AD_____

Award Number: DAMD17-03-1-0451

TITLE: A Role for TIMP-1 in Breast Cancer Progression

PRINCIPAL INVESTIGATOR: James A. Cardelli, Ph.D
Dr. Rebecca Bigelow

CONTRACTING ORGANIZATION: Louisiana State University Health Sciences Center
Shreveport, LA 71130

REPORT DATE: January 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-01-2007	Final	1 Jul 2003 – 31 Dec 2006

4. TITLE AND SUBTITLE

A Role for TIMP-1 in Breast Cancer Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0451

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

James A. Cardelli, Ph.D
Dr. Rebecca Bigelow

Email: jcarde@lsuhsc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Louisiana State University Health Sciences Center
Shreveport, LA 71130

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In order to assess the role of TIMP-1 in breast cancer development, we created several MCF-7 and MDA-MB-231 cell lines which overexpress TIMP-1 as well as MDA-MB-231 cells which underexpress TIMP-1 using shRNA. Compared to vector control cells, MCF-7 TIMP-1(+), MDA-MB-231 TIMP-1(+) cells and MDA-MB-231 (-) cells displayed no change in proliferation, and these cells did not demonstrate activation of the p38, ERK, AKT or FAK signaling pathways. Additionally, exogenously supplied TIMP-1 did not activate these signaling pathways in either MCF-7 or MCF10A cells, contrary to published reports. Also, TIMP-1 overexpression inhibited MCF-7 motility in modified Boyden Chamber assays, whereas no differences in invasion were observed in MDA-MB-231 (-) cells compared to vector controls. *In vivo* xenograft studies in SCID/bg mice revealed that TIMP-1 was able to stimulate tumorigenesis of MCF-7 cells, potentially by stimulating angiogenesis as noted by the increase in CD34 positive blood vessels. No differences in tumor growth were observed with MDA-MB-231 TIMP-1 shRNA cells compared to control cells. Finally, Affymetrix array analysis revealed that TIMP-1 overexpression did have an effect on gene expression in MCF-7 and MDA-MB-231 cells.

15. SUBJECT TERMS
Breast Cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	19	USAMRMC

Table of Contents

INTRODUCTION

The main goal of our research was to determine how TIMP-1 contributed to breast cancer progression, particularly invasion. This MMP inhibitor has historically been considered an anti-cancer protein, but a number of published studies now demonstrate this protein can also act to promote cancer progression (1-4). We originally hypothesized that TIMP-1 could stimulate invasion of breast tumor cells via activation of Ras and other downstream pathways. We further proposed that a new version of TIMP-1 could be genetically engineered that would still inhibit MMP function, but would be inactive in promoting cancer progression. The following represents a complete final report of our studies during the past three years. We have completed tasks one and two, and unfortunately due to unexpected results, task three was altered, as described below.

BODY (Review of data obtained July 1, 2004 to March 1, 2007)

Overview of this Section:

We received reviewers' comments back on our paper entitled "TIMP-1 Induces Epithelial to Mesenchymal Transition in MCF10A Breast Epithelial Cells" that was submitted to Cancer Research. Although they found the study compelling, their main concern was the incomplete nature of the story. In particular, they were concerned that we did not measure TIMP-1 levels in the cell-lines that were generated by another laboratory to overexpress TIMP-1. We therefore re-examined our approach, and attempted to create our own MCF10A TIMP-1 over expressing clones as described below and unfortunately, developed technical difficulties. We decided to change our model system to the MCF-7 and MDA-MB-231 breast carcinoma cell lines, and generated both under and overexpressing TIMP-1 stable cell lines.

The following section summarizes our approaches and successes over the past three years and is divided into a section on publishable data which will be used in two separate publications, as well as additional preliminary data which may or may not be used in publications.

I. Publishable Data

Generation of TIMP-1 MCF-7 and MDA-MB-231 Overexpressing Cell-Lines and MDA-MB-231 underexpressing cell-lines (Relevant to Task 1).

One of our original tasks was to characterize MCF10A cells (received from Dr. Kim at Wayne State University) which overexpressed TIMP-1. A variety of cellular and biochemical assays revealed that these cells had undergone epithelial mesenchymal transition, a process leading to invasion. This was a very exciting finding and we proposed in our Cancer Research manuscript that EMT driven by overexpression of TIMP-1 could explain the bad prognosis for breast cancer patients expressing high levels of TIMP-1. Unfortunately, the cells stopped overexpressing TIMP-1, and we decided to create our own lines. Unfortunately, while attempting to create new TIMP-1 overexpressing MCF10A cells, we noticed that simply treating MCF10A cells with neomycin occasionally caused a change in the cell's morphology consistent with EMT, suggesting the selection for clones was driving the EMT process. As a result, we decided that the MCF10A model was not conducive for continued studies. Therefore, we concentrated our efforts on studying the role of TIMP-1 in breast MCF-7 and MDA-MB-231 cells-lines. We needed to generate our own MCF-7 cells overexpressing TIMP-1, due to the fact that, Dr.

Thorgeirsson, whom we were originally to receive the cells from, no longer had them. We have successfully completed this task and generated 2 sets of overexpressing pools as well as clones that overexpress low (< 250 ng/ml), intermediate (250 – 500 ng/ml) and high (> 500 ng/ml) amounts of TIMP-1 (Figure 1). We also created MDA-MB-231 cells which overexpress TIMP-1. We generated 3 sets of pools, rather than clones, due to the possibility of clonal variation. The pools ranged in levels of secreted TIMP-1 from 100 ng/ml to 300 ng/ml in serum conditions. We also generated vector control cell-lines resistant to the antibiotic.

We also decided to assess the effect of reduced TIMP-1 on cell signaling, proliferation, invasion and tumor growth. We began by using siRNA oligos purchased from BD Biosciences transfected into MDA-MB-231 cells to determine which sites decrease TIMP-1 protein at maximal levels. We then used those oligo sequences to create a lentiviral system to make stable shRNA TIMP-1 cells which have lower levels of TIMP-1 compared to GFP shRNA control cells. The TIMP-1 shRNA cells had approximately five-fold less secreted TIMP-1 compared to GFP control shRNA cells (Figure 2).

TIMP-1 Overexpressing MCF-7 Cells Oversecrete VEGF (Relevant to Task 2)

Various cell-lines and pools of overexpressing cells were analyzed for the production of VEGF, a protein known to be important in the regulation of angiogenesis. Fresh media was placed on these cells, and harvested 24 hours later. We used a commercial ELISA kit to measure the level of VEGF. Figure 1 indicates that the extracellular level of VEGF correlated significantly with the level of extracellular TIMP-1 secreted (Figure 1). Our results are consistent with previous published results (5) and support an important role for TIMP-1 overexpression and the regulation of tumor angiogenesis (see below). We do not yet know the intracellular signaling pathways induced by TIMP-1 to control expression of VEGF.

Interestingly, our MDA-MB-231 TIMP-1 shRNA cells also had higher levels of VEGF compared to their respective GFP shRNA controls (Figure 2). This was noted at both the RNA and protein level and was a surprising result considering our results with the overexpressing MCF-7 TIMP-1 cells.

Analysis of Motility and Proliferation of MCF-7 and MDA-MB-231 cells Over or under-expressing TIMP-1 (Relevant to Task 1)

We used the standard filter insert method to determine if cells overexpressing TIMP-1 were more motile or invasive *in vitro*. Interestingly, the TIMP-1 overexpressing MCF-7 cells were less motile compared to vector control cells, which did not support our original hypothesis that TIMP-1 overexpression stimulated changes in motility and invasion (Figure 3). Additionally, TIMP-1 did not have an effect on proliferation of MCF-7 cells (data not shown). Similarly, the MDA-MB-231 overexpressing and underexpressing TIMP-1 cells proliferated at the same rate compared to their respective controls (data not shown). Additionally, the MDA-MB-231 shRNA TIMP-1 cells showed no difference in an *in vitro* invasion assay compared to the GFP shRNA control cells (data not shown). We are currently assessing the ability of the MDA-MB-231 overexpressing TIMP-1 cells to invade through an artificial basement membrane as compared to the pcDNA control cells.

Analysis of Signaling Pathways in MCF-7 Breast Tumor Cells Overexpressing TIMP-1 (Relevant to Task 2)

It has been published that TIMP-1 overexpression in MCF10A breast epithelial cells stimulates the FAK, AKT, p44/42 ERK and p38 signaling pathways and it is suggested that activation of these pathways promotes inhibition of apoptosis (6-7). Western blot analysis was performed to determine the affect of TIMP-1 expression on pathways known to contribute to cancer progression in our MCF-7 TIMP-1 overexpressing cells. We examined low, medium and high overexpressing clones and pooled populations under both serum and serum-free

conditions by western blot analysis with antibodies specific for the phosphorylated, active state of FAK, AKT, p38 and p44/42. In our hands, TIMP-1 overexpression in MCF-7 cells did not consistently activate FAK, AKT, p38 or p44/42 signaling under either serum or serum-free conditions (Figure 4a). We hypothesized that the failure of TIMP-1 to stimulate these pathways could be due to cell-context dependency. Therefore, we tried to recapitulate what has been published in the literature by treating 5 or 48 hour serum-starved MCF10A cells with purified TIMP-1 protein in a dose response from 10 to 500 ng/ml and analyzed lysates from 10, 30 or 60 minutes of treatment. Cells were treated with serum-containing media or HGF (30 ng/ml) as a positive control. Under all experimental conditions, we found that TIMP-1 was unable to activate the p44/42 or FAK signaling pathways (Figure 4b). As a final experiment, conditioned media from the MCF-7 TIMP-1 clones was added to MCF-7 vector control cells and lysates taken over an 8 hour time course. Again, we found that TIMP-1 was unable to activate the p44/42 or FAK pathways, unlike what has been published in the literature (Figure 4c). We then determined the effect of decreased TIMP-1 levels on these signaling pathways by transient transfection of siRNA oligos to either a Luciferase control or TIMP-1 in MDA-MB-231 cells. As seen if Figure 4d, reduced levels of TIMP-1 had no effect on signaling in these experimental conditions. We finally assessed the effect of stable under or overexpression of TIMP-1 on these signaling pathways in MDA-MB-231 cells under both, serum or serum-free conditions. Again, similar to our previous experiments in MCF-7 cells and MCF10A cells, alterations in the level of TIMP-1 had no effect on signaling through p44/42, p38, FAK or the AKT pathways (data not shown).

Despite this negative data, we feel that this is still extremely important to report due to the fact that TIMP-1 has been reported to affect these signaling pathways in MCF10A cells. We question the physiological relevance of these studies, since they were primarily done under 48 hour serum-starved conditions and western blot analysis for signaling was only performed with one clone when assessing the effect of TIMP-1 overexpression on MCF10A cells (6-7).

Affymetrix Analysis (Relevant to task 2)

Affymetrix microarray analysis was performed with the vector control and TIMP-1 overexpressing MCF-7 pooled populations to determine any potential differences in gene expression. Alterations in expression patterns of genes potentially involved in breast cancer progression were confirmed through RT-PCR analysis in all of the individual clones. TIMP-1 overexpression resulted in increased levels of PDE4-B, caveolin-1, caveolin-2, amphiregulin and S100A7 (psoriasin) and decreased levels of cystatin A (Figure 5). These results are especially interesting in regards to some findings which suggest that high caveolin-1 and psoriasin levels are associated with breast cancer. Additionally, amphiregulin, a ligand for the EGF Receptor family, is known to stimulate tumorigenesis and angiogenesis. The mechanism by which TIMP-1 is able to effect gene expression in MCF-7 cells, however, is currently unclear.

We also performed Affymetrix microarray analysis on the MDA-MB-231 TIMP-1 under and overexpressing cells. Interestingly, the genes which showed statistical differences in the MCF-7 TIMP-1 overexpressing cells did not appear to be differentially regulated in the MDA-MB-231 under or overexpressing TIMP-1 cells. This suggests that the effects of TIMP-1 may be cell context dependent, especially in regards to the tumorigenicity of the model system. This also could explain some of the disparities which appear in the literature. Currently we are repeating the affymetrix analysis on the MDA-MB-231 under and overexpressing TIMP-1 cells to perform statistical analysis (Data not shown).

Animal Studies to Determine if TIMP-1 Overexpressing Cell-Lines are more Angiogenic and Invasive in vivo (Relevant to Task 1c and 1d)

We have performed two mouse experiments with our MCF-7 vector control and TIMP-1 cells in 6-8 week old female, SCID/bg mice. In our first experiment, we injected a vector control line and the low and high expressing TIMP-1 clones. During the second experiment, pooled populations of control and TIMP-1 cells were used. Unfortunately, during the initial experiment the vector control cells failed to form palpable tumors. However, it is noted that the high expressing MCF-7 TIMP-1 clone (TIMP-1 #7) grew at a faster rate compared to the low expressing TIMP-1 clone (TIMP-1 #4) (Figure 6). During our second experiment with pooled populations of pcDNA and TIMP-1 MCF-7 cells, results demonstrated that TIMP-1 stimulated tumor growth compared to control mice. Unlike what was hypothesized, however, we did not see significant invasive growth. Rather, the TIMP-1 tumors had a significantly higher number of cd34 positive vessels compared to vector control tumors (Figure 7), suggesting that TIMP-1 promotes tumorigenesis by stimulating angiogenesis. Unfortunately, due to small animal numbers, the tumor volume results were not statistically significant (data not shown). We are therefore, currently repeating the MCF-7 experiment with a large number of mice to obtain statistically significant values, and the studies should be complete in a month.

We have also performed *in vivo* mouse xenograft experiments with the MDA-MB-231 shRNA GFP and TIMP-1 cells. Interestingly, there was no difference in tumor volume or growth over the course of the experiment (Figure 8). We are currently performing the tumor xenograft experiment with the MDA-MB-231 overexpressing cells.

II. Additional Preliminary Data

The Effect of TIMP-1 on Endothelial Cell Properties *In Vitro*

Conditioned media from the MCF-7 TIMP-1 overexpressing cells and vector controls has been used in several *in vitro* assays to determine the effect of TIMP-1 on endothelial cell tubulogenesis and proliferation. Preliminary studies suggest that TIMP-1 present in the conditioned media has no effect on human endothelial cell morphogenesis on matrigel. This data agrees with studies using endothelial cells embedded in a fibrin-matrix and treated with recombinant TIMP-1 protein. Under these conditions, TIMP-1 also did not have an effect on tubulogenesis. We have additional preliminary evidence to also suggest the TIMP-1 has no effect on endothelial cell proliferation.

Despite these negative results, our *in vivo* data showing the ability of TIMP-1 to stimulate angiogenesis is quite convincing. TIMP-1's effect on angiogenesis may only be apparent *in vivo.*

KEY RESEARCH ACCOMPLISHMENTS:

1. Generated multiple MCF-7 clonal cell-lines overexpressing TIMP-1.
2. Determined that TIMP-1 overexpression correlated with overexpression of VEGF.
3. Demonstrated that the p44/42, p38, AKT and FAK pathways were <u>not</u> activated in MCF-7 TIMP-1 overexpressing cells, and that exogenously supplied TIMP-1 protein did not stimulate these pathways in MCF10A cells, contrary to published literature.
4. Demonstrated that MCF-7 TIMP-1 overexpressing cells did not proliferate faster and were less invasive compared to vector control cells.
5. TIMP-1 overexpression in MCF-7 cells resulted in higher levels of Amphiregulin, Psoriasin, PDE-4B and Caveolin-1 and -2 and lower levels of Cystatin A.
6. MCF-7 TIMP-1 cells grew at a faster rate *in vivo* and the TIMP-1 tumors were larger than vector controls. This was due to an increase in angiogenesis.
7. TIMP-1 did not affect tubulogenesis of human endothelial cells grown on matrigel. TIMP-1 also did not effect endothelial cell proliferation.

8. Created MDA-MB-231 shRNA pools which have lower levels of TIMP-1 compared to GFP control cells.
9. Demonstrated that the p44/42, p38, AKT and FAK pathways were not altered in MDA-MB-231 shRNA TIMP-1 cells compared to GFP shRNA control cells.
10. MDA-MB-231 shRNA TIMP-1 cells were similar to GFP shRNA control cells in regards to proliferation, invasion and tumor growth in SCID/bg mice.
11. Generated MDA-MB-231 TIMP-1 overexpressing cells and pcDNA vector control cells.
12. Demonstrated no difference in p44/42, p38, AKT and FAK signaling pathways between MDA-MB-231 pcDNA and TIMP-1 overexpressing cells.

REPORTABLE OUTCOMES:

We are in the process of preparing a manuscript on the MCF-7 pcDNA and TIMP-1 overexpressing cells and are awaiting completion of the final MCF-7 mouse xenograft study. Additionally, a second manuscript regarding the MDA-MB-231 data is in preparation following completion of the Affymetrix analysis and the tumor xenograft study. Both manuscripts will be submitted during this calendar year.

RLH Bigelow, JA Cardelli. 2004. TIMP-1 Induces Epithelial to Mesenchymal Transition in MCF10A Breast Epithelial Cells. Abstract for the *"95th meeting for the American Association for Cancer Research"*. Orlando, FL.

RLH Bigelow, JA Cardelli. 2004. TIMP-1 Induces Epithelial to Mesenchymal Transition in MCF10A Breast Epithelial Cells. Abstract and poster for Era of Hope Breast Cancer Research Meeting.

RLH Bigelow, BJ Williams, JL Carroll, JA Cardelli. TIMP-1 Promotes Tumorigenesis in MCF-7 Cells and Affects Angiogenesis. Manuscript in preparation.

RLH Bigelow, BJ Williams, L Jones, JA Cardelli. The Effect of TIMP-1 Modulation in MDA-MB-231 Cells. Manuscript in preparation.

CONCLUSIONS

During the past three years, we have created several different breast epithelial cell lines, MCF-7s and MDA-MB-231s, which either overexpressed or underexpressed levels of TIMP-1 to assess the effect of TIMP-1 on breast cancer progression. Our original hypothesis was that TIMP-1 stimulated Ras signaling, thereby affecting tumor growth and invasion. However, our studies have demonstrated that in our hands, alterations in TIMP-1 levels did not impact cell signaling through the p44/42 ERK, p38, AKT or FAK pathways in MCF-7, MDA-MB-231 or MCF10A cells, unlike what has been previously published in MCF10A cells. We feel that this is an extremely important finding, considering that several publications have come out recently implying the importance of TIMP-1 affecting cell signaling in MCF10A cells, thereby inhibiting apoptosis (6-7). The fact that we can not recapitulate these published findings suggests that possibly there is cell context dependent effects in regards to the effect of TIMP-1 or rather TIMP-1 may have more pronounced effects under serum-starved conditions, though we question the physiological relevance of these results.

Despite the fact that we could find no effect of TIMP-1 on signaling pathways, TIMP-1 was still capable of affecting gene expression. Affymetrix array analysis revealed that overexpression of TIMP-1 induced changes in 49 genes at 2.5 fold or greater ($p < 0.01$). The genes fell into numerous classes including signal transduction, apoptosis, cell motility/adhesion, calcium homeostasis and transport. RT-PCR confirmation revealed that TIMP-1 stimulated expression of amphiregulin, PDE4-B, psoriasin and decreased levels of Cystatin A in all clones analyzed. Additionally, caveolin-1 and -2 levels were increased in the majority of the clones compared to pcDNA vector control cells. These changes are interesting in that amphiregulin, a ligand for the EGF family of receptors, is known to stimulate tumorigenesis, whereas levels of Cystatin A, an inhibitor of proteases, are decreased in breast cancer and inversely associated with tumorigenicity (8-11). Additionally, caveolin-1 and -2 have been implicated in cancer progression (12-14). Interestingly, however, preliminary Affymetrix analysis of the MDA-MB-231 under and overexpressing TIMP-1 cells did not show similar changes in gene expression compared to the MCF-7 TIMP-1 overexpressing cells. This suggests that TIMP-1's effects may be cell context dependent, such that it affects cells which are in the early stages of malignancy differently compared to cells originating in a later stage of malignancy.

We additionally have evidence to suggest that TIMP-1 may stimulate tumor growth and angiogenesis in MCF-7 cells, potentially through regulation of VEGF levels. This data agrees with published reports from the Thorgeirsson group (5). Preliminary studies demonstrate that a reduction of TIMP-1 levels has no effect on tumor growth in MDA-MB-231 cells. Studies are currently underway to assess the effect of overexpression of TIMP-1 in MDA-MB-231 cells.

Unfortunately, we did not complete our third task, originally intended to make point mutations in TIMP-1 to find a mutated version that is incapable of stimulating invasion, simply because TIMP-1 in our hands did not stimulate Ras signaling or induce invasion. Additionally, the results we obtained from the MDA-MB-231 underexpressing cells was surprising, suggesting that a decrease in TIMP-1 levels may not be sufficient to inhibit tumor growth, most likely due to redundancy in TIMP family function.

It is difficult to explain the contradictory evidence that exists in the literature concerning TIMP-1 function and cancer. The outcome of TIMP-1 activity may depend on its local tissue or serum concentration, such that at low physiological concentrations TIMP-1 may function as an inhibitor of MMP activity, while at higher levels, as seen in tumors, TIMP-1 may stimulate angiogenesis, proliferation and inhibit apoptosis. TIMP-1's dual effects on tumorigenesis may be the result of stage specific expression. Many studies have shown that TIMP-1 functions during late stages of tumor progression by inhibiting tumor metastasis, invasion and angiogenesis (15-18), however, a recent study of TIMP-1 K14-HPV16 transgenic mice demonstrated that TIMP-1 enhances tumorigenesis by increasing keratinocyte proliferation and chromosomal aberrations (19). TIMP-1 had no effect on metastasis development (19). Alternatively, the outcome of TIMP-1 overexpression may be dependent on the genotype of the tumor, where the presence of additional mutations may synergize with the pro-malignant function of TIMP-1 promoting tumorigenesis (20). Our data argues that there may be stage specific effects. Additionally, decreasing TIMP-1 levels in an invasive condition, as in our MDA-MB-231 underexpressing lines, may not have tumor reducing effects, arguing against agents which target TIMP-1 *in vivo*.

Despite the fact that our intended hypothesis was not correct, we believe our findings are important for theTIMP-1 field, especially in regards to the lack of effect on cell signaling that we observed as well as no effect on tumor growth with reduced TIMP-1 levels in MDA-MB-231 cells. Our data is intended to be published in two separate manuscripts, one focused on the results with the MCF-7 TIMP-1 overexpressing cells and the second centered on the data from the MDA-MB-231 under and overexpressing cells.

REFERENCES

1. McCarthy, K., Maguire, T., McGreal, G., McDermott, E., O'Higgins, N., and Duffy, M. J. High levels of tissue inhibitor of metalloproteinase-1 predict poor outcome in patients with breast cancer. Int J Cancer, *84:* 44-48, 1999.
2. Nakopoulou, L., Giannopoulou, I., Stefanaki, K., Panayotopoulou, E., Tsirmpa, I., Alexandrou, P., Mavrommatis, J., Katsarou, S., and Davaris, P. Enhanced mRNA expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in breast carcinomas is correlated with adverse prognosis. J Pathol, *197:* 307-313, 2002.
3. Ree, A. H., Florenes, V. A., Berg, J. P., Maelandsmo, G. M., Nesland, J. M., and Fodstad, O. High levels of messenger RNAs for tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) in primary breast carcinomas are associated with development of distant metastases. Clin Cancer Res, *3:* 1623-1628, 1997.
4. Brummer, O., Athar, S., Riethdorf, L., Loning, T., and Herbst, H. Matrix-metalloproteinases 1, 2, and 3 and their tissue inhibitors 1 and 2 in benign and malignant breast lesions: an in situ hybridization study. Virchows Arch, *435:* 566-573, 1999.
5. Yoshiji H, Harris SR, Raso E, Gomez DE, Lindsay CK, Shibuya M, Sinha CC., Thorgeirsson UP. Mammary carcinoma cells over-expressing tissue inhibitor of metalloproteinases-1 show enhanced vascular endothelial growth factor expression. Int J Cancer. 1998 Jan 5;75(1):81-7.
6. Li, G., Fridman, R., and Kim, H. R. Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. Cancer Res, *59:* 6267-6275, 1999.
7. Liu, X. W., Bernardo, M. M., Fridman, R., and Kim, H. R. Tissue inhibitor of metalloproteinase-1 protects human breast epithelial cells against intrinsic apoptotic cell death via the focal adhesion kinase/phosphatidylinositol 3-kinase and MAPK signaling pathway. J Biol Chem, *278:* 40364-40372, 2003.
8. Zajc, I., Sever, N., Bervar, A., and Lah, T. T. Expression of cysteine peptidase cathepsin L and its inhibitors stefins A and B in relation to tumorigenicity of breast cancer cell lines. Cancer Lett, *187:* 185-190, 2002.
9. Lah, T. T., Kokalj-Kunovar, M., Strukelj, B., Pungercar, J., Barlic-Maganja, D., Drobnic-Kosorok, M., Kastelic, L., Babnik, J., Golouh, R., and Turk, V. Stefins and lysosomal cathepsins B, L and D in human breast carcinoma. Int J Cancer, *50:* 36-44, 1992.
10. Ma, L., Gauville, C., Berthois, Y., Millot, G., Johnson, G. R., and Calvo, F. Antisense expression for amphiregulin suppresses tumorigenicity of a transformed human breast epithelial cell line. Oncogene, *18:* 6513-6520, 1999.
11. LeJeune, S., Leek, R., Horak, E., Plowman, G., Greenall, M., and Harris, A. L. Amphiregulin, epidermal growth factor receptor, and estrogen receptor expression in human primary breast cancer. Cancer Res, *53:* 3597-3602, 1993.
12. Williams, T. M. and Lisanti, M. P. Caveolin-1 in oncogenic transformation, cancer, and metastasis. Am J Physiol Cell Physiol, *288:* C494-506, 2005.
13. Engelman, J. A., Zhang, X. L., and Lisanti, M. P. Genes encoding human caveolin-1 and -2 are co-localized to the D7S522 locus (7q31.1), a known fragile site (FRA7G) that is frequently deleted in human cancers. FEBS Lett, *436:* 403-410, 1998.
14. Hayashi, K., Matsuda, S., Machida, K., Yamamoto, T., Fukuda, Y., Nimura, Y., Hayakawa, T., and Hamaguchi, M. Invasion activating caveolin-1 mutation in human scirrhous breast cancers. Cancer Res, *61:* 2361-2364, 2001.
15. Guedez, L., McMarlin, A. J., Kingma, D. W., Bennett, T. A., Stetler-Stevenson, M., and Stetler-Stevenson, W. G. Tissue inhibitor of metalloproteinase-1 alters the tumorigenicity of Burkitt's lymphoma via divergent effects on tumor growth and angiogenesis. Am J Pathol, *158:* 1207-1215, 2001.
16. Ikenaka, Y., Yoshiji, H., Kuriyama, S., Yoshii, J., Noguchi, R., Tsujinoue, H., Yanase, K., Namisaki, T., Imazu, H., Masaki, T., and Fukui, H. Tissue inhibitor of metalloproteinases-

1 (TIMP-1) inhibits tumor growth and angiogenesis in the TIMP-1 transgenic mouse model. Int J Cancer, *105:* 340-346, 2003.
17. Rigg, A. S. and Lemoine, N. R. Adenoviral delivery of TIMP1 or TIMP2 can modify the invasive behavior of pancreatic cancer and can have a significant antitumor effect in vivo. Cancer Gene Ther, *8:* 869-878, 2001.
18. Yamazaki, M., Akahane, T., Buck, T., Yoshiji, H., Gomez, D. E., Schoeffner, D. J., Okajima, E., Harris, S. R., Bunce, O. R., Thorgeirsson, S. S., and Thorgeirsson, U. P. Long-term exposure to elevated levels of circulating TIMP-1 but not mammary TIMP-1 suppresses growth of mammary carcinomas in transgenic mice. Carcinogenesis, *25:* 1735-1746, 2004.
19. Rhee, J. S., Diaz, R., Korets, L., Hodgson, J. G., and Coussens, L. M. TIMP-1 alters susceptibility to carcinogenesis. Cancer Res, *64:* 952-961, 2004.
20. Soloway, P. D., Alexander, C. M., Werb, Z., and Jaenisch, R. Targeted mutagenesis of Timp-1 reveals that lung tumor invasion is influenced by Timp-1 genotype of the tumor but not by that of the host. Oncogene, *13:* 2307-2314, 1996.

SUPPORTING DATA

FIGURES

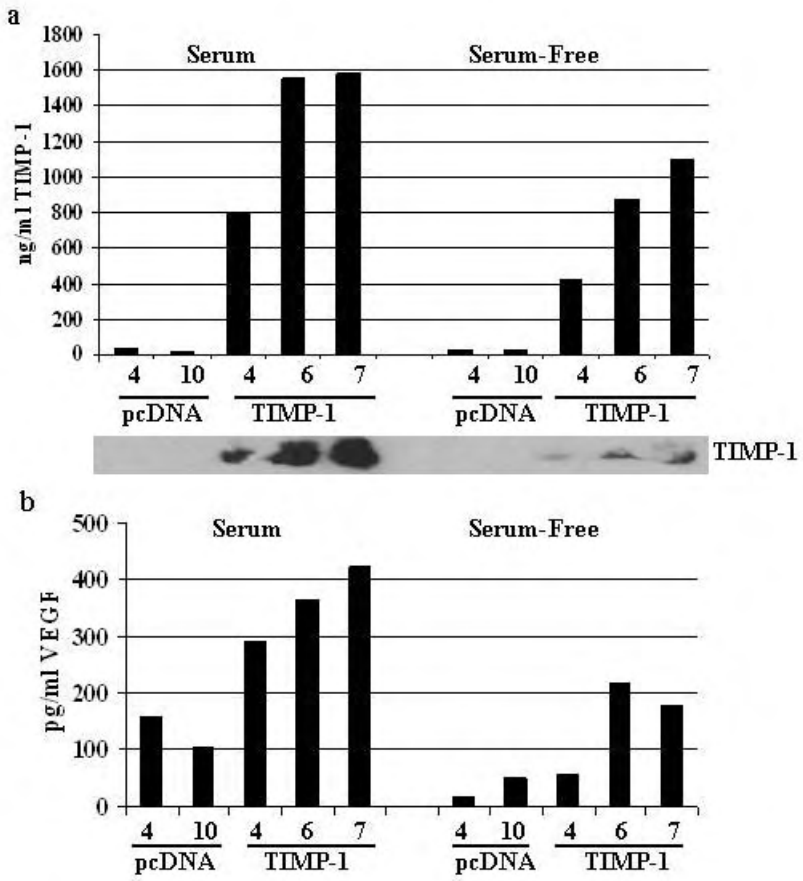

Figure 1: MCF-7 TIMP-1 cells overexpress VEGF. MCF-7 pcDNA and TIMP-1 cells were plated to 60% confluency (pcDNA clone #4 and #10, TIMP-1 clone #4, #6 and #7). The media was changed to either serum-free or serum containing media and incubated overnight. Conditioned media was harvested the following day and used for ELISA for TIMP-1 (a) and VEGF levels (b). Results are expressed in ng/ml for TIMP-1 and pg/ml for VEGF. Western blot was also performed with the conditioned media to assess levels of TIMP-1 and confirm the ELISA data.

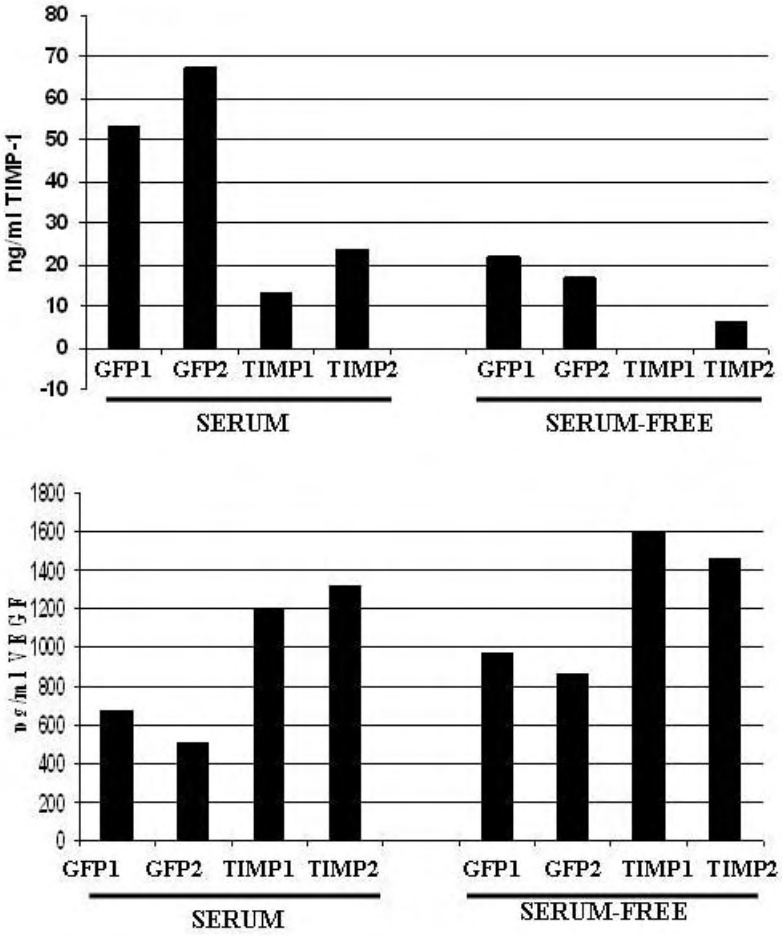

Figure 2: MDA-MB-231 shRNA TIMP-1 cells overexpress VEGF. MDA-MB-231 GFP control and TIMP-1 shRNA cells were plated to 60% confluency (GFP shRNA pool #1 and #2 and TIMP-1 shRNA pool #1 and #2). The media was changed to either serum-free or serum containing media and incubated overnight. Conditioned media was harvested the following day and used for ELISA for TIMP-1 (a) and VEGF levels (b). Results are expressed in ng/ml for TIMP-1 and pg/ml for VEGF

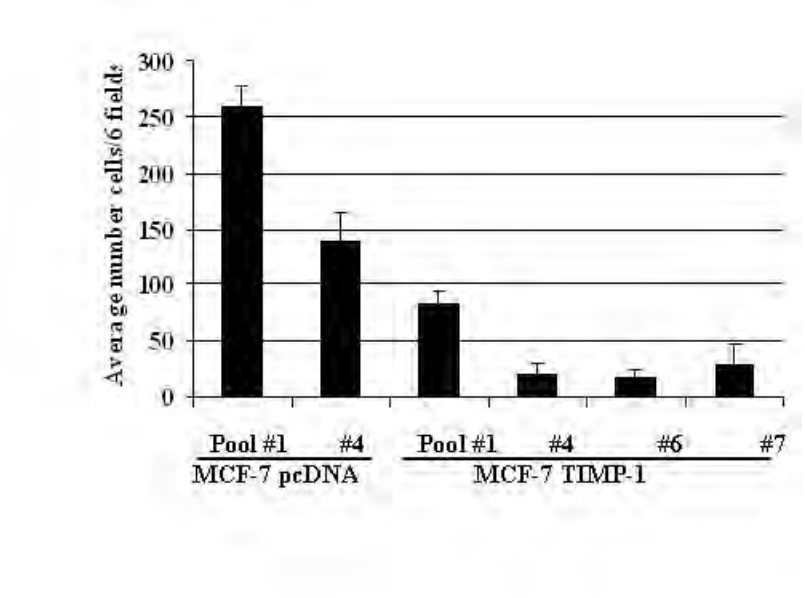

Figure 3: TIMP-1 overexpression in MCF-7 cells inhibits cell motility. MCF-7 pcDNA (pool #1, clone #4) and TIMP-1 (pool #1, clone #4, #6 and #7), $2x10^4$, cells were plated on top of the Transwell inserts in 300 µl media. 600 µl media was added to the bottom of the well and the cells were incubated overnight. Cells were fixed and the cells remaining on the top were removed with a cotton swap. The remaining cells were stained with crystal violet and the number of cells in 5 random cells was counted. TIMP-1 blocked cell motility of MCF-7 cells. The experiment was performed in triplicate and repeated twice.

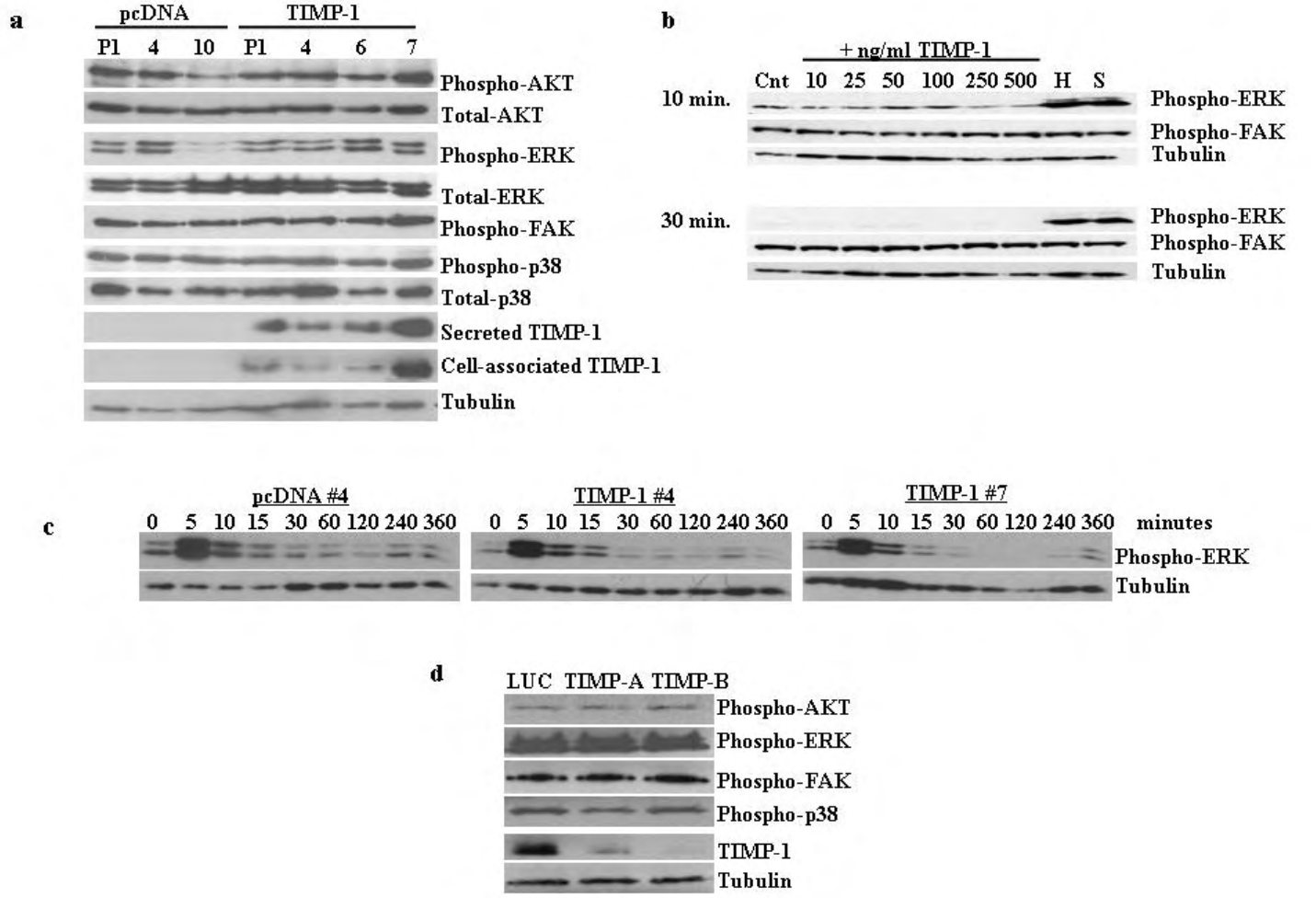

Figure 4: TIMP-1 does not activate the ERK, FAK, p38 or AKT signaling pathways.
a) MCF-7 pcDNA (pool #1- P1, clone #4, clone #10) and TIMP-1 (pool #1- P1, clone #4, clone #6, clone #7) cells were plated at 60% confluency and the following day the cells were synchronized in G0/G1 by serum-starvation overnight. The media was replaced with complete media and incubated overnight. Conditioned media and lysates were taken and run by western blot analysis and probed with the indicated antibodies. Overexpression of TIMP-1 in MCF-7 cells did not result in changes in pathway signaling. b) MCF10A ($3x10^4$) were plated in a 24 well plate and serum starved for 5 hours. TIMP-1 was added at the indicated concentrations in serum-free media. Fresh serum-free media without TIMP-1 was added to the control well. As positive controls, hepatocyte growth factor, 30 ng/ml (HGF) and serum-containing media (S) was added as positive controls. The cells were incubated for 10 or 30 minutes and lysates taken, run by western blot analysis and probed with the indicated antibodies. Exogenous TIMP-1 did not affect ERK or FAK signaling. c) MCF-7 pcDNA #4 cells were plated at $3x10^4$ in 24 well plates. Serum containing conditioned media from pcDNA #10, TIMP-1 #4 and TIMP-1 #7 (0.5 mls) was added to each well. Lysates were taken at the indicated timepoints, run by western blot analysis and probed with the indicated antibodies. Exogenous TIMP-1 secreted into conditioned media did not have an effect on phospho-ERK activity. d) MDA-MB-231 cells were transiently transfected with one of two shRNA plasmids to downregulate TIMP-1 levels (pSIREN-DNR-TIMP1 A or B) or a non-specific Luciferase shRNA control plasmid. Lysates and conditioned media were taken 3 days after transfection and probed with the indicated antibodies. Decreasing TIMP-1 levels in MDA-MB-231 cells did not affect phosphorylation of AKT, ERK, p38 or FAK.

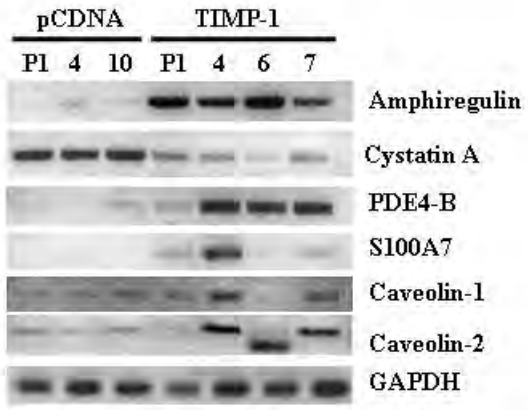

Figure 5: RT-PCR confirmation of genes from Affymetrix arrays. Genes which revealed differences by Affymetrix analysis were confirmed by RT-PCR analysis in all MCF-7 pcDNA (P1- pool #1, clone #4 and #10) and TIMP-1 clones (P1- pool #1, clone #4, #6 and #7). TIMP-1 increased levels of amphiregulin, PDE4-B and S100A7 (psoriasin). TIMP-1 decreased levels of Cystatin A. Caveolin-1 and -2 were also increased in the majority of the TIMP-1 clones. Caveolin-1 was not detectable in MCF-7 TIMP-1 #6 cells under the PCR conditions used and Caveolin-2 ran at a lower than predicted level. GAPDH was used as a load control. Please note that the levels of GAPDH for TIMP-1 pool #1 are slightly lower compared to the other lanes.

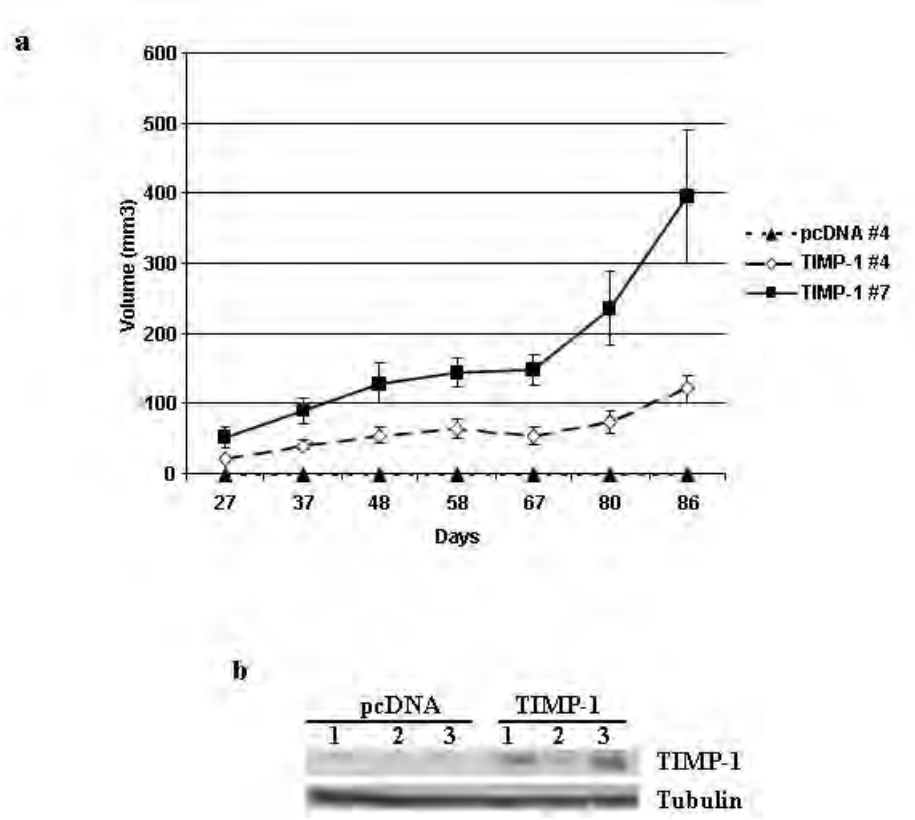

Figure 6: MCF-7 TIMP-1 cells grew faster in xenograft studies. a) MCF-7 pcDNA #4, TIMP-1 #4 and #7 cells ($5x10^6$) were injected subcutaneously in SCID/bg mice (n=8/group) in the presence of a 90-day release estrogen pellet. The mice were monitored daily and tumors measured approximately every 10 days. On the final day, the mice were sacrificed and the tumors were harvested for further analysis. TIMP-1 overexpression stimulated MCF-7 tumor growth. Data represents the average tumor volume per group +/- standard error of the mean. . b) Western blot analysis of MCF-7 pcDNA pool #1 and TIMP-1 pool #1 tumor xenografts. The MCF-7 TIMP-1 tumors retained TIMP-1 expression throughout the duration of the experiment.

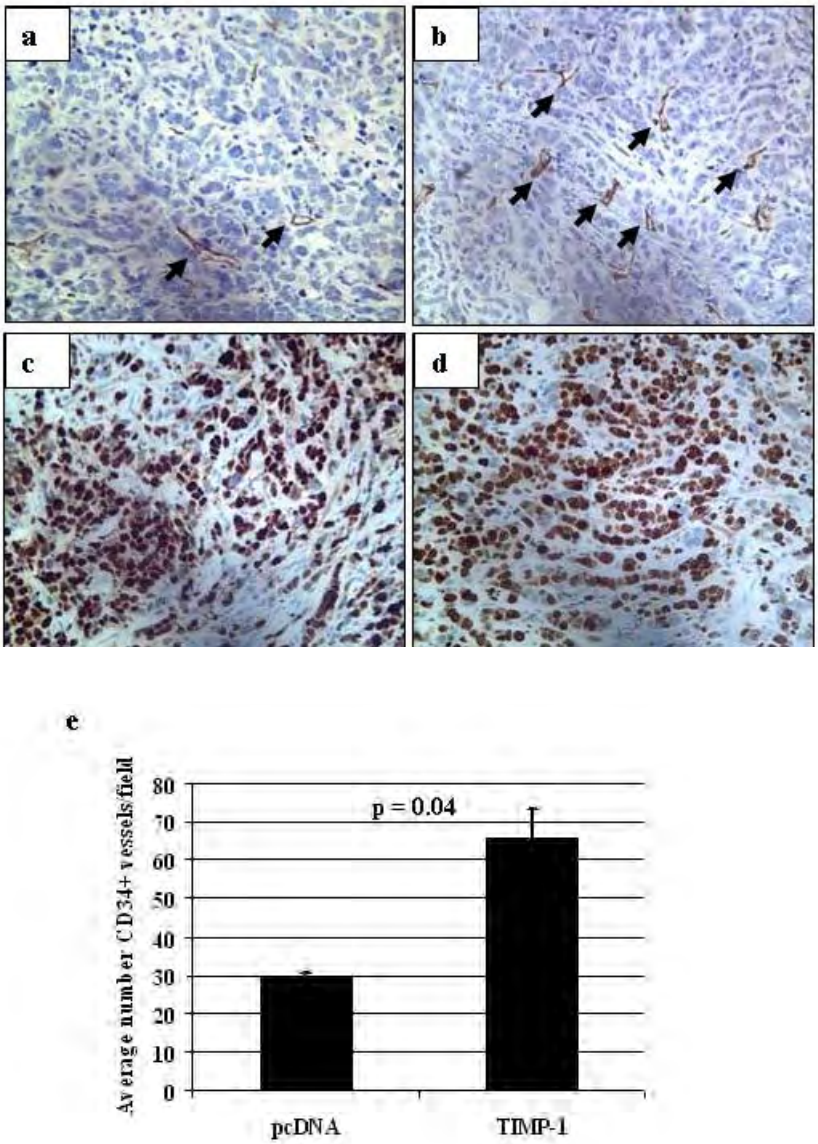

Figure 7: MCF-7 TIMP-1 tumors have a higher number of CD34 positive vessels. MCF-7 pcDNA pool #1 (a, c) and TIMP-1 pool #1 (b, d) tumors were stained by immunohistochemistry for CD34 (a, b) and Ki67 (c, d). The TIMP-1 tumors had a higher number of CD34 positive vessels compared to the vector control tumors. Arrows point to CD34 positive vessels. There was no change in proliferation, as noted by Ki67 staining. Representative images are shown. e) The number of CD34 positive vessels were counted in each tumor. The average number of CD34 positive vessels per field was higher in the TIMP-1 tumors compared to the vector control tumors

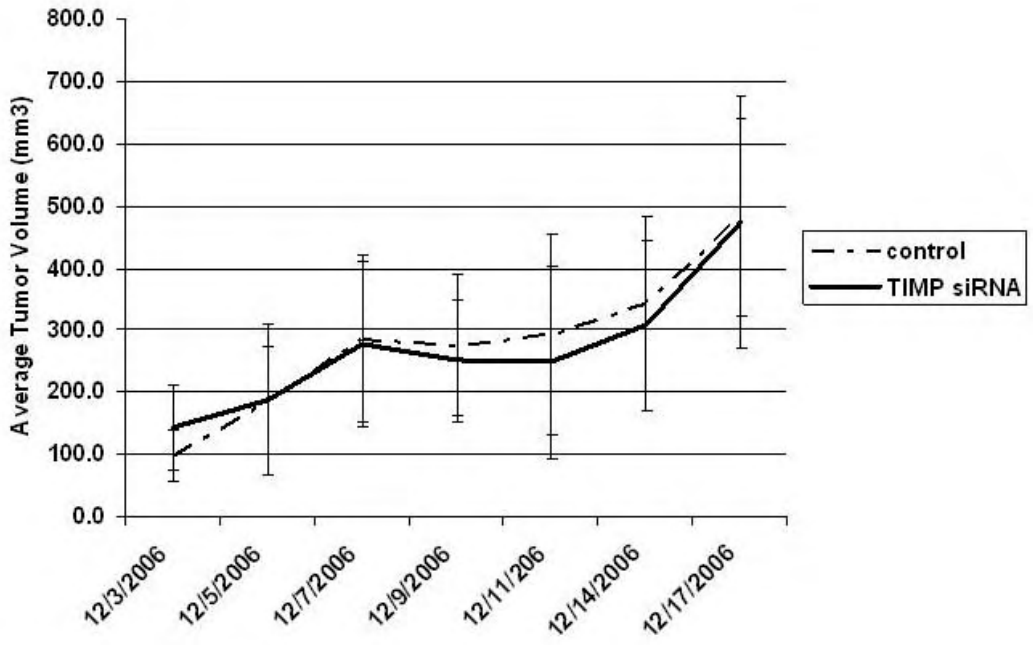

Figure 8: MDA-MB-231 GFP shRNA and TIMP-1 shRNA cells grew at the same rate in SCID/bg mice. a) MDA-MB-231 GFP shRNA pool #1 and TIMP-1 shRNA pool #1 cells ($5x10^6$) were injected into the mammary fat pad with matrigel in SCID/bg mice (n=8/group). The mice were monitored daily and tumors measured approximately every 2-3 days. On the final day, the mice were sacrificed and the tumors were harvested for further analysis. Reduced levels of TIMP-1 in MDA-MB-231 cells had no effect on tumor volume compared to GFP control cells. Data represents the average tumor volume per group +/- standard deviation. Western blot analysis of tumor lysates confirmed a decrease in TIMP-1 levels in the TIMP-1 shRNA tumors (data not shown).