD63 and CD44. Therefore the possibility exists that TIMP-1 activates these effects via binding to a cell surface receptor which could be either a CD63/b1 integrin complex or a CD44/proMMP-9 complex. Further studies will be necessary in order to verify a surface receptor for TIMP-1 on HUVECs, and to tie it more strongly to the activated pathways and functionalities.

Chapter 6, in part, is currently being prepared for submission for publication of the material. Frimodig, Jane; Gough, David. The dissertation author was the primary investigator and author of this material.

Figure 6-10 is reprinted from Methods in Enzymology, Vol 248, Murphy, Gillian; Willenbrock, Frances, Tissue inhibitors of metalloendopeptidases, 496-510, 1995, with permission from Elsevier.

Table 6-2 is reprinted with permission from John Wiley and Sons, International Journal of Cancer, Mammary carcinoma cells over-expressing tissue inhibitor of metalloproteinases-1 show vascular endothelial growth factor expression, Yoshiji, Hitoshi; Harris, Steven R.; Raso, Erzsebet, et al. Dec 6, 1998, 81-87, Table II page 86.

Chapter 7: Paracrine Effects of PrTuSC *in Vivo*

7.1 Introduction

In vivo cancer studies used the dorsal skinfold window chamber in rodents as early as 1943 (Algire and Chalkley 1945). Endrich modified the chamber for the unanesthetized hamster (Endrich, Asaishi et al. 1980); Intaglietta's lab perfected the model for the study of microcirculation of the awake hamster; Jain has used it intensively for cancer vascularization studies; Dewhirst in North Carolina and Menger and Messmer in Germany have used it for *in vivo* studies of vascularization of transplanted beta islets, for biomaterials studies, and for a range of other studies (Leunig, Yuan et al. 1992; Torres Filho, Leunig et al. 1994; Brown, Campbell et al. 2001; Menger, Laschke et al. 2002). It has been used in our lab to study oxygen sensors and oxygen diffusion in tissue (Makale, Lin et al. 2003; Makale, Jablecki et al. 2004; Makale, Chen et al. 2005).

A paper concerning the use of the dorsal skin-fold window chamber on the nude mouse for microvascular studies has been particularly helpful, as this is the animal model used in this research (Lehr, Leunig et al. 1993). The nude mouse is unable to produce T-cells, (which are the main mode of immune system attack against foreign body invasion (i.e. transplants)), due to thymic aplasia. Human cells can therefore be implanted into this model without fear of T-cell attack.

The dorsal skin-fold model has been used extensively to study neovascularization, and most of the work has focused on cancer vasculature. *In vivo* fluorescent microscopy facilitates examination of cells placed in the animal. With this tool, implanted cells can be localized and vessel diameter, density, tortuosity, and permeability can be quantified. *In vivo* fluorescent videography enables one to examine functionality, i.e. the blood cells can be seen moving. Therefore their velocity and functional vessel density can be quantified. Leukocytes (white blood cells) can also be tagged with a fluorescent marker (Rhodamine 6G or acridine orange) to discover if they are rolling, adhering and extravasating or not; in this way the

degree of inflammation in the subject can be evaluated. A caveat: the window chamber preparation is essentially a chronic wound so it is most likely never free from inflammation during its lifetime, but a minimum of inflammation is desirable.

We used this tool to examine the *in vivo* behavior of PrTuSC B+T and iPS-PrTuSC. Unfortunately, primary PrTuSC would not survive when transplanted into the window chamber. In this work cells contained in collagen and alginate disks, and alginate beads containing angiogenic proteins were transplanted into the window chamber and their effect on angiogenesis was observed.

7.2. Materials and Methods

7.2.1. Materials

eGFP HFFs (enhanced green fluorescent protein human foreskin fibroblasts) and eGFP MCF7 human breast carcinoma cell line were the kind gift of Rita Finones and Martin Haas at the Haas Lab, UCSD. Human Umbilical Vein endothelial cells (HUVECs) were obtained from Lonza (BioWhittaker, Switzerland). Exogenous growth factors used were: TIMP-1 from human neutrophils from Alexis Biochemicals, AXXORA, LLC, San Diego CA, purity $\geq 92\%$ by SDS-page and western blot and rhIL-8 expressed in *E. coli*, I-1645 Sigma-Aldrich (St. Louis MO). PKH26 fluorescent cell stain, FITC-dextran, rhodamine 6G and alginate were from Sigma. Nude mice were purchased from UCSD Animal Care Program and Charles Rivers Laboratories (Cambridge, MA). Mice were treated in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. Protocols were approved by the University of California San Diego Institutional Animal Care and Use Committee. Mice were female for the MCF7 implantation but otherwise males and between 26-32 g at the time of purchase. The mice were anesthetized using ketamine/medetomidine cocktail (50 mg ketamine and 1 mg/ medetomidine (Domitor, Pfizer)/100 g body weight) injected intraperitoneally with a 30 ga $\frac{1}{2}$ " needle. Parts for the air-jet bead fabricating apparatus and nylon hardware for the window chambers were purchased from Small Parts (Lexington KY). Disposable 2 mm Tru-Punch biopsy punch was by Sklar (West Chester, PA).

Titanium window chambers were fabricated by the UCSD campus research machine shop. Delrin window chambers were fabricated by Mark Frimodig. Delrin and other plastic stock was purchased from Plastics International (Eden Prairie, MN).

7.2.2. Cells in Beads

Cells were initially immobilized in alginate beads. Alginate (Alginic acid sodium salt, from brown algae, viscosity of 2% solution at 25°C about 250 cps, Sigma A2158, St. Louis MO) was prepared by adding 2% w/v to 0.9 sterile NaCl solution and mixing gently for 3 days. This is because it takes time for the alginate to absorb water and swell. Next, the alginate was sterilized by filtration through a 0.45 µm syringe filter followed by a 0.22 µm syringe filter (Millipore). Cells were trypsinized, counted, pelleted by centrifugation at 1200 rpm for five minutes, then resuspended in the required quantity of sterile alginate to give the desired cell density. In some cases, cells were stained with the fluorescent cell stain PKH26, per manufacturer's instructions, immediately prior to encapsulation. Finally the cell/alginate solution was loaded into a 1 ml syringe and extruded through a 30 ga needle into a 0.10 M calcium chloride bath. The Ca²⁺ ions serve to cross-link the COO⁻ moieties on the alginate, forming a gel. The alginate stream from the 30 ga needle breaks into spheres under the demands of surface tension to form perfect beads when they are immersed in the CaCl₂ bath. Beads were then washed three times in PBS and placed in medium in a 37°C 5% CO₂ humidified incubator until needed.

Cell densities in alginate used in studies by others ranged from 1x10⁶ cells/ml alginate (Steinert, Weber et al. 2003; Khattak, Spataro et al. 2006) up to 40 x 10⁶ cells/ml for chondrocytes (Wong 2004). We optimized cell density *in vitro* using eGFP HFF cells. Cells encapsulated at densities from 1 to 20 x 10⁶ cells/ml had about the same acceptable survival rates after 10 days *in vitro* (about 85% viability).

Using the syringe and 30 ga needle only allowed us to make beads in the size range of 600-1000 µm diameter, but we wanted smaller beads to put into the window chamber, as

larger beads were crushed in the $\sim 500\ \mu\text{m}$ fluid-filled space between the cover glass and the tissue. Therefore, a coaxial air jet needle was devised. Pressurized air was passed through a pressure gauge, flow meter, filter (Millipore), and finally through a custom made 28 ga needle into the side of a glass tee containing the 28 ga needle. The alginate was loaded into a syringe which attached to a hub at the top of tee, where it accessed the 28 ga hypo needle. The syringe hub was glued into an opening drilled into a bakelite cap which threaded onto the glass tee. The bottom leg of the glass tee was fitted with a 16 ga stainless steel hypo tube air jacket, which had 3 holes radially drilled in it for screws to hold the 28 ga needle centered inside, 1 cm from the bottom end of the air jacket. The 28 ga needle was placed inside a larger needle with the same inside diameter as the small needle's outside diameter, to give enough structural strength so the 28 ga needle would not be bent. The air jacket and needle were fitted into a glass tee that allowed alginate to be fed into the needle through the top of the tee, while pressurized air entered the tube through the side of the tee. The side entry was connected with tygon tubing to a low pressure gauge, valve, and air filter then finally to the pressurized air source (either house air or a tank of pressurized air). The end of the needle must be 1 mm inside from the end of the surrounding air jacket tube at the exit end. This way, the pressurized air breaks up the alginate stream making smaller beads. Inside needle diameter and air flow were the two main parameters controlling final bead size. Air pressure of about 5 psi and a distance of 5 cm from the needle outlet to the top surface of the bath gave the best results. With the airjet apparatus, we were able to make uniform beads $200\ \mu\text{m}$ in diameter and smaller. These fit better into the window chamber, where they must fit into the space between the exposed tissue side of the skin and the chamber window. However, there were several problems with the beads which led us to choose a different geometry. One problem was that the volume of the $200\ \mu\text{m}$ bead was so small that even at the 10 million cells/ml density, only $\sim 42,000$ cells could fit into $200\ \mu\text{m}$ diameter bead. When paracrine effects are being studied, more cells may be needed to secrete enough protein to show an effect. If we made a bigger bead to contain more cells, there were issues with the bead

pressing on the blood vessels causing pressure-induced changes in the vasculature that were undesirable in this assay, and with the bead simply being crushed as well. Therefore we decided an alginate disk might be a better geometry for the window chamber.

To make alginate disks, alginate/cell solution was poured onto a flat surface (in this case the bottom of a 6-well tissue culture dish), and a flat object (a glass cover-slip) was placed on top and weighted in place. Three stainless steel wires laid around the perimeter of the bottom of the well, of a known thickness, functioned as spacers to produce the desired thickness of alginate film (in this case ~0.5 mm). 0.10 M CaCl_2 solution was added to the well and incubated for 10-20 minutes. Then the CaCl_2 was aspirated. Next the weight and coverglass were removed and disks were made using a 2 mm sterile biopsy punch.

The newly formed disks were placed in a 100 μm cell filter and washed three times with PBS, then placed in medium in a humidified 37°C 5% CO_2 incubator until implantation.

7.2.3. Cells in Collagen Disks

Recombination Disks were a kind gift of the Haas lab. Each disk consisted of a total of ~20 SCC/PrTuSC colonies (SCC = Stem Cell Center) combined with 10^5 urogenital sinus mesenchyme (UGM) cells in a collagen matrix. Tissue recombination with UGM mimics the embryonic microenvironment, in which the urogenital sinus develops into urinary and reproductive organs, and has proven its utility in enabling human prostate cells to differentiate *in vivo*. The disks were 4-5 mm in diameter and about 0.5 mm thick. In the experiments we also used some collagen disks containing PrTuSC with no UGM added.

7.2.4. Proteins in Alginate Disks

To test for paracrine effects on neovascularization, exogenous proteins were immobilized in alginate disks and implanted into the window chamber. The alginate disks were made in the same way as described previously with cells, except instead of adding the cells, exogenous TIMP-1 or IL-8 were added to the alginate solution prior to forming the film.

7.2.5. The Dorsal Skin Fold Window Chamber

Makale et al provided a detailed description of the window chamber surgery (Makale 2007; Makale 2008), but that surgical technique will be briefly summarized here.

7.2.5.1 Surgery

Nude mice were anesthetized using 50 mg ketamine and 1 mg medetomidine per 100 g animal body weight, injected intraperitoneally. The window chamber consists of two matching titanium halves (Figure 7-3), between which a fold of the elastic back skin of the mouse is sandwiched (Figure 7-1). The left side of the window chamber (Figure 7-3A right) shows a small technical modification with three nylon bolts held in place with threaded stainless steel spacers (about 0.5 mm thick) to prevent the sandwiched skin fold or any implanted constructs from being squeezed in the window chamber. Prior to surgery, the back skin of the animal was thoroughly soaked in betadine which was then wiped clean with alcohol pads. The surgery was performed under sterile conditions in a biosafety cabinet to minimize the risk of infection. A fold of the backskin was first raised and suspended from three sutures attached to two “goal posts” on either side of the surgical stage. One half of the window chamber was suspended by bolt at its top from the skin fold. Two temporary sutures were placed at the top of the window chamber to secure it to the skinfold during surgery. Then using a #20 blade scapel and 4 mm biopsy punch, holes were made through both skin thicknesses of the skin fold to accomodate two bolts at the bottom sides of the window chamber. These two bolts were then pulled through the skin fold and temporarily secured with bulldog clips following the application of triple antibiotic ointment to the bolt holes. Next, a circle of 15 mm diameter was carefully removed from the top skin layer of the exposed side of the skin fold, followed by removal an inner facia layer with iridectomy scissors. This procedure leaves the inside layer of skin muscle (M. cutaneous max.) exposed and gives a clear view of the

underlying blood vessels (Figure 7-1). During surgery, the exposed tissue was irrigated with sterile saline containing 20% tobramycin to prevent drying out. Finally, the second half of the window chamber, holding a circular cover glass in its center (Figure 7-3A left), was placed over the side where the skin was removed and affixed to the other side of the chamber with the three bolts. The skin was further secured with sutures along the top of the window chamber. The exposed muscle layer and its vasculature could be viewed through the cover glass (Figure 7-1). The window chamber was fixed permanently to the skin by sutures at the top and sides of the chamber. The animals were given an antidote (Antisedan™, atipamezole hydrochloride, Pfizer) to expedite revival after surgery. Animals were carefully observed during the recovery process and daily after the surgery to ensure minimal stress.

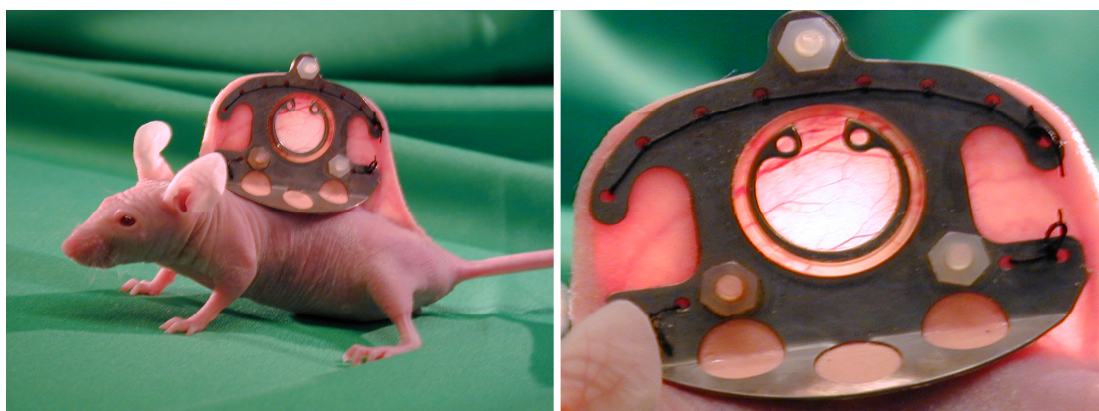


Figure 7-1.

The dorsal skin fold window chamber.

7.2.4.2. Chamber Modifications

Several changes were made in the window chamber over the course of this work to improve performance. The window chamber has been used extensively in our lab on hamsters. Although the mouse window chamber was scaled down from the hamster version, the titanium, even though being a metal with excellent strength to weight ratio (4.43 g/cm^3) was still too heavy for the mouse. One to two weeks after surgery, the delicate back skin of the nude mice would stretch to the extent that the chamber would flop completely over to one

side, which no doubt compromised circulation. First, in order to make the chamber lighter, the stainless steel hardware (nuts and bolts) was replaced with nylon hardware (1.14 g/cm^3 as opposed to stainless steel, about 7.5 g/cm^3). Figure 7-1 shows the titanium window chamber fitted with nylon hardware. This lightened the chamber by 0.7 g, but it was still too heavy for the mice. We decided to try a polymer since these materials average about one g/cm^3 and can be selected for the desired qualities. A database of potential polymers for the window chamber was developed. Desired properties were first of all biocompatibility, then strength, low moisture absorbance, chemical resistance, and heat resistance (autoclavable). First we had the machine shop make a prototype of Duracon (an acetyl copolymer or POM), but this material had several disadvantages. It was somewhat brittle, in addition it autofluoresced which was unacceptable for fluorescent imaging. Finally Delrin (the DuPont tradename for polyoxymethylene, from Plastics International), a polyacetal homopolymer (1.42 g/cm^3) that is FDA approved, was selected (Figure 7-2). To save costs, a family member fabricated the window chambers out of Delrin, based on the design of an acrylic window chamber which had been a gift of Dr. Ruijuan Xiu of Peking Union Medical College, Institute of Microcirculation, Beijing China. The design was slightly modified to reinforce the top bolt area. This design was quite successful, being able to last on the mouse for 5-6 weeks before beginning to flop. Originally we used the Duracon coverglass-holding ring fitting made by the machine shop which was a part of the window chamber requiring precision, but when those became brittle and cracked (after 2-3 surgeries) we went back to the old metal part. Figure 7-3 shows the titanium window with the Duracon coverglass-holding ring fitting which is transparent (The coverglass sits on this ring, and the stainless steel metal retaining ring holds the coverglass in place). Overall the window chamber was still light enough to prevent flopping. Upon weighing the titanium and the Delrin window chambers, it was discovered that the Delrin chamber actually weighed 0.5 g more than the titanium chamber. This is because the plastic chamber needed to be thicker in order to maintain the minimal stiffness. However, the plastic chamber

still did not flop over like the titanium chamber did. This could have been due to a better distribution of center of gravity in the plastic chamber.

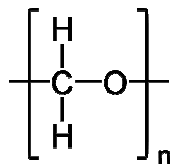


Figure 7-2.

Delrin (polyoxymethylene).

Performance of the window chamber skin fold is totally dependent on perfusion. Accordingly, it was desirable to minimize the amount of destruction to the bottom part of the skin fold through which blood vessels must remain intact to provide blood flow to the top part of the skin fold. For this reason we stopped putting additional sutures in this area. To keep the skin from falling, a few more stitches had to be added to the top of the window chamber. This however allowed perfusion that could minimize inflammation and maintain viability of the back skin for up to 6 weeks.

To prevent the back skin from being squeezed too much, we designed a spacer nut which was a m2 stainless steel threaded nut milled down to 1.5 mm high (the spacing between the chamber halves) and 3 mm in diameter. It was important to minimize the size of the bolt-holes in the back skin for the reasons mentioned above. The spacers are also important in that they keep the bolts immobilized in the half of the window chamber that is placed initially during surgery, before the second half is added and the bolts can be secured by nylon nuts on the outside of the window chamber.

Now the only problem with the Delrin chamber was, the mice seemed to enjoy chewing on the flanges. Diligent applications of Mavala Stop (Mavala International, Switzerland), a very bad-tasting nail polish designed to stop nail-biting, and Fisher tape combined to minimize this problem.

7.2.5.3. Postsurgical Care and Observations

The weight of the mice was monitored. The mice were observed daily to insure that they were eating, drinking and moving around their cages normally.

7.2.5.4. Bead and Disk Implantation

Three days after surgery the mice were anesthetized again as described before, and the retaining ring which holds the cover glass in place and the cover glass were removed. Beads, alginate disks containing cells or protein, or recombination disks were placed into the window chamber under sterile conditions directly on the exposed tissue muscle layer, before sealing the window with a new sterile cover glass and the retaining ring. Implanted cells in the window chamber could be observed immediately *in vivo* with a microscope. Figure 7-3B shows the imaging stage apparatus for microscopic observation of the window chamber. In order to image the window chamber, the mouse is immobilized in the acrylic tube with the window chamber protruding through a slit on top of the tube. To keep the window chamber stable during microscopy, it is fixed over an acrylic block with a square hole in the middle and can be viewed with a long working distance objective.

7.2.5.5. Monitoring the window chamber

The mice were examined under the microscope (Olympus BX51W1 with 7700 OptiQuip Lamp house with 200 W mercury/xenon lamp (Ushio) and OptiQuip 1600 power supply using four different filter cubes: Olympus U-MBF3 (bright field); Olympus U-MNB2, excitation 470-490 emission 515 filter cube for imaging FITC and eGFP; and OMEGA xF108-2, excitation 525 and emission 595, for imaging Rhodamine 6G and PKH26 cell stains; and an

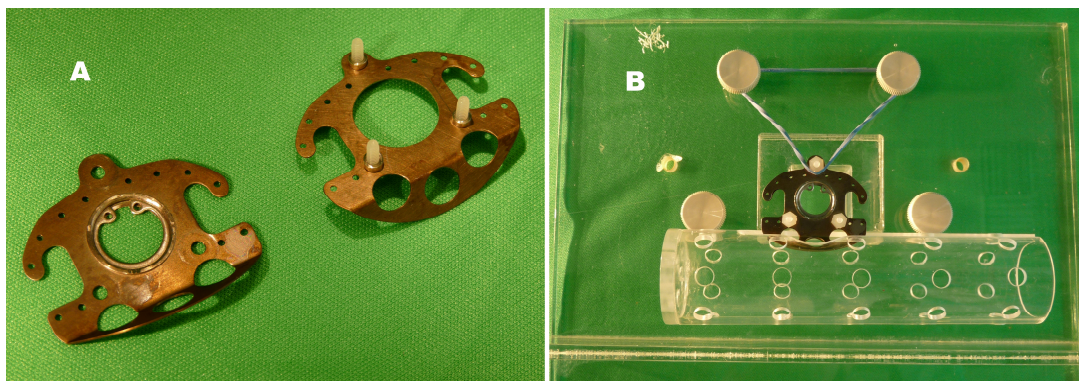


Figure 7-3.

Titanium window chamber and acrylic microscope stage.

A) The titanium dorsal skinfold window chamber. B) Microscope stage set-up showing tube in which mouse is immobilized. The window chamber protrudes through the tube as shown so the window chamber can be imaged. The microscope stage was the kind gift of Dr. Ruijuan Xiu, PUMC, Institute of Microcirculation, Beijing, China.

Olympus UMDIC3 transmitted light Nomarski differential interference contrast filter cube for imaging blood vessels. The mice were photographed three times a week for the duration of the window chamber with an Olympus E-620 digital camera mounted on the microscope.

Once a week (usually Monday), 50-100 μ l of 5% FITC-dextran (500 kDa, Sigma), a vascular tracer, was injected into the tail vein using a 30 ga needle, and videos were taken using a COHU 4915-2000/0000 high performance monochrome CCD camera and Pinnacle Studio Plus V. 10.8 software. Also once a week, but on a different day (usually Friday), 100 μ m of 1% Rhodamine 6G (Sigma) 1.45 mg/10 ml in sterile injectible saline, was injected via tail vein to label the leukocytes, and videos were taken.

Inflammation was monitored by labeling of the leukocytes with Rhodamine 6G. The window chamber was videoed in the region of the implant and also at control regions away from the implant. A fairly straight segment of blood vessel, usually a small vein or venule, was chosen and measured using NIH ImageJ. These dimensions were used to calculate the inside surface area of the blood vessel in the region of interest. The quantity of fluorescently labeled leukocytes either adhering, rolling or flowing freely past was counted in the region of interest

for a given period of time. These quantities were normalized per surface area unit of blood vessel and time to give quantities indicative of inflammation.

FITC-dextran consists of a fluorophore (fluorescein isothiocyanate) attached to a polysaccharide (dextran) of known molecular weight used to label plasma. It was used to help visualize the blood vessels and to ascertain their functionality. Red blood cells appear as dark moving spots against the brightly stained plasma on the monochrome CCD camera. We used three molecular weights: 500 kDa, 250 kDa and 150 kDa. The smaller molecular weights diffuse out of the blood vessels more quickly but the larger ones have a longer residual in the tissue where they can give unwanted background to images being taken at later dates. The blood vessels in regions of interest were photographed using the Olympus UMDIC3 transmitted light Nomarski differential interference contrast filter cube. This filter makes the blood vessels stand out very well and these photos could be used for image analysis using ImageJ free NIH software. For image analysis the photos were opened in ImageJ and split into RGB. The red and blue channels were thrown away and the green channel was modified to eliminate background, binarized, and subjected to particle count. This counts the pixels that were black (ie dark, blood vessels) in the region of interest, so it can be used to obtain blood vessel area as a percentage of total area. Regions of interest that were within the bead or disk were selected for analysis, as well as control areas away from the bead or disk.

7.3 Results

7.3.1 Cells in the Window Chamber

Fluorescent protein eGFP-expressing Human Foreskin Fibroblasts (eGFP HFF) and MCF7 breast carcinoma in alginate beads, PKH26-stained HUVECs, and PrTuSC B+T in collagen disks and recombination disks were placed into the window chamber in various surgeries. Details and results are described in Tables 7-1 and 7-2.

Fibroblasts in alginate beads induced angiogenesis (Figure 7-4), but the HUVECs, MCF7 cells in an alginate bead or PC-3 prostate carcinoma cells implanted in collagen did not seem to.

Most notably, PrTuSC B + T in collagen disks survived in the window chamber, yet did not induce any noticeable angiogenesis. PrTuSC B+T cells in a recombination disk, however, induced striking angiogenesis in the window chamber (Figure 7-5). Figure 7-6 shows a graph of blood vessel density in this window chamber.

PrTuSC iPS cells survived and filled in the window chambers. They induced angiogenesis but it was not easily visualized due to the overproliferation of the cells.

Table 7-1.
Cells in alginate beads and disks

mouse	encapsulation	cells	cell density (cells/ml)	blank (control)	results
NM38	2% alginate bead	double-stained HFF	don't know	no	cells in beads stimulated angiogenesis, see Figure 7-4
NM42	1% and 2% alginate beads	none	--	--	small neovascularization on beads
NM52	2% alginate bead	doubled-stained MCF-7	1.5×10^6	yes on right	beads too small, not enough cells for effect; small neovascularization equal on control and cell beads
NM55	2% alginate disk	none	--	--	very small amount neovascularization around disk
NM57	2% alginate disk	eGFP-HFF and PKH-26 EC	5×10^6 each		small amount neovascularization around disk

Table 7-2.
Cells in collagen disks

mouse	disk	cells	blank (control)	results
NM43	collagen	p12B+T	no	some p12B+T cells survived but did not stimulate neovascularization
NM43	collagen	PC3	no	PC3 cells survived but did not induce any neovascularization
NM48	collagen + UGM	p74t2:4G (iPS)	yes	cells grew throughout chamber and stimulated angiogenesis
NM49	collagen + UGM	p72t2:5G (iPS)	yes	cells multiplied and filled in chamber; stimulated angiogenesis
NM50	collagen + UGM	p74t7:B+T	yes	cells remained localized and stimulated angiogenesis; control side did not stimulate angiogenesis, see Figure 7-6

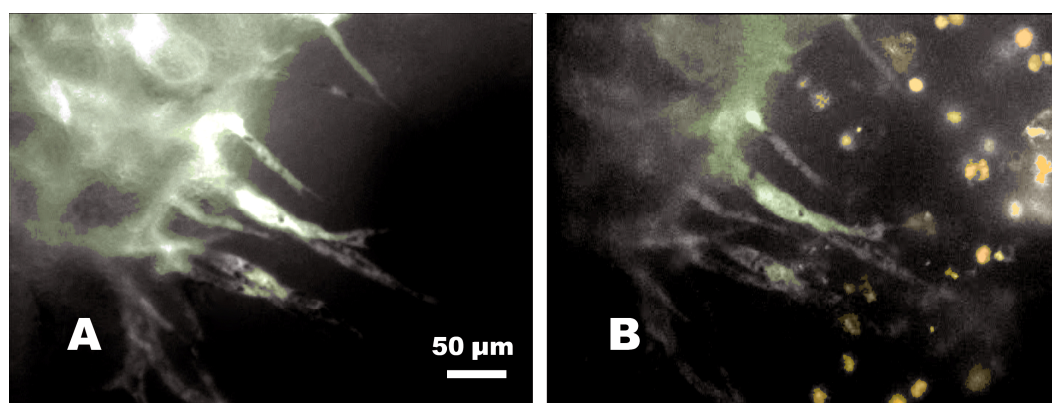


Figure 7-4.

Human foreskin fibroblasts immobilized in an alginate bead in window chamber.

A) Blood vessels sprouting towards the cells are perfused with vascular tracer FITC. B) a different wavelength filter reveals the fluorescently stained cells.

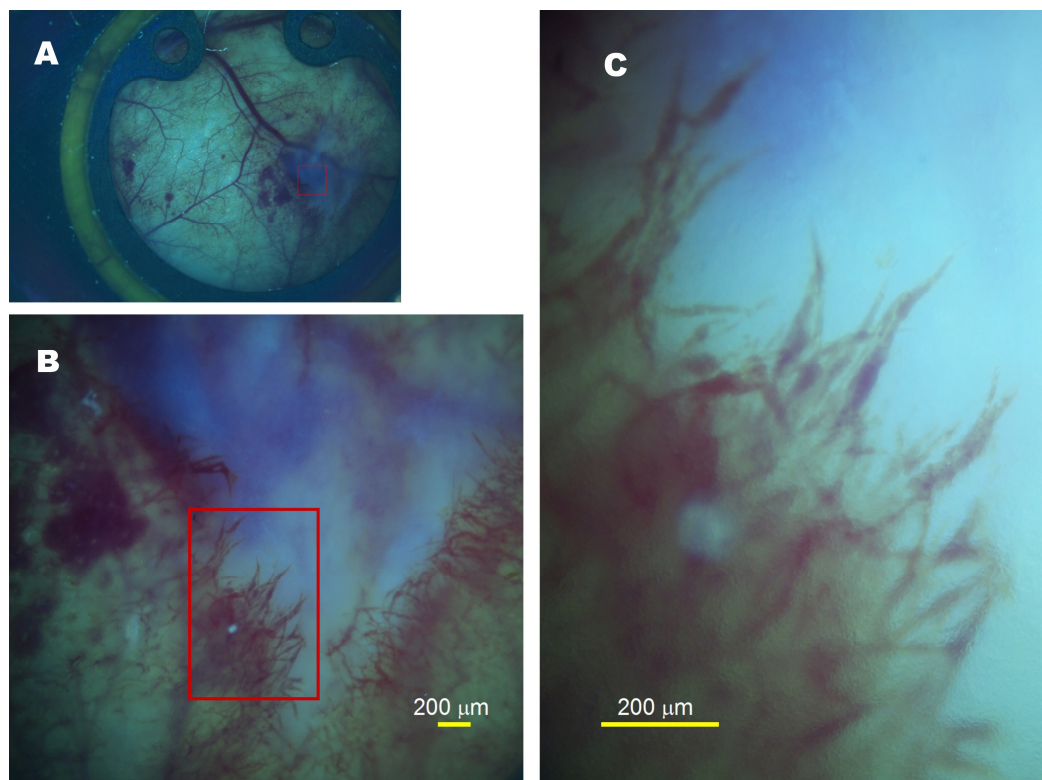


Figure 7-5.

Angiogenesis induced by PrTuSC B+T cells in a collagen recombination disk with rat UGM in the window chamber (NM50).

A) the entire window chamber with recombination disk at center right side. B) close-up of sprouting into disk, the cloudy area is the disk. C) close-up of blood vessel sprouts.

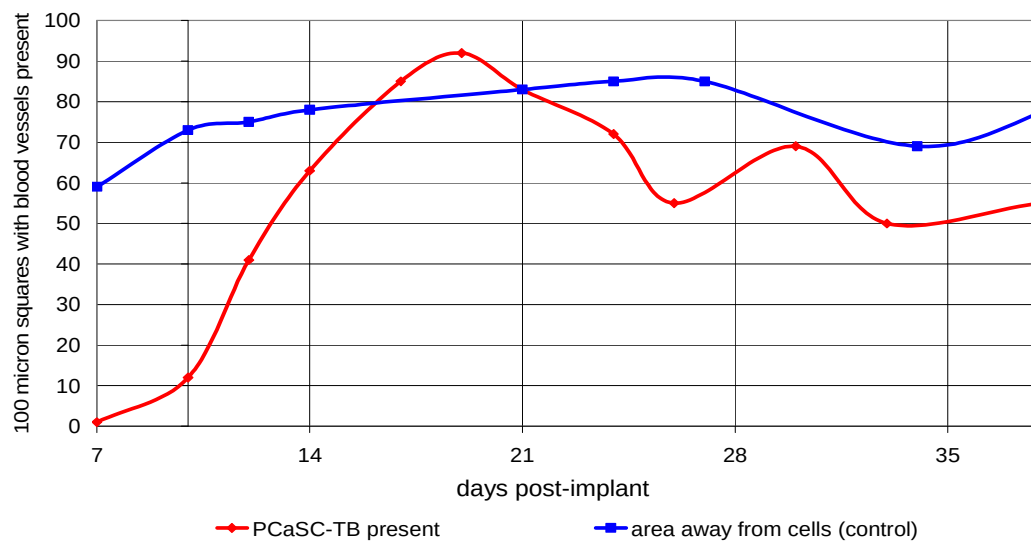


Figure 7-6.

Changes in blood vessel density due to the presence of PrTuSC recombination disk in the window chamber (NM50).

7.3.2 Disks Containing Protein in the Window Chamber

Alginate disks containing 100 ng/ml human II-8 and 100 ng/ml TIMP-1 stimulated angiogenesis in the window chamber. Table 7-3 gives a synopsis of protein-containing disks that were implanted into the window chamber. II-8 induced angiogenesis by Day 5 post-graft and TIMP-1 by Day 7. Figure 7-7 shows blood vessels sprouting into the disk, and Figure 7-8 is a graph of blood vessel density under the TIMP-1 disk, the control disk with no protein, and in an area away from the disks. The blood vessel density differed significantly between these three cases.

Table 7-3.

Proteins in alginate disks in the window chamber.

mouse	protein	blank	Results
NM56	100 ng/ml II-8	yes	strong angiogenesis in II-8 bead by Day 10; none apparent in control disk
NM59	100 ng/ml II-8 (2 disks)	no	strong inflammatory-like angiogenic response around both disks by Day 4
NM60	100 ng/ml TIMP-1	yes	General inflammation in window chamber by Day 5, strong angiogenesis around TIMP-1 disk
NM61	100 ng/ml TIMP-1	yes	more angiogenesis in TIMP-1 disk than control, see Figure 7-8
NM62	100 ng/ml TIMP-1 + 100 ng/ml II-8	yes	some angiogenesis, no difference between disk with protein and control (blank)

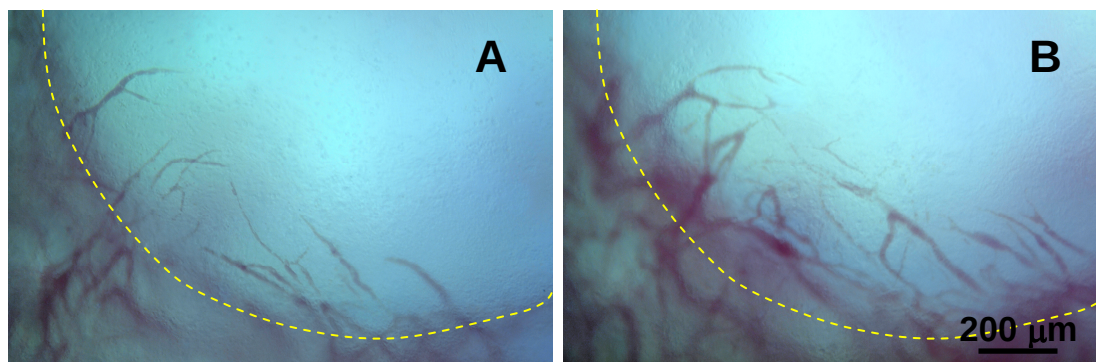


Figure 7-7.

Blood vessels sprouting into an alginate disk containing TIMP-1 in the window chamber (NM60).
The dashed line shows the outline of the disk.

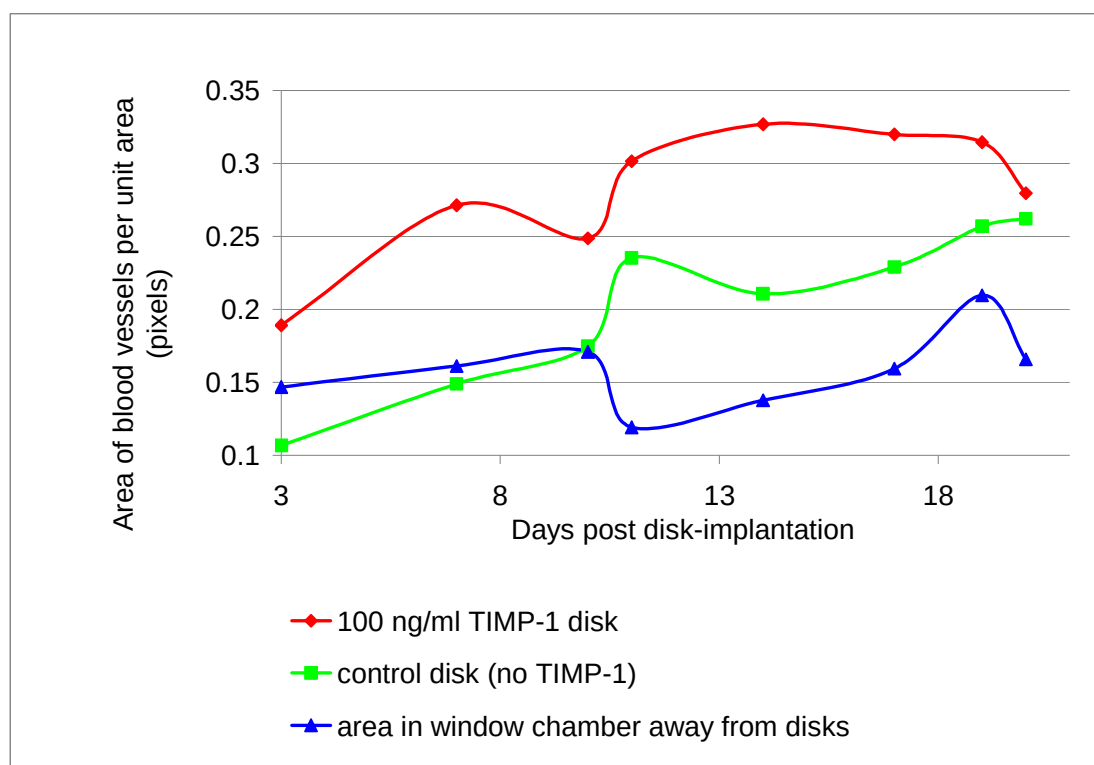


Figure 7-8.

Changes in blood vessel density induced by the presence of an alginate disk containing 100 ng/ml TIMP-1 in the window chamber (NM61).

7.4. Discussion

7.4.1 *In Vivo* studies

We found that PrTuSC B+T cells immobilized in a collagen disk survived in the window chamber. If UGM was included in the collagen disk, the cells seemed to be capable of stimulating normal angiogenesis. Since we did not do a control with only UGM cells, however, we do not know how much this regulation might have depended on them. UGM are known to exert a 'normal' regulatory influence over PrTuSC, and Haas lab showed this when PrTuSCs in recombination disks implanted into the kidney capsule of SCID mice grew into 'normal', not tumorigenic, prostate organs. PrTuSC B+T implanted into the window chamber in collagen disks without UGM did not form apparent neovascularization, although the cells appeared to survive. This implies that the UGM may have been an important component of the recombination disk in influencing angiogenesis. iPS PrTuSC are not a very good cell model to study angiogenesis in the window chamber, as they were subject to overproliferation and filled the chamber and apparently, neovascularization was not stimulated in parallel.

PrTuSC were found to express relatively high quantities of IL-8 and TIMP-1 into their cell culture medium. Accordingly we put these proteins in alginate disks in the window chamber to study their effect on angiogenesis. Both seemed to stimulate neovascularization, but judging by the leukocyte assay (data not shown), which is an index of inflammation, this condition was relatively high in the neighborhood of the disks and may have been an additional unwanted stimulus to neovascularization. It could be that some impurity found its way into the disks in these experiments even though every precaution was taken. In contrast, inflammation index as measured by leukocyte activity remained low at all times in the window chambers with cells implanted. At any rate, alginate disks containing IL-8 and TIMP-1 both stimulated angiogenesis in the window chamber.

7.4.2. Cell Encapsulation

Alginate is the classic cell encapsulation material. It has been extensively studied and characterized. It is a disaccharide, consisting of guluronic and mannuronic acids (Figure 7-9), which is isolated from seaweed. Usually the cells are encapsulated simply by suspending them in an alginate solution, then shooting the solution through a small gauge needle into a CaCl_2 bath. The carboxyl groups on the guluronic acids are crosslinked by the Ca^{2+} ions in the bath, forming a spherical bead (Gu, Amsden et al. 2004). The beads have very good biocompatibility, especially if the alginate is very pure (DeVos, DeHaan et al. 1997). Alginate has been used to encapsulate beta islets from the pancreas (Lim and Sun 1980; DeVos, DeHaan et al. 1996), fibroblasts (Tobias, Dhoot et al. 2001), fetal kidney cells transfected to express endostatin (Read, Farhadi et al. 2001) and mesenchymal progenitor cells (Steinert, Weber et al. 2003).

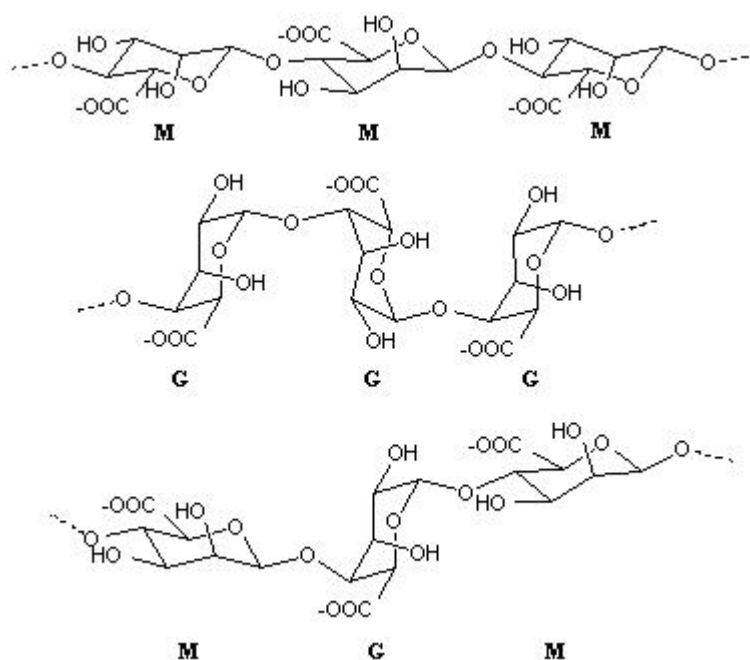


Figure 7-9.

Alginate oligosaccharides. M stands for mannuronic and G for guluronic acid units.

Alginate has also been investigated for the encapsulation of many growth factors and other biological products, including VEGF (Gu, Amsden et al. 2004), sperm (Herrler, Eisner et

al. 2006), fibrinogen and hyaluronic acid (Lindenhayn, Perka et al. 1999). When VEGF was encapsulated in alginate beads, a fairly steady release rate of six ng/ml/day could be sustained for two weeks *in vitro* (Gu, Amsden et al. 2004). When 150 ng TGF- β 1 was encapsulated in 200 μ l alginate pellets and stored in PBS, 63% $\pm$ 30% eluted within the first few hours, then release rate plateaued with about 22% left inside the pellets for five days. When implanted into brain embolisms in dogs, 50% of the protein eluted out in the first hour and 80% by 24 hours. Only 6% remained in the alginate after 1 week. (Raymond, Metcalfe et al. 2003). Another study used BSA as a model protein and studied its elution from alginate beads and films at different CaCl_2 (crosslinker) concentrations. Alginate films eluted from 19-46% of their BSA within nine days and from 83-95% within 36 days (Hermes and Narayani 2002). FGF had the highest *pI* of proteins evaluated in a study by Downs et al. (9.6) and the highest entrapment efficiency in alginate beads. Alginate beads containing 5-15 ng of TGF- α , bFGF or EGF were injected subcutaneously in mice; all resulted in increased neovascularization in 14 days while simple injection of the angiogenic proteins did not (Downs, Robertson et al. 1992).

Chondrocytes were kept in alginate beads *in vitro* for 14 days. 43% of the IL-8 made by cells during this time was retained in beads (Sanchez, Mateus et al. 2002). Tumor cells encapsulated in RGD-modified alginate secreted significantly more IL-8 than cells in 2D cultures, but VEGF and bFGF expression was the same as in 2D. *In vivo*, the encapsulated cells made smaller and less blood vessels and less bone marrow cell recruitment occurred when cells were engineered to secrete a IL-8 blocking antibody. The IL-8 effect, due to more integrin signaling, was independent of VEGF activity (Fischbach, Kong et al. 2009).

Taken together, these studies show that growth factors encapsulated in alginate remain active and are generally released in an initial burst release, followed by a slow gradual release for time periods comparable to the duration of the window chamber model on the nude mouse.

While encapsulated cells remain viable, they do not seem to actively proliferate in alginate beads, according to our observations and to a study that found by the third day in beads, 92.5% of human mesenchymal stem cells had entered G_0G_1 phase (Ma, Hung et al. 2003).

The main reason for encapsulation has been to protect allogenic or xenogenic grafts from the host immune system. This was not much of an issue in these studies as immunodeficient mice were used. Other reasons for encapsulation include immobilization, 3-D effects on cell growth, and ease of recovery. In the case of stem cells, a major motivation for encapsulation is the fact that adult stem cells do not survive well outside their niche in an undifferentiated state. Therefore encapsulation may be necessary to insure survival and integrity of the transplanted stem cell population. Immobilization is also crucial to window chamber studies in order to keep cells in a visible area. Unimmobilized eGFP fibroblasts implanted in the window chamber in a cell suspension in preliminary studies survived, but they dissipated all over the chamber, making it difficult to monitor their proliferation or their effects on neovascularization.

Studies have found that when cells are encapsulated in alginate beads larger than 500 μm in diameter, a necrotic center forms, due to insufficient oxygen supply (DeVos, DeHaan et al. 1996). However the stem cells that were used in this research, as mentioned previously, prefer an hypoxic environment; therefore it is predicted that the total size of these beads can be greater than 500 μm without detriment to these particular cells, and maybe even to their advantage. At any rate, due to space restrictions in the window chamber, we limited bead diameter and alginate disk thickness to 500 μm .

Both PrTuSC, which we have found secrete angiogenic proteins, and fibroblasts, which are known to secrete angiogenic proteins, induced neovascularization when implanted into the window chamber under specific conditions. The PrTuSC had to be co-implanted with UGM to have this effect. Also, exogenous proteins (TIMP-1 and IL-8) encapsulated in alginate disks stimulated neovascularization in the window chamber, although they also seemed to

stimulate some inflammation as well. For IL-8, which is an inflammatory cytokine, this is a natural response, but the TIMP-1 results require verification.

Chapter 7, in part, is currently being prepared for submission for publication of the material. Frimodig, Jane; Li, Hongwei; Xiu, Ruijuan; Gough, David. The dissertation author was the primary investigator and author of this material.

Chapter 8: Conclusion

This research has found that prostate tumor stem cells isolated from early prostate cancer probably do not contribute to tumor angiogenesis by differentiating into blood vessels; but that they produce relatively high quantities of two proteins implicated in angiogenesis, Il-8 and TIMP-1; that TIMP-1 significantly induces proliferation in HUVECs at concentrations of 200-1000 ng/ml and similar concentrations result in small and insignificant increases in tube-forming and migration; and that the proliferation response is induced via activation of PI3K/Akt and possibly MEK/ERK MAPK pathways. It is possible that HUVECs use a receptor complex of CD44 to dock proMMP-9 bound to TIMP-1 for activation of biological response. This would explain why the high blood concentration of TIMP-1 found in cancer and many other disease states is not activating endothelial cells systemically, only at tumors, as tumor ECs have been found to express more CD44. This would also imply an autocrine loop in PrTuSC as they also express CD44. Although colocalization on TIMP-1 stimulated HUVECs was higher for proMMP-9 and TIMP-1 than for CD63 and TIMP-1, it is still possible that the other receptor complex for TIMP-1 consisting of CD63 and $\beta 1$ integrin is used by ECs, as they express both of these proteins. Implantation of collagen recombination disks containing rat urogenital sinus mesenchyme and PrTuSC cells transfected to overexpress Bmi-1 and TERT caused formation of normal (not tumor) blood vessels in a nude mouse window chamber. Il-8 and TIMP-1 contained in alginate disks and beads induced angiogenesis in the window chamber, but results for TIMP-1 were difficult to dissect from inflammation effects.

Since the preponderance of research points to TIMP-1 being anti-angiogenic, anti-migratory, and anti-tumor, targeting TIMP-1 may not result in an efficacious therapy in cancer, but may be worth pursuing in fibrosis or other disease states. In addition to inhibiting ECM breakdown via its inhibition of MMPs, TIMP-1 has a host of more subtle biological functions. These functions are a) complicated and often opposing each other, and b) probably extremely time- and microlocation- dependent. The mystery remains as to why blood levels of TIMP-1

are so elevated in so many diseases. It may just be that upregulation of MMPs or other proteins in these diseases causes a feed-back response in TIMP-1 transcription.

In the case of cancer, we might see TIMP-1 effects on endothelial cells found in this and other research, such as increased proliferation and independence from contact inhibition and anoikis, to come into play in the rapidly proliferating and disorganized tumor vasculature. These effects also might be a contributing factor to the aforementioned irregularities of cancer vasculature. TIMP-1's possible synergy with VEGF, an angiogenic protein that is upregulated in tumors, might contribute to these effects. Increased blood levels of TIMP-1 found in many dangerous cancers might serve partly to promote cancer vasculature; however there must be some mechanism such as a receptor specific to tumor endothelial cells to explain why these effects are not systemic.

The Haas lab's PrTuSC cultures are proposed to consist of a mixed population of stem and transit amplifying cells. We know this mixed population of primary cells produces TIMP-1, but it would be instructive to find out how much TIMP-1 is produced by the stem cells and how much by the transit amplifying cells.

An interesting development in the course of this study was the discovery of how many times the two possibly angiogenic proteins found cross pathways. For example, they both use PI3K/Akt and MEK/ERK MAPK pathways for their mitogenic effects. In addition, Il-8 activates its two receptors CXCR1 and CXCR2 on endothelial cells via Rho and Rac signaling pathways, and Rac activation has been found to result in upregulation of TIMP-1 (Engers, Springer et al. 2001; Schraufstatter, Chung et al. 2001).

This research has shown that PrTuSC do indeed have a strong angiogenic potential, due to their expression of angiogenic proteins and their stimulation of angiogenesis *in vivo*. PrTuSC made relatively high quantities of TIMP-1 and Il-8 and that TIMP-1 has the ability to stimulate proliferation in endothelial cells via PI3K/Akt and possibly MEK/ERK MAPK pathways. Dysregulation of contact inhibition and normal morphogenic cues and increased proliferation and possibly survival in the Matrigel tube-forming assay points to involvement of

cell-cell and cell-ECM adhesion pathways involving proteins such as FAK, Rho, Rac, and p27, although this research did not investigate these. It is possible TIMP-1 activates these effects via binding to a cell surface receptor which could be either a CD63/ β 1 integrin complex or a CD44/proMMP-9 complex. Our data points tentatively to the latter. TIMP-1 involvement in angiogenesis is probably very dynamic and locally enacted. We can hypothesize that TIMP-1 helps stimulate EC proliferation early in angiogenesis, but does not play a role in migration and vessel assembly based on our research. It is probably that TIMP-1 contributes to the stabilization of newly formed blood vessels by inhibiting MMPs so that basement membrane and ECM can be rebuilt. Further studies will be necessary to confirm the receptor for TIMP-1 on endothelial cells, and to elucidate the pathways involving cell adhesion and migration, which are probably involved in a very dynamic and localized role for TIMP-1 in angiogenesis.

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