

AD \_\_\_\_\_

Award Number: DAMD17-97-1-7282

TITLE: A Developmental Approach to Characterizing the Tissue-Invasion Gene Program in Breast Cancer

PRINCIPAL INVESTIGATOR: Stephen J. Weiss, M.D.

CONTRACTING ORGANIZATION: University of Michigan  
Ann Arbor, Michigan 48109-1274

REPORT DATE: September 2001

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020904 024

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                 |                                                                |                                                                          |                               |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|----------------------------------------------------------------|--------------------------------------------------------------------------|-------------------------------|
| <b>1. AGENCY USE ONLY (Leave blank)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                 | <b>2. REPORT DATE</b><br>September 2001                        | <b>3. REPORT TYPE AND DATES COVERED</b><br>Final (11 Aug 97 - 10 Aug 01) |                               |
| <b>4. TITLE AND SUBTITLE</b><br>A Developmental Approach to Characterizing the Tissue-Invasion Gene Program in Breast Cancer                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                                                                 |                                                                | <b>5. FUNDING NUMBERS</b><br>DAMD17-97-1-7282                            |                               |
| <b>6. AUTHOR(S)</b><br>Stephen J. Weiss, M.D.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                 |                                                                |                                                                          |                               |
| <b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b><br>University of Michigan<br>Ann Arbor, Michigan 48109-1274<br><br>E-Mail: SJWEISS@umich.edu                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                 |                                                                | <b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>                          |                               |
| <b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b><br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                 |                                                                | <b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>                  |                               |
| <b>11. SUPPLEMENTARY NOTES</b><br>Report contains color                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                 |                                                                |                                                                          |                               |
| <b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b><br>Approved for Public Release; Distribution Unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                 |                                                                |                                                                          | <b>12b. DISTRIBUTION CODE</b> |
| <b>13. ABSTRACT (Maximum 200 Words)</b><br><p>The changes in the gene program of neoplastic cells that regulate the expression of an invasive phenotype are largely undefined. Direct comparisons of the gene expression profile displayed in normal and carcinomatous breast tissues have provided insights into the mechanisms underlying tumor progression. However, attempts to identify the gene products differentially expressed during invasion <i>in vivo</i> have been hampered by the fact that only a small percentage of the cells recovered from a tumor mass are actively engaged in invasive behavior at the time of isolation. Because tissue remodeling induced during mammary gland involution bears homology to early stages of carcinogenesis, the involuting mammary gland may be used to identify genes that control matrix turnover in cancerous states. To this end, we propose to <i>i) screen involuting versus resting mouse mammary glands by oligonucleotide microarray for differentially expressed gene products associated with matrix remodeling, ii) evaluate potential role of differentially expressed gene products in regulating cell invasion in vitro and/or in vivo and iii) evaluate role of differentially expressed gene products in regulating breast cancer cell invasion.</i></p> |                                                                 |                                                                |                                                                          |                               |
| <b>14. SUBJECT TERMS</b><br>Breast Cancer                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                 |                                                                | <b>15. NUMBER OF PAGES</b><br>24                                         |                               |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                 |                                                                | <b>16. PRICE CODE</b>                                                    |                               |
| <b>17. SECURITY CLASSIFICATION OF REPORT</b><br>Unclassified                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | <b>18. SECURITY CLASSIFICATION OF THIS PAGE</b><br>Unclassified | <b>19. SECURITY CLASSIFICATION OF ABSTRACT</b><br>Unclassified | <b>20. LIMITATION OF ABSTRACT</b><br>Unlimited                           |                               |

## Table of Contents

|                                   |       |
|-----------------------------------|-------|
| Cover.....                        | 1     |
| SF 298.....                       | 2     |
| Table of Contents.....            | 3     |
| Introduction.....                 | 4     |
| Body.....                         | 5-8   |
| Key Research Accomplishments..... | 8     |
| Reportable Outcomes.....          | 8     |
| Conclusions.....                  | 8     |
| References.....                   | 8-11  |
| Appendices.....                   | 11-12 |

## I. INTRODUCTION

Current evidence suggests that breast carcinoma cells invade local tissues and metastasize by i) altering their cell-cell and cell-matrix interactions, ii) displaying an aberrant motile phenotype, and iii) either synthesizing, or inducing the synthesis of, proteolytic enzymes that degrade the structural barriers established by the extracellular matrix<sup>1-3</sup>. The complex changes in the gene program of neoplastic cells that regulate the expression of this phenotype are largely undefined, but increased interest has focused on identifying those genes that are specifically overexpressed in human breast cancer<sup>(e.g., 3-10)</sup>. Such information not only provides new insights into the cellular factors that control tissue-invasive behavior, but may also lead to improvements in patient diagnosis and to the more rational design of therapeutic interventions<sup>3-10</sup>. Consistent with this rationale, direct comparisons of the gene expression profile displayed in normal versus neoplastic breast cancer cell lines, or normal and carcinomatous breast tissues, have provided a number of novel insights into the mechanisms and processes underlying tumor progression<sup>6-11</sup>. Interestingly, despite the power of the analytical techniques employed for these purposes, the number of differentially expressed genes identified thus far are - at first glance - perplexingly small, despite the striking changes known to occur in cellular behavior<sup>(e.g., 7,8)</sup>. However, analyses of breast cancer cell lines grown *in vitro* or static tumor masses recovered from *in vivo* sites of disease may be problematic. First, comparisons between normal and neoplastic breast cancer cell lines grown atop plastic substrata *in vitro* will not recapitulate the complex interactions known to occur across the carcinoma-mesenchymal cell axis *in vivo*<sup>1,2</sup>. Indeed, many of the most interesting gene products that have been associated with the expression of tissue-invasive phenotypes in breast cancer tissue are synthesized by surrounding stromal cells rather than the tumor itself<sup>2,3,10</sup>. Secondly, while the gene expression patterns identified in tissues recovered from *in vivo* sites clearly circumvent the limitations inherent in the *in vitro* studies, only a small percentage of the cells recovered from a tumor mass at a single, fixed time point would be expected to be actively engaged in invasive behavior. Given the many similarities between developmental/tissue repair processes and malignant growth (re; the ability of cancer cells inappropriately recapitulate developmental programs associated with epithelial-mesenchymal cell transitions or repair programs associated with wound healing<sup>12,13</sup>), we have considered the possibility that the *in situ* induction of a synchronous matrix remodeling program in normal tissues would allow for the more efficient isolation of those gene products critical to cancer cell invasion. Indeed, recent studies have demonstrated that gene expression patterns associated with the tissue remodeling program induced during the involution of the normal lactating mammary gland bear considerable overlap with those detected in the early stages of carcinogenesis (e.g., stromelysin-1, stromelysin-3, urokinase-type plasminogen activator, tissue inhibitor of metalloproteinases<sup>14-16</sup>). Hence, we propose to use the involuting mammary gland explant model as a means to rapidly enrich for, and identify, the subset of genes that control the disassembly of the extracellular matrix in cancerous states. Furthermore, by selectively identifying the subset of gene products that regulate invasion of breast cancer cells, new diagnostics as well as novel targets for therapeutic intervention can be rapidly identified.

## II. BODY

In the original proposal, we intended to generate cDNA libraries from control versus involuting mammary gland explants. However, the high rate of cell apoptosis in control glands precluded

analysis and emphasis was shifted to analyses of gene expression patterns in tissues recovered from lactating versus involuting glands *in vivo* by oligonucleotide array (see approved "Revised Statement of Work"). Each of the proposed aims have now been completed as described below.

**Task 1. Screen involuting versus resting (i.e., lactating) mammary glands by oligonucleotide array for differentially expressed gene products associated with matrix remodeling.**

*a. Documentation of involution program in mammary explants.* To demonstrate that an involution program was successfully engaged in mammary gland tissue, glands were excised from lactating (day 10) or involuting glands (day 3 post-weaning). Tissue sections were then processed for H and E staining, apoptosis (as assessed by TUNEL staining) or for the dissolution of the subepithelial basement membrane by immunofluorescence (as assessed by staining for type IV collagen or laminin). As shown in Figure 1, the milk-engorged ductal system of lactating mice rapidly collapsed 3 days post-weaning. Coincident with this event, an increase in apoptosis was noted which paralleled a major loss and/or fragmenting of basement membrane-associated type IV collagen or laminin (Fig. 1). As the matrix-degrading involution program had been successfully engaged, tissues from the mice were collected and subjected to oligonucleotide array analysis as described below.

*b. Collection mRNA and performance of oligonucleotide microarray analysis.* In three experiments, RNA was isolated from glands with a Qiagen RNassay mini-kit and cRNA prepared for hybridization as described<sup>17,18</sup>. Oligonucleotide arrays (Gene Chip, Affymetrix) representing a total of 30,000 EST cluster sequences and/or full-length genes were used for hybridization according to the manufacturer's instructions. Arrays were then scanned using an Affymetrix confocal scanner and analyzed using Gene Chip 3.0 Software (Affymetrix). Expression data from the Affymetrix arrays were analyzed using a statistically based analysis methodology that estimates expression levels and provides confidence intervals for these estimates. It also allows for the normalization of array-based expression data to control for variations due to non-biological factors such as array-to-array variability, and variations in sample quality. For each gene, the presence or absence of a transcript was determined by testing the Null hypothesis. Briefly, the arrays included a set of probes derived from non-eukaryotic ("foreign") organisms (e.g., bacterial and bacteriophage sequences) which were defined as the "null set". This null set thus defines the intensity of nonspecific background/cross-hybridization. This null intensity distribution is modeled by a parametric statistical distribution. Since intensity is a positive random variable, this null distribution is modeled by either a Gamma or a Weibull class distribution. Once the parametric null distribution is determined, we computed the p-value for the hypothesis that the observed hybridization intensity values are also a random sample from the null distribution. Target genes with low p-values (i.e., not likely to have come from the same distribution as the null genes) are classified as present. The p-value provides a continuous measure of the confidence in the presence of a gene in the target sample. We also include a mathematical method to standardize the gene-expression levels between different samples, based on exogenous gene spikes, added at known concentrations, that constitute a calibration set<sup>18</sup>. Genes scored as "positive" (i.e., induced) in involuting tissues were i) more highly expressed in each of 3 independently performed experiments and ii) expressed at levels  $\geq 2.5$  than those detected in lactating glands in at least 2 of the 3 experiments.

Following analysis of the approximately 100 genes whose expression was upregulated as defined, a series of proteolytic enzymes were identified (Appendix I). In the matrix metalloproteinase family, stromelysin-1 was the only upregulated member whose expression reached significance (Appendix I). However, the membrane-anchored metalloproteinase, MT1-MMP, was also identified though its expression level did not increase by the requisite 2.5-fold in 2/3 samples. However, given the positive trend, samples from involuting tissues were examined by RT-PCR at both 3 and 5 days post-weaning. Significantly, MT1-MMP mRNA was dramatically upregulated at the latter time point (Fig. 2). In addition, message levels for three matrix-destructive cathepsins were also found to be upregulated during involution, i.e., the aspartyl proteinase cathepsin D, and the cysteinyl proteinases, cathepsins L and S.

Table I  
Fold-Increase in Gene Expression

|             | EXP #1 | EXP #2 | EXP #3 |
|-------------|--------|--------|--------|
| cathepsin D | 4.7    | 3.9    | 2.3    |
| cathepsin L | 4.1    | 2.9    | 1.8    |
| cathepsin S | 7.4    | 6.2    | 2.3    |

**Task 2. Evaluate the potential role of differentially expressed gene products in regulating cell invasion *in vitro* and/or *in vivo*.**

With regard to the MMPs, both stromelysin-1 and MT1-MMP are expressed in breast cancer<sup>19,20</sup>. Thus, we sought to determine whether either of these enzymes might serve as basement membrane-degrading enzymes. To this end, we developed a model system wherein an immortalized epithelial cell line deposits an intact basement membrane *in vitro* (containing type IV collagen, laminin and heparan sulfate proteoglycan; data not shown) atop a dense layer of type I collagen (Fig. 3). Following lysis of the overlying epithelium, COS-1 cells that have been engineered to overexpress human stromelysin-1 were then allowed to adhere to the denuded basement membrane. Following a 5 d incubation period, the basement membrane retained its normal structure as assessed by transmission electron microscopy (Fig. 4). However, in marked contrast, cells transfected with MT1-MMP not only rapidly degraded the underlying substratum, but also conferred the recipient cells with invasive activity (Fig. 5). While MT1-MMP could conceivably mediate basement membrane degradation directly, the proteinase can also process the MMP zymogen, gelatinase A (or MMP-2), to its active form<sup>21</sup>. Further, gelatinase A has been posited to serve as a basement membrane degrading enzyme itself<sup>22</sup>. However, COS-1 cells do not express gelatinase A and the experiments described above were performed in the absence of exogenous gelatinase A (i.e., serum contains gelatinase A, but this can be removed by gelatin-affinity chromatography)<sup>21</sup>. Nonetheless, to rule out a role for gelatinase A directly, COS-1 cells were stably transfected with gelatinase A and cultured atop the denuded basement membrane as described. While no change in basement membrane structure was noted (data not shown), the cells only secreted gelatinase A in its latent form (see below). To test the ability of active gelatinase A to exert proteolytic activity in this system, a chimeric enzyme was designed wherein a basic recognition motif (RXKR, where R = Arg, X = any amino acid, K = Lys) for the proprotein convertase, furin, was inserted directly upstream of the catalytic domain of gelatinase A between N<sup>109</sup> and Y<sup>110</sup> (we described this general approach previously for other MMPs in ref.

23). In this manner, progelatinase is cleaved within the *trans*-Golgi network to a fully active form prior to secretion. Indeed, as shown in Figure 6, supernatants of transfected cells released active gelatinase A as assessed by gelatin zymography. Nonetheless, cells overexpressing active gelatinase A did not display basement membrane-degrading activity (Fig. 6). Likewise, the closely related gelatinase, MMP-9, was also unable to degrade the underlying basement membrane when expressed in its fully active form (the basic recognition motif was inserted between R<sup>106</sup> and F<sup>107</sup>; Fig. 6). Thus, only MT1-MMP, an MMP upregulated during the involution cycle, arms expressing cells with the ability to degrade the basement membrane construct used in these studies. Nonetheless, as the basement membrane was generated *in vitro*, concerns could be raised that basement membranes assembled *in vivo* display unique properties relative to their susceptibility to degradation. Consequently, we have extended these studies to analyze the ability of MT1-MMP to degrade basement membrane recovered from animal tissues. As shown in Figure 6, an intact basement membrane is located beneath the mesothelial cell layer of the peritoneum. Hence, resident cells were lysed by freeze-thawing and the denuded peritoneal basement membrane overlaid with MT1-MMP expressing COS-1 cells. As predicted, the MT1-MMP transfected cells displayed a proteolytic activity similar, if not identical, to that observed with the "*in vitro*" basement membrane constructs (Fig. 7). Further, the invasive/degradative activity was completely blocked in the presence of the synthetic MMP inhibitor, BB-94 (Fig. 7).

While cathepsin D has been previously associated with human breast cancer and shown to express matrix destructive activity *in vitro*<sup>24,25</sup>, roles for cathepsins L and S in regulating extracellular matrix turnover are less clear. Though these enzymes are usually confined to the lysosomal system (they are unstable at neutral pH), we have recently characterized cathepsin L and S as matrix-destructive enzymes in human macrophages<sup>26</sup>. However, whereas macrophages are able to secrete the active enzymes extracellularly into an acidic pericellular environment maintained by H<sup>+</sup>-pumps targeted to the plasma membrane, we have been unable to identify other cell types capable of displaying this phenotype<sup>26</sup>. As such, we have been unable to demonstrate the ability of cathepsin L or S to confer degradative activity in transfected COS cells. Given this limitation, we instead attempted to determine whether the involution program in cathepsin L/S-deleted mice was affected. However, these animals did not breed well in our facility which precluded attempts to study the effects of the L/S-deficient state on the involution program. Further, in the absence of direct evidence to support their extracellular secretion from the mammary epithelium (or tumor cells), we would not be able to rule out the possibility that the proteinases were only required for intracellular, lysosomal degradation and not extracellular proteolysis.

**Task 3. Evaluate the role of differentially expressed gene products identified in the involution screen to regulate breast cancer invasion.**

To determine the ability of MT1-MMP to regulate tumor cell invasion, we first examined the ability of invasion-incompetent MCF-7 cells to penetrate the basement membrane before or after transfection with MT1-MMP. As shown in Figure 8, MCF-7 breast cancer cells seeded atop the basement membrane construct did not display invasive activity as assessed by phase contrast microscopy or in H-E sections. However, following transfection with MT1-MMP (but not stromelysin-1), the cells displayed striking invasive activity (Fig. 8). As human breast cancer tissue expresses MT1-MMP *in vivo*<sup>27</sup>, the invasive activity of MT1-MMP-positive tumor cells

were examined (i.e., the MB-231 cell line). Significantly, when those cells were cultured atop the peritoneal basement membrane, the underlying matrix was degraded (as assessed by scanning electron microscopy; Fig. 9). In the presence of recombinant TIMP-2, a potent MT1-MMP inhibitor, degradation and invasion were completely blocked over a 7 d assay period (Fig. 9). In preliminary experiments performed with a neutralizing monoclonal antibody directed against MT1-MMP, invasive activity was likewise inhibited completely. Taken together, these data suggest that MT1-MMP may be the critical proteinase involved in basement membrane degradation and invasion by breast carcinoma cells.

### **III. KEY RESEARCH ACCOMPLISHMENTS**

- Gene program associated with mammary gland involution program characterized.
- Membrane type-1 matrix metalloproteinase identified as key basement membrane degrading enzyme.
- Membrane type-1 matrix metalloproteinase identified as pro-invasive factor for human breast cancer cells and potential target for therapeutic intervention.

### **IV. REPORTABLE OUTCOMES**

- Presentation at Gordon Conference on Matrix Metalloproteinases, 2001.
- New funding obtained to assess role of membrane-type matrix metalloproteinases in breast cancer development from Komen Breast Cancer Foundation.
- Manuscript in preparation, "Regulation of Basement Membrane Invasion by MT1-MMP", Kevin Hotary and Stephen J. Weiss.

### **VI. CONCLUSIONS**

With the identification of suitable mammary gland tissues for isolating gene products differentially expressed during matrix-remodeling events, the model system has been used to identify genes that likely control the disassembly of the matrix during tumor invasion and metastasis. Furthermore, by selectively identifying critical gene products that regulate invasion, new diagnostics as well as novel targets for therapeutic intervention may be identified.

### **VII. REFERENCES**

1. Liotta, L.A., Steeg, P.S., and Stetler-Stevenson, W.G. Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. *Cell* 64:327-336, 1991.
2. MacDougall, J.R., and Matrisian, L.M. Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. *Cancer Metast Rev.* 14:351-362, 1995.

3. Heppner, K.J., Matrisian, L.M., Jensen, R.A., and Rodgers, W.H. Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response. *Am J. Pathol.* 149:273-282, 1996.
4. Wolf, c., Rouyer, N., Lutz, Y., Adida, C., Lorient, M., Bellocq, J.P., Chambon, P., and Basset, P. Stromelysin 3 belongs to a subgroup of proteinases expressed in breast carcinoma fibroblastic cells and possibly implicated in tumor progression. *Proc. Natl. Acad. Sci.* 90:1843-1847, 1993.
5. Okada, A., Bellocq, J.P., Rouyer, N., Chenard, M.P., Rio, M.C., Chambon, P., and Basset, P. Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. *Proc. Natl. Acad. Sci.* 92:2730-2734, 1995.
6. Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C., and Chambon, P. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature* 348:699-794, 1990.
7. Byrne, J.A., Tomasetto, C., Garnier, J.M., Rouyer, N., Mattei, M.G., Bellocq, J.P., Rio, M.C., and Basset, P. A screening method to identify genes commonly overexpressed in carcinomas and the identification of a novel complementary DNA sequence. *Cancer Res.* 55:2896-2903, 1995.
8. Tomasetto, C., Regnier, C., Moog-Lutz, C., Mattei, M.G., Chenard, M.P., Lidereau, R., Basset, P., and Rio, M.C. Identification of four novel human genes amplified and overexpressed in breast carcinoma and localized to the q11-q21.3 region of chromosome 17. *Genomics* 28:367-376, 1995.
9. Puente, X.S., Pendas, A.M., Llano, E., Velasco, G., and Lopez-Otin, C. Molecular cloning of a novel membrane-type matrix metalloproteinase from a human breast carcinoma. *Cancer Res.* 56:944-949, 1996.
10. Basset, P., Wolf, C., and Chambon, P. Expression of the stromelysin-3 gene in fibroblastic cells of invasive carcinomas of the breast and other human tissues: a review. *Breast Cancer Res. Treatment* 24:185-193, 1993.
11. Aou, A., Anisowicz, A., Hendrix, M.J.C., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. *Science* 263:526-529, 1994.
12. Sutherland, H., Dougherty, G., and Dedhar, S. Developmental biology and oncology: two sides to the same coin? *New Biologist* 2:970-973, 1990.
13. Cross, M., and Dexter, T.M. Growth factors in development, transformation, and tumorigenesis. *Cell* 64:271-280, 1991.

14. Li, F., Strange, R., Friis, R.R., Djonov, V., Altermatt, H.J., Saurer, S., Niemann, H., and Andres, A.C. Expression of stromelysin-1 and TIMP-1 in the involuting mammary gland and in early invasive tumors of the mouse. *Int. J. Cancer* 59:560-568, 1994.
15. Lund, L.R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M.J., Dano, K., and Werb, Z. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* 122:181-193, 1996.
16. Talhouk, R.S., Bissell, M.J., and Werb, Z. Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. *J. Cell Biol.* 118:1271-1281, 1992.
17. Clark, E.A., Golub, T.R., Lander E.S., and Hynes, R.O. Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* 406:532-535, 2000.
18. Punturieri, A., Filippov, S., Allen, E., Caras, I., Murray, R., Reddy, V. and Weiss, S.J. Regulation of elastinolytic cysteine proteinase activity in normal and cathepsin K-deficient human macrophages. *J. Exp. Med.* 192:789-799, 2000.
19. Sternlicht, M.D., Lochter, A., Sympton, C.J., Huey, B., Rougier, J.-P., Gray, J.W., Pinkel, D., Bissell, M.J., and Werb, Z. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 98:137-146, 1999.
20. Ueno, H., Nakamura, H., Inoue, M., Imai, K., Noguchi, M., Sato, H., Seiki, M., and Okada, Y. Expression and tissue localization of membrane-types 1, 2, and 3 matrix metalloproteinases in human invasive breast carcinomas. *Cancer Res.* 57:2055-2060, 1997.
21. Hotary, K., Allen, E., Punturieri, A., Yana, I., Weiss, S.J. Regulation of cell invasion and morphogenesis in a 3-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2 and 3. *J. Cell Biol.* 149:1309-1323, 2000.
22. Nagase, H., and Woessner, Jr., J.F. Matrix metalloproteinases. *J Biol Chem* 274:21491-21494, 1999.
23. Pei, D., and Weiss, S.J. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Nature* 375:244-247, 1995.
24. Capony, F., Rougeot, C., Montcourrier, P., Cavailles, V., Salazar, G., and Rochefort, H. Increased secretion, altered processing, and glycosylation of pro-cathepsin D in human mammary cancer cells. *Cancer Res.* 49:3904-3909, 1989.
25. Montcourrier, P., Mangeat, P.H., Salazar, G., Morisset, M., Sahuguet, A., and Rochefort, H. Cathepsin D in breast cancer cells can digest extracellular matrix in large acidic vesicles. *Cancer Res.* 50:6045-6054, 1990.

26. Punturieri, A., Filippov, S., Allen, E., Caras, I., Murray, R., Reddy, V., Weiss, S.J. Regulation of elastinolytic cysteine proteinase activity in normal and cathepsin K-deficient human macrophages. *J. Exp. Med.* 192:789-800, 2000.
27. Fata, J.E., Leco, K.J., Voura, E.B., Yu, H.-Y.E., Waterhouse, P., Murphy, G., Moorehead, R.A., and Khokha R. Accelerated apoptosis in the TIMP-3-deficient mammary gland. *J Clin Invest* 108:831-841, 2001.

## VIII. APPENDICES

### Bibliography - None

### Personnel

Stephen J. Weiss, M.D.

Kevin Hotary, Ph.D.

### Figure Legends

*Figure 1. Induction of Mammary Gland Involution Program.* Lactating (10 days) or involuting (3 days post-weaning) glands were isolated from wild-type mice and tissues processed for H and E staining and apoptosis (TUNEL) as well as immunostaining for laminin and type IV collagen as described<sup>27</sup>. Lactating glands were milk-engorged and showed no significant apoptosis. In addition, basement membranes were laminin- and type IV collagen- positive. Following 3 days of involution, glandular structures collapsed and apoptotic cells were observed as yellow-green staining. Laminin staining decreased significantly while type IV collagen immunoreactivity decreased coincident with a thickening/blurring of basement membrane structure.

*Figure 2. RT-PCR Analysis of MT1-MMP Expression in Mouse Mammary Glands.* RNA was isolated from virgin, 10 d lactating, 1 d involuting or 5 d involuting glands. Reverse transcription was performed as described<sup>28</sup>. The arrow at left marks the position of the MT1-MMP standard.

*Figure 3. Basement Membranes Synthesized by MDCK Epithelial Cells are Deposited Atop a 3-Dimensional Type I Collagen Gel During a 3 Week Culture Period.* In panel A, TEM analysis shows an epithelial cell depositing a ~90 nm thick basement membrane which is more readily observed after the overlying cell layer has been lysed (panel B). Panels C and D are scanning electron micrographs showing the type I collagen gel upon which the intact basement membrane is deposited, respectively.

*Figure 4. Basement Membrane-Invasive Potential of Stromelysin-1.* Stromelysin-1 transfected cells cultured atop an intact basement membrane (arrows) were unable to confer degradative or invasive activity as visualized by transmission electron microscopy.

*Figure 5. MT1-MMP-Dependent Basement Membrane Degradation/Invasion.* Control cells did not penetrate the basement membrane as assessed by TEM or SEM analysis following a 5 d incubation period. (upper left and right panels). In contrast, MT1-MMP-transfected cells perforated the BM in representative TEM and SEM samples, respectively. Arrows indicate the position of the basement membrane.

*Figure 6. Active MMP-2 or MMP-9 Do Not Confer Basement Membrane-Invasive Activity.* COS-1 cells were transfected with MMP-2 or MMP-9 chimeric constructs and supernatants analyzed for MMP activation by gelatin zymography (yellow arrow in left panel depicts position of proenzyme and the red arrow marks the position of the fully active, processed enzyme) or for basement membrane degradation (right side) by scanning electron microscopy.

*Figure 7. MT1-MMP-Dependent Degradation of an In Vivo-Generated Basement Membrane.* In the upper panel, the ~90 nm thick peritoneal basement membrane is visualized by TEM. In the three lower panels, SEM analysis demonstrates that MT1-MMP-transfected cells, but not control vector transfectants, degraded the underlying basement membrane by a process that was blocked by the synthetic MMP inhibitor, BB-94 (5  $\mu$ M).

*Figure 8. MT1-MMP Regulates Basement Membrane Invasion of Transfected MCF-7 Cells.* Wild-type or MT1-MMP-transfected MCF-7 cells were cultured atop a basement membrane construct which overlaid a 3-D type I collagen gel in a Transwell insert as described. Following a 5 d incubation period, invasion by MT1-MMP transfectants was observed by phase contrast microscopy (left panel) or in H and E sections (right panel). The control MCF-7 cells which do not express MT1-MMP, did not display invasive activity.

*Figure 9. Basement Membrane Invasion by Human MB-231 Breast Cancer Cells.* Peritoneal basement membranes (top panel) were used as a substratum for MB-231 cells during a 5 d incubation period. Cells were then lysed and the underlying basement membrane viewed by scanning electron microscopy. MB-231 cells left large holes in the basement membrane leaving the underlying interstitial matrix exposed (middle panel). Culturing the MB-231 cells atop the basement membrane in the presence of the MMP inhibitor, TIMP-2 (5  $\mu$ g/ml), left the basement membrane largely intact (bottom panel).

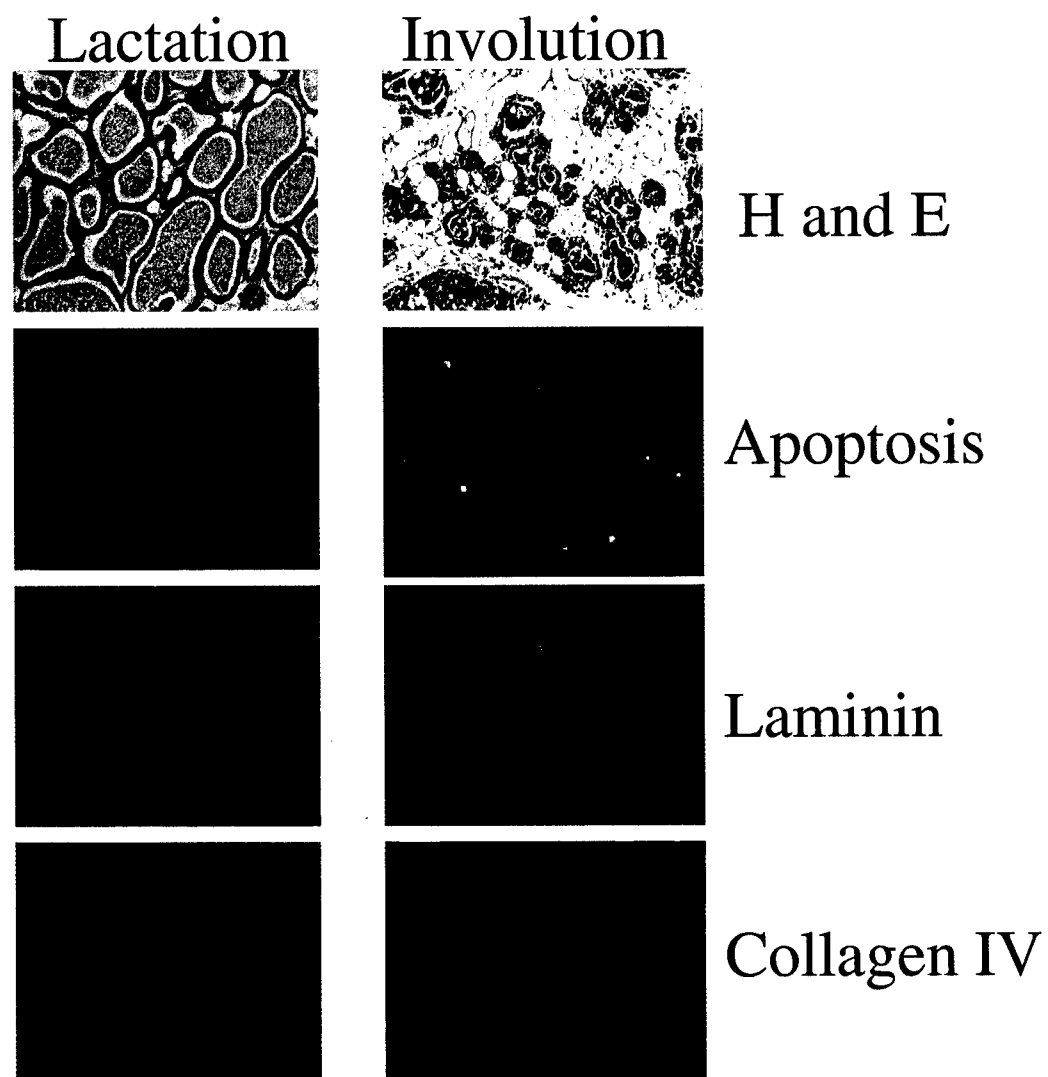


Figure 1

## RT-PCR Analysis of MT1-MMP Expression

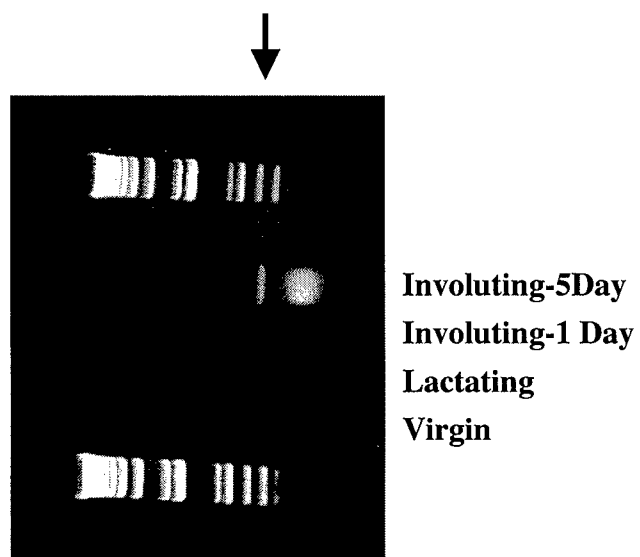
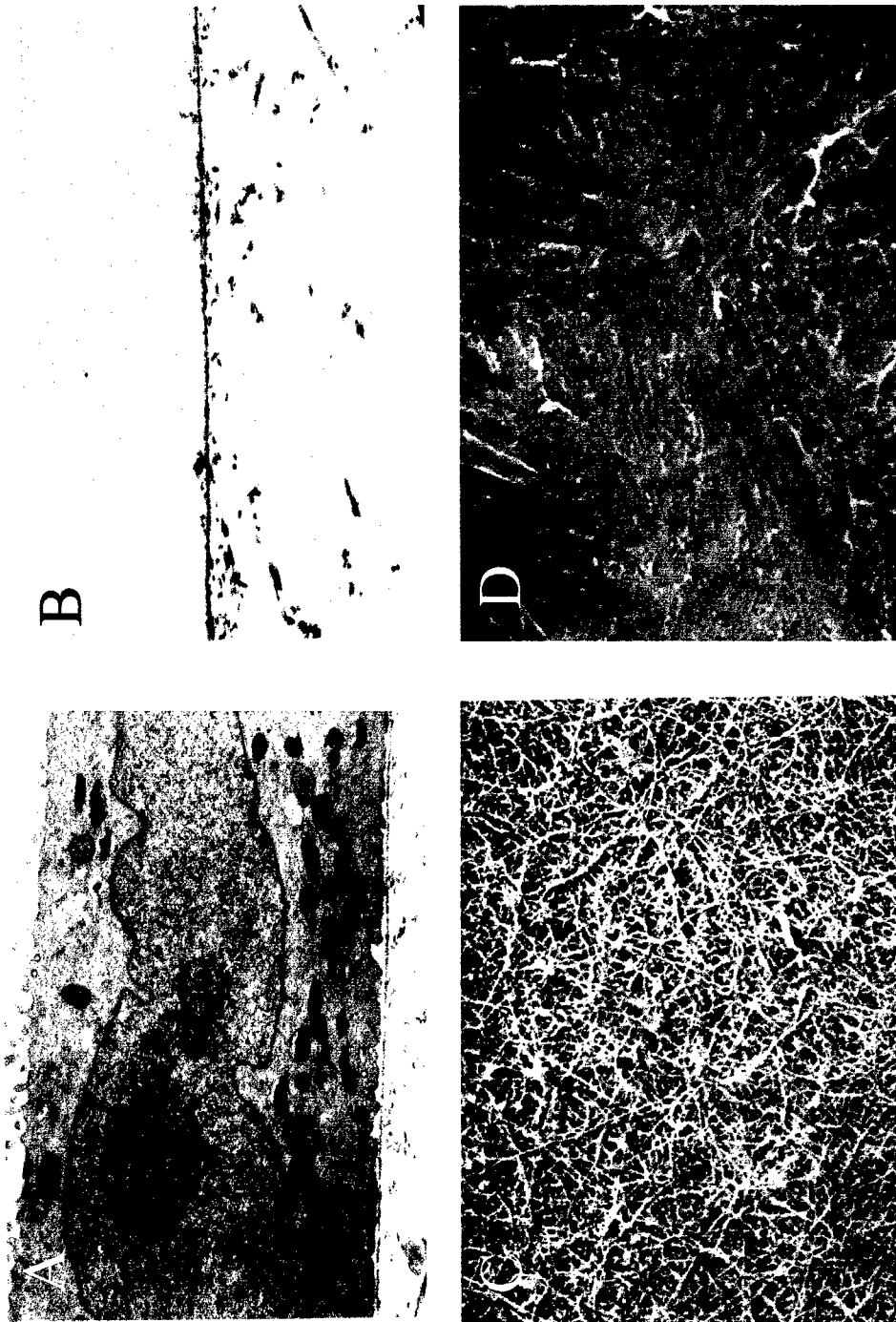


Figure 2

Figure 3



# Stromelysin-1 Does Not Confer Basement Membrane-Invasive Activity

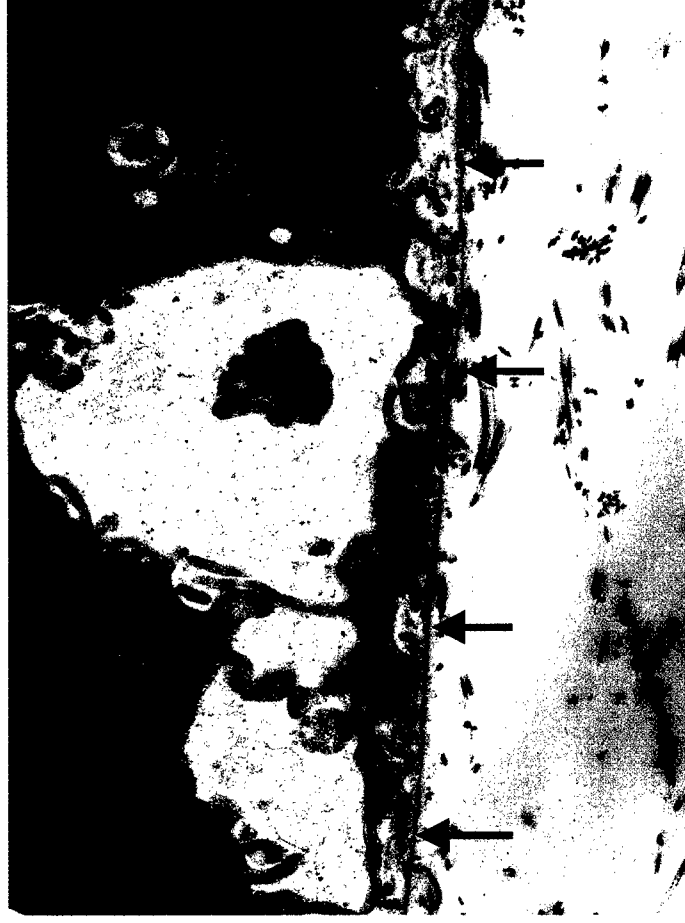


Figure 4

# Basement Membrane Invasion Mediated By MT1-MMP

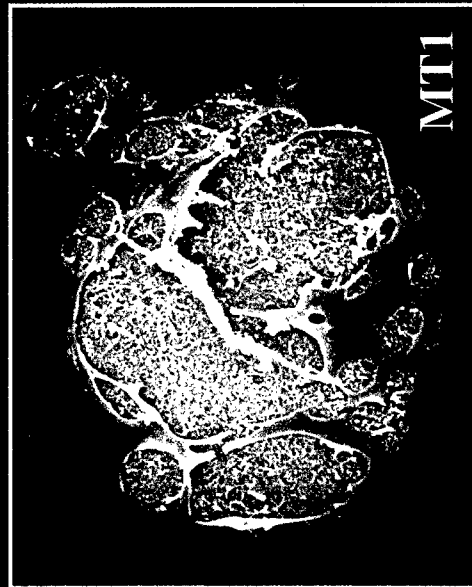
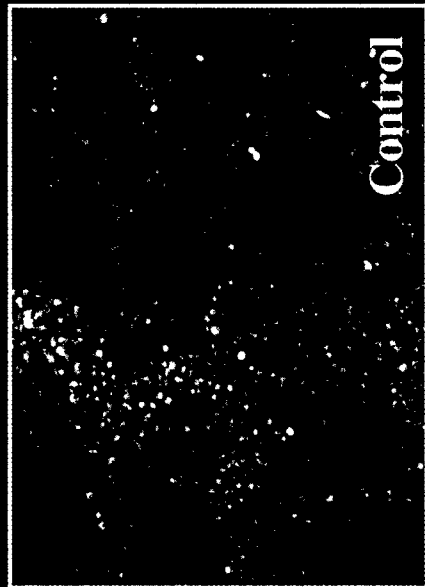


Figure 5

## Fully Active MMP-2 and MMP-9 Do Not Invade Basement Membrane

Proprotein convertase recognition sequence (RXXR) inserted between Pro domain and catalytic domain - generates active enzyme.

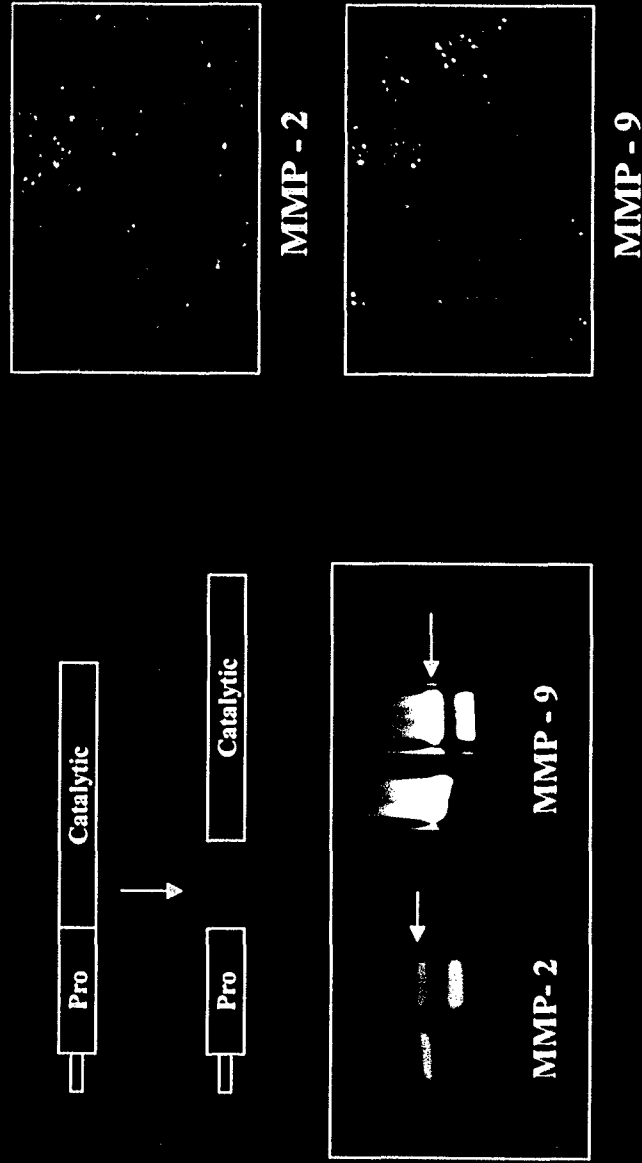


Figure 6

# Basement Membrane Invasion By MT1-MMP



Figure 7



+BB-94

Control

MT1-MMP

# MT1-MMP Regulates Basement Membrane Invasion of MCF-7 Cells

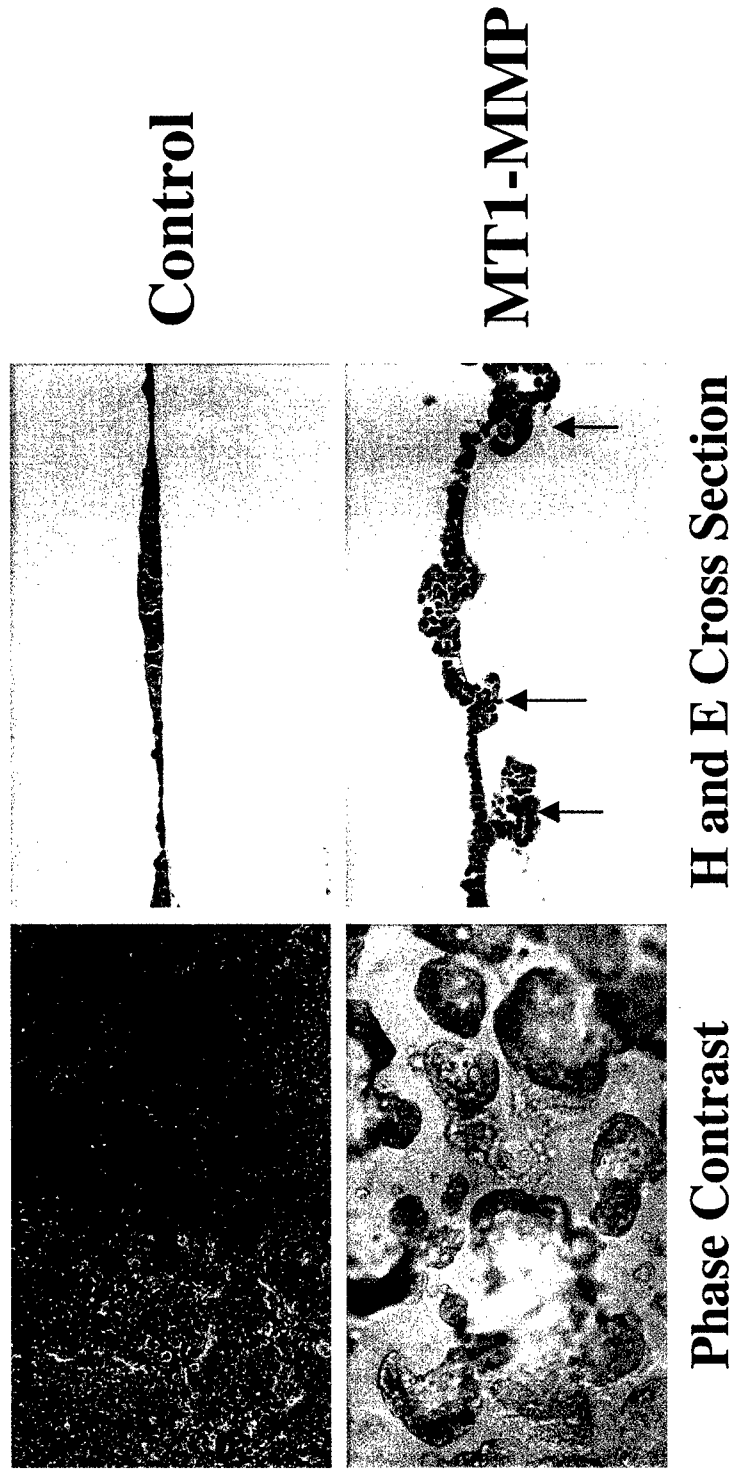
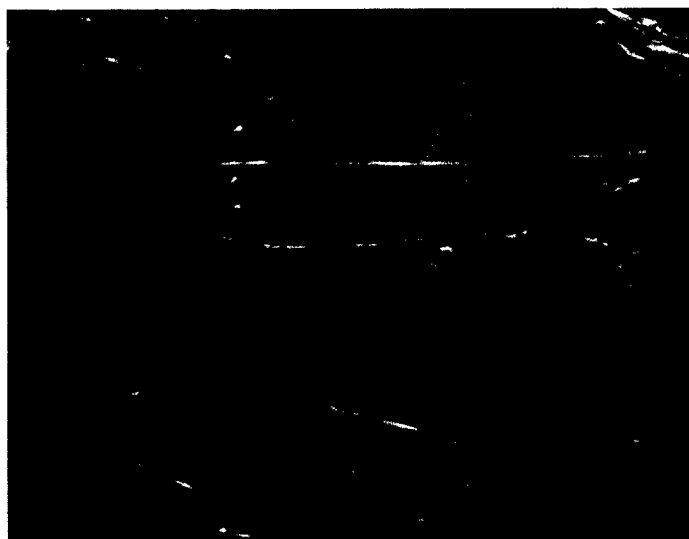
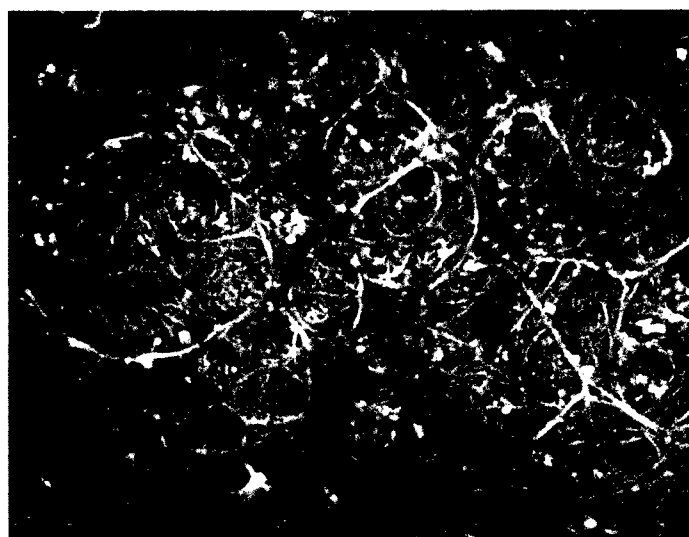


Figure 8

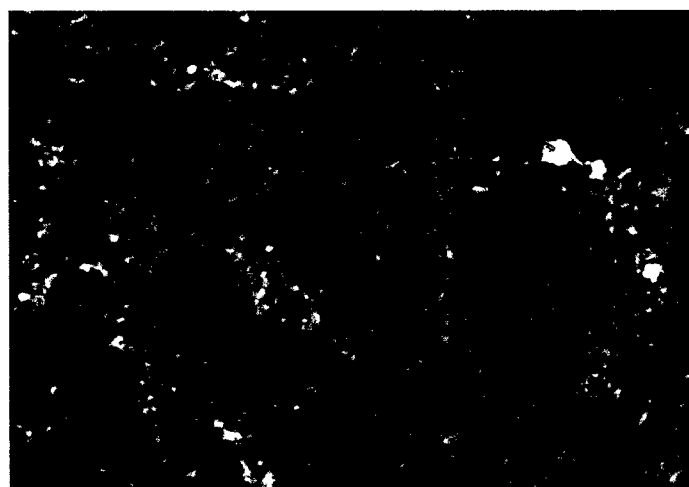
# **MT1-MMP-Positive MB-231 Cells Display Invasive Activity**



**Control**



**MB-231**



**MB-231  
+ TIMP-2**

**Figure 9**

# APPENDIX

| CHIP PROBESET    | Inv A | Inv C | Inv D | Lac J | Lac K | Lac N | DESC                                                                                                                                                                      |
|------------------|-------|-------|-------|-------|-------|-------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| A 108235_s       | 3894  | 4535  | 3521  | 915   | 643   | 652   | Mus musculus clusterin mRNA, complete cds.                                                                                                                                |
| A 112447_s       | 1861  | 2287  | 2039  | 943   | 297   | 297   | Mus musculus insulin-like growth factor binding protein 5 (IGFBP5) mRNA, complete cds.                                                                                    |
| A AA09861_f      | 1694  | 1926  | 1090  | 705   | 390   | 442   | vt35a01.r1 Barstead Mu proximal colon MPLRB6 Mus musculus cDNA clone 1165032 5', mRNA sequence.                                                                           |
| B Msa.1271.0_s   | 1168  | 1258  | 1105  | 479   | 260   | 280   | House Mu; Musculus domesticus mRNA for lactoferrin, complete cds                                                                                                          |
| A d11468_s       | 1047  | 1148  | 1114  | 181   | 358   | 417   | Mu gene for immunoglobulin alpha heavy chain, switch region and constant region complete sequence.                                                                        |
| A u73004_s       | 799   | 778   | 695   | 186   | 89    | 105   | Mus musculus secretory leukocyte protease inhibitor mRNA, complete cds.                                                                                                   |
| B x52886_s       | 768   | 628   | 682   | 337   | 161   | 145   | M.musculus mRNA for cathepsin D.                                                                                                                                          |
| B ET62985_f      | 730   | 995   | 787   | 169   | 293   | 364   | M.musculus mRNA (1B5) for IgA V-D-J-heavy chain.                                                                                                                          |
| B X00496_s       | 725   | 600   | 593   | 339   | 69    | 33    | Mu Ia-associated invariant chain (Ii) mRNA fragment.                                                                                                                      |
| B Msa.22134.0_s  | 534   | 480   | 465   | 312   | 69    | 66    | Homologous to sp P13983: EXTENSIN PRECURSOR (CELL WALL HYD                                                                                                                |
| B Msa.739.0_s    | 526   | 643   | 632   | 307   | 127   | 142   | C57BL/6J ob/ob hemoglobin mRNA, complete cds                                                                                                                              |
| B X62940_s       | 499   | 421   | 439   | 196   | 98    | 67    | M.musculus TSC-22 mRNA.                                                                                                                                                   |
| A U62386_s       | 483   | 393   | 352   | 39    | 38    | 54    | Mus musculus immunoglobulin heavy and light chain variable region mRNA, complete cds.                                                                                     |
| A M73329_s       | 481   | 520   | 477   | 342   | 44    | 98    | Mu phospholipase C-alpha (PLC-alpha) mRNA, complete cds.                                                                                                                  |
| B w08453_s       | 417   | 293   | 436   | 191   | 55    | 67    | W08453 mb50a09.r1 Soares Mu p3NNMF19.5 Mus musculus cDNA clone 332824 5' similar to gb:565738 DESTRIN (HUMAN); TIGR cluster TC34375                                       |
| B x67141         | 415   | 778   | 805   | 383   | 59    | 35    | M.musculus Pva mRNA for parvalbumin.                                                                                                                                      |
| B X13605_s       | 406   | 274   | 364   | 219   | 50    | 40    | Murine mRNA for replacement variant histone H3.3.                                                                                                                         |
| B ET63295_f      | 395   | 318   | 326   | 31    | 126   | 126   | M.domesticus IgM variable region.JPIR:S26747 (Ig heavy chain J region JH4 - Mu                                                                                            |
| A aa711217_s     | 349   | 372   | 391   | 230   | 46    | 72    | vt0b10.r2 Barstead Mu irradiated colon MPLRB7 Mus musculus cDNA clone 1176475 5' similar to SW:NB2M_BOVIN Q02365 NADH-UBIQUINONE OXIDOREDUCTA                             |
| B x51438_s       | 342   | 185   | 290   | 167   | 22    | 32    | Mu mRNA for vimentin.                                                                                                                                                     |
| B Msa.6056.0_s   | 324   | 353   | 413   | 215   | 19    | 28    | Homologous to sp P09912: INTERFERON-INDUCED PROTEIN 6-16 PRECURSOR (IFI-6-16).                                                                                            |
| B Msa.41264.0_s  | 309   | 494   | 535   | 239   | -8    | -11   | Homologous to sp P04462: MYOSIN HEAVY CHAIN, PERINATAL SKELETAL MUSCLE (FRAGMENT).                                                                                        |
| B ET61925_f      | 299   | 364   | 344   | 72    | 92    | 118   | Mus musculus anti-DNA immunoglobulin light chain IgG, antibody 363s.71, partial cds.                                                                                      |
| B ET62705_f      | 291   | 198   | 200   | 39    | 79    | 77    | Mus musculus anti-DNA antibody heavy chain variable region mRNA, partial cds.                                                                                             |
| B Msa.22488.0_s  | 244   | 194   | 221   | 106   | 32    | 30    | Homologous to sp Q02765: CATHEPSIN S PRECURSOR (EC 3.4.22.27).                                                                                                            |
| B x06115_s       | 226   | 128   | 168   | 95    | 45    | 33    | Mu mRNA for E-cadherin (= uvomulin, = L-CAM, = cell-CAM 120/80, = Avc-1).                                                                                                 |
| B x61433_s       | 223   | 211   | 227   | 120   | 49    | 22    | M.musculus mRNA for sodium/potassium ATPase beta subunit.                                                                                                                 |
| B AFFX-MURINE_b1 | 216   | 128   | 169   | 87    | 10    | 20    | MURINE_b1                                                                                                                                                                 |
| A J04992_f       | 200   | 324   | 312   | 212   | 23    | 30    | Mu fast fiber tropinin 1 mRNA, complete cds.                                                                                                                              |
| B ET63288_f      | 194   | 172   | 170   | 26    | 67    | 65    | M.domesticus IgM variable region.JPIR:PH0975 (Ig heavy chain V region (clone 163.72) - Mu (fragment)                                                                      |
| B x14194_s       | 190   | 131   | 193   | 51    | 1     | -7    | Mu mRNA for entactin.                                                                                                                                                     |
| B ET63358_f      | 178   | 174   | 161   | 17    | 29    | 27    | M.domesticus IgK variable region.JPIR:PH1046 (Ig light chain V region (clone 202.9) - Mu (fragment))PIR:PH1048 (Ig light chain V region (clone 165.49) - Mu (fragment))PI |
| B Msa.88.0_s     | 173   | 150   | 153   | 109   | 27    | 21    | Mu mRNA for osteoblast specific factor 2 (OSF-2)                                                                                                                          |
| B Msa.41890.0_f  | 171   | 297   | 276   | 170   | 22    | 25    | Homologous to sp P10469: TROPOMYOSIN ALPHA CHAIN, SMOOTH MUSCLE (FRAGMENT).                                                                                               |
| B Msa.2614.0_g   | 169   | 187   | 178   | 87    | 40    | 45    | Mu mRNA for perlecan (AA 5 - 441)                                                                                                                                         |
| B w54482         | 154   | 60    | 103   | 44    | 19    | 25    | W54482 md08e09.r1 Soares Mu embryo NBME13.5 14.5 Mus musculus cDNA clone 367816 5' similar to WP:TI2A2.7 CE01400 .; TIGR cluster TC15447                                  |
| B ET62941_f      | 152   | 254   | 258   | 66    | 68    | 83    | M.musculus antibody light chain variable region (318bp).                                                                                                                  |
| B Msa.2642.0_f   | 144   | 284   | 216   | 185   | 4     | 1     | Mus musculus adenine nucleotide translocase-1 (Ant1) mRNA, complete cds                                                                                                   |
| A m27009_s       | 143   | 101   | 88    | 57    | -17   | -15   | Mu alpha-1 acid glycoprotein (Ags-2) mRNA, complete cds.                                                                                                                  |

|   |                |     |     |     |     |     |     |                                                                                                                                                  |
|---|----------------|-----|-----|-----|-----|-----|-----|--------------------------------------------------------------------------------------------------------------------------------------------------|
| B | x62600_s       | 139 | 54  | 79  | 36  | 6   | -10 | M.musculus mRNA for C/EBP beta.                                                                                                                  |
| B | x64837_s       | 139 | 88  | 83  | 66  | 26  | 6   | M.musculus Oat mRNA for ornithine aminotransferase.                                                                                              |
| A | aa690738_s     | 138 | 118 | 114 | 29  | -32 | -31 | vu57b03.r1 Soares Mu mammary gland NbMMG Mus musculus cDNA clone 1195469 5' mRNA sequence.                                                       |
| A | aa260736_s     | 130 | 98  | 93  | 67  | 15  | 14  | AA260736 va02a11.r1 Soares Mu lymph node NbMLN Mus musculus cDNA clone 721724 5' TIGR cluster TC22696                                            |
| B | x15591_s       | 128 | 98  | 110 | 54  | 14  | 7   | Mu cta-2-alpha mRNA, homolog, to cysteine protease proregion.                                                                                    |
| B | ET61285_f      | 126 | 70  | 74  | 6   | 25  | 24  | Mus musculus anti-DNA immunoglobulin heavy chain variable region, clone 4B2, partial cds.                                                        |
| A | aa265871       | 124 | 116 | 117 | 103 | -7  | 6   | AA265871 mz70h03.r1 Soares Mu lymph node NbMLN Mus musculus cDNA clone 718805 5' TIGR cluster TC34843                                            |
| B | Msa..21652.0_f | 123 | 82  | 114 | 51  | 10  | 8   | Homologous to sp P09568: LYMPHOCYTE ANTIGEN LY-6C.2LY-6C.1 PRECURSOR.                                                                            |
| B | w30230_g       | 123 | 40  | 78  | 40  | 8   | 7   | W30230 mc26f11.r1 Soares Mu p3NNMF19.5 Mus musculus cDNA clone 349677 5' TIGR cluster TC26484                                                    |
| B | w82831_i       | 122 | 54  | 83  | 16  | -8  | -5  | W82831 mf07e10.r1 Soares Mu p3NNMF19.5 Mus musculus cDNA clone 404394 5' TIGR cluster TC23212                                                    |
| A | aa254768       | 112 | 94  | 99  | 48  | 0   | -5  | AA254768 mz75h08.r1 Soares Mu lymph node NbMLN Mus musculus cDNA clone 719295 5'                                                                 |
| B | Msa..728.0     | 111 | 117 | 159 | 82  | -16 | -15 | M.musculus glucose transporter 2 mRNA, complete cds                                                                                              |
| A | M60474_f       | 110 | 105 | 94  | 51  | 2   | 7   | Mu myristoylated alanine-rich C-kinase substrate (MARCKS) mRNA, complete cds.                                                                    |
| B | x66402_s       | 109 | 69  | 77  | 25  | -3  | -10 | M.musculus mRNA for stromelysin 1.                                                                                                               |
| A | aa684097       | 107 | 110 | 103 | 68  | 10  | 16  | vm66d02.s1 Knowles Solter Mu 2 cell Mus musculus cDNA clone 1003203 5' mRNA sequence.                                                            |
| A | u34920_s       | 107 | 82  | 85  | 18  | -21 | -18 | Mus musculus white homolog (white) mRNA, complete cds.                                                                                           |
| B | Msa..39985.0_f | 107 | 229 | 259 | 141 | -8  | -7  | Homologous to sp P49438: TROPOMYOSIN ALPHA CHAIN, MAJOR BRAIN ISOFORM.                                                                           |
| A | c75983_rc_f    | 99  | 112 | 165 | 46  | 29  | 29  | C75983 Mu 3.5-dpc blastocyst cDNA Mus musculus cDNA clone J0001E09 3' similar to Unannotated data. mRNA sequence.                                |
| B | ET61749_f      | 97  | 80  | 73  | 18  | 27  | 23  | Mus musculus Ig 6C3.B8 heavy chain mRNA, specific for rat (Mu) cytochrome c, partial cds.                                                        |
| A | M27008         | 95  | 63  | 72  | 76  | 3   | -18 | Mu alpha-1 acid glycoprotein (Agi-1B) mRNA, complete cds.                                                                                        |
| A | aa690872_f     | 93  | 73  | 80  | 68  | -5  | 0   | vc32c11.r1 Barstead Mu proximal colon MPLRB6 Mus musculus cDNA clone 1164788 5' similar to gb:D00762 PROTEASOME COMPONENT C8 (HUMAN); mRNA sequ  |
| B | Msa..43191.0_s | 92  | 74  | 75  | 54  | -4  | 3   | M.musculus integrin associated protein mRNA, complete CDS (EXTRACTED 3'UTR)                                                                      |
| B | Msa..10564.0   | 90  | 58  | 68  | 24  | 16  | 21  | Homologous to sp P09117: FRUCTOSE-BISPHOSPHATE ALDOLASE (EC 4.1.2.13) C (BRAIN).                                                                 |
| A | D49744_s       | 87  | 85  | 89  | 59  | 3   | 4   | Mu mRNA for farnesyltransferase alpha subunit, complete cds.                                                                                     |
| A | M74227_s       | 85  | 71  | 80  | 37  | 9   | 7   | Mu cyclophilin C (cyp C) mRNA, complete cds.                                                                                                     |
| B | Msa..727.0_s   | 82  | 55  | 51  | 33  | 2   | 11  | Mu glutathione S-transferase (GST Yc) mRNA, complete cds                                                                                         |
| A | M63695_s       | 82  | 48  | 54  | 7   | -25 | -23 | Mu CD1.1 mRNA, complete cds.                                                                                                                     |
| B | x56548_s       | 80  | 52  | 69  | 22  | -12 | -10 | M.musculus Np-b mRNA for purine-nucleoside phosphorylase.                                                                                        |
| A | D50032_s       | 79  | 73  | 47  | 18  | 13  | 9   | Mu mRNA for TGN38B, complete cds.                                                                                                                |
| A | U25844_s       | 79  | 77  | 63  | 27  | -19 | -32 | Mus musculus serine proteinase inhibitor (SPI3) mRNA, complete cds.                                                                              |
| A | U58883_s       | 79  | 59  | 38  | 44  | -7  | -8  | Mus musculus putative SH3-containing protein SH3PI2 mRNA, partial cds.                                                                           |
| B | ET61918_f      | 79  | 95  | 113 | 6   | 27  | 42  | Mus musculus anti-DNA immunoglobulin light chain IgM mRNA, antibody 363p-202, partial cds.                                                       |
| A | AA274696_s     | 78  | 51  | 63  | 38  | 6   | -3  | AA274696 vc05b03.r1 Soares Mu lymph node NbMLN Mus musculus cDNA clone 765581 5' TIGR cluster TC38052                                            |
| B | x83932_s       | 73  | 84  | 134 | 69  | 7   | 8   | M.musculus mRNA for ryanodine receptor type 1.                                                                                                   |
| B | z11997_s       | 70  | 60  | 87  | -1  | -21 | -22 | M.musculus mRNA for non-histone chromosomal high-mobility group 1 protein.                                                                       |
| A | r75131_rc_s    | 69  | 117 | 102 | 66  | -1  | -7  | R75131 MDB1078 Mu brain; Stratagene Mus musculus cDNA 3'end. TIGR cluster TC17356                                                                |
| B | ET62942_f      | 69  | 82  | 79  | 9   | 24  | 28  | M.musculus antibody light chain variable region (324bp).                                                                                         |
| A | AA170444       | 69  | 65  | 56  | 34  | 15  | 12  | AA170444 ms90f10.r1 Soares Mu 3NBMS Mus musculus cDNA clone 618859 5' similar to SW:UBAL_HUMAN P41226 UBIQUITIN-ACTIVATING ENZYME E1 HOMOL       |
| A | aa253918       | 69  | 73  | 91  | 13  | -25 | -25 | AA253918 mw07h06.r1 Soares Mu 3NME12 5 Mus musculus cDNA clone 670043 5' similar to gb:Z21507 ELONGATION FACTOR 1-DELTA (HUMAN); TIGR cluster TC |
| A | AA259399_s     | 67  | 54  | 54  | 63  | -5  | -1  | AA259399 va51b02.r1 Soares Mu 3NME12 5 Mus musculus cDNA clone 734859 5' TIGR cluster TC35817                                                    |

|   |               |    |     |     |    |     |                                                                                                                               |
|---|---------------|----|-----|-----|----|-----|-------------------------------------------------------------------------------------------------------------------------------|
| B | Msa.41380.0_s | 66 | 166 | 181 | 95 | 6   | 12 Homologous to sp Q08043: ALPHA-ACTININ 3, SKELETAL MUSCLE ISOFORM (F-ACTIN CROSS LINKING PROTEIN).                         |
| B | v00727_s      | 61 | 26  | 69  | 21 | 14  | 10 Mu c-fos oncogene.                                                                                                         |
| A | j05479_s      | 60 | 50  | 50  | 28 | -2  | 4 Mu calcineurin catalytic subunit mRNA, complete cds.                                                                        |
| A | AA597258      | 60 | 46  | 50  | 44 | 2   | 3 vo36a09.r1 Barstead Mu irradiated colon MPLRB7 Mus musculus cDNA clone 1051960 5' mRNA sequence.                            |
| A | L04961_s      | 58 | 44  | 49  | 27 | -15 | 3 Mu nuclear-localized inactive X-specific transcript (Xist) mRNA.                                                            |
| B | Msa.3557.0_s  | 56 | 70  | 59  | 29 | -8  | -5 Homologous to sp P36955: PIGMENT EPITHELIUM-DERIVED FACTOR PRECURSOR (PEDF) (EPC-1).                                       |
| A | AA203803_s    | 56 | 56  | 44  | 39 | 9   | 2 AA203803 mu61b05.r1 Soares Mu lymph node N0MLN Mus musculus cDNA clone 643857 5' TIGR cluster TC25395                       |
| A | aa500554_s    | 49 | 55  | 59  | 32 | 13  | 3 AA500554 vi86a01.r1 Stratagene Mu skin (#937313) Mus musculus cDNA clone 919080 5' TIGR cluster TC39733                     |
| A | af023463_s    | 49 | 54  | 58  | 38 | 8   | 6 Mus musculus peroxisomal phytyl-CoA alpha-hydroxylase (PAHX) mRNA, complete cds.                                            |
| B | BT62260_f     | 43 | 160 | 162 | 22 | 30  | 39 Mus musculus immunoglobulin light chain variable region mRNA, partial cds.                                                 |
| A | M74495_s      | 41 | 87  | 88  | 49 | 5   | 4 Mu adenylosuccinate synthetase mRNA, complete cds.                                                                          |
| A | c76162_rc_f   | 32 | 75  | 104 | 12 | 11  | 9 C76162 Mu 3.5-dpe blastocyst cDNA Mus musculus cDNA clone 10004G06 3' similar to Rat insulin-1 (ins-1) gene, mRNA sequence. |