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Award Number: W81XWH-07-1-0424

TITLE: Annexin II-dependent mechanism of breast cancer progression

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REPORT DATE: June 2008

TYPE OF REPORT: Annual

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
30-06-2008	Annual	1 JUN 2007 - 31 MAY 2008

4. TITLE AND SUBTITLE

Annexin II-dependent mechanism of breast cancer progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0424

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Drexel University
Philadelphia, PA 19102

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

Angiogenesis and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of anti-cancer agents. Invasive and metastatic cancers of the breast are distinguished by their propensity of newly formed blood vessels (neoangiogenesis). Neoangiogenesis is a significant independent prognostic indicator in early stage breast cancer (1). Delineating the molecular mechanism(s) of neoangiogenesis may provide new insights into the biology of breast cancer progression and metastasis and may provide novel prognostic and therapeutic tools. Recently, the plasminogen (PLG)/plasmin (PL) system was demonstrated to play an important role in breast cancer progression and metastasis. Experimental studies in animal models combined with extensive clinicopathological data provide a compelling case indicating that proteins of PLG/ PL pathways play a key role in breast cancer progression and metastasis(2). In this context, enzymes of the PLG/PL pathway have been reported to have prognostic value in breast cancer and are associated with poor prognosis both for overall and disease free survival(2). In fact these molecules have been associated with a high rate of relapse for patients with breast cancer Preliminary studies in animal model demonstrated that PLG gene deficient mice (PLG-/-) display inhibition of tumor invasion, lymph node metastasis and angiogenesis supporting the idea that PL is required for angiogenesis, tumor growth and metastasis(3). Despite established role in tumor angiogenesis, growth and metastasis it is still unclear

15. SUBJECT TERMS
Annexin II, angiogenesis, breast cancer, plasmin

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	9	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION: Angiogenesis and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of anticancer agents. Both angiogenesis and metastasis require a proteolytic cascade that involves serine, cysteine, and metalloproteases. This proteolytic cascade degrades the extracellular matrix (ECM) and the basement membrane that surround blood vessels (1, 2). During angiogenesis, the resulting lesion in the basement membrane allows endothelial/epithelial cells to extend into the neighboring tissues and form new blood vessels. During metastasis, cancer cells penetrate through the degraded basement membrane, become implanted in the underlying tissues, and subsequently form secondary tumors. Thus, cell migration, invasion, and proliferation rely on a proteolytic/antiproteolytic balance based on cell-surface-restricted reactions. The plasminogen/plasmin system, a serine protease pathway that is known to regulate angiogenesis, also plays a critical role in breast cancer (3). Analysis of the enzymes of the plasminogen/plasmin system suggests that high levels of urokinase-type plasminogen activator (uPA) in breast tumors predict poor survival (4, 5) and are significantly associated with a high rate of relapse (4). Breast cancer growth and metastasis require extensive angiogenesis (6). Microvessel density in the area of the most intense neovascularization in invasive breast carcinoma is an independent and highly significant prognostic indicator for overall and relapse-free survival in patients with early-stage breast carcinoma (6).

Cell surface receptors for plasminogen activators (PA) (7) or cellular binding sites for plasminogen (8) are positioned for localized generation of plasmin. Plasmin is a strong serine protease that plays an important role in the proteolytic cascade (1). This protease acts directly by hydrolyzing components of the basement membrane such as fibrin, type IV collagen, fibronectin, and laminin and also acts indirectly by activating other enzymes in the cascade such as matrix metalloproteases, collagenases, and vascular endothelial growth factors (9). Degradation of the basement membrane by plasmin is a multistep process. For example, during the first step in fibrin hydrolysis, plasminogen, which is the inactive precursor to plasmin, binds to receptors via a lysine-binding site that is also known as kringle1-4 (10). Next, plasminogen is converted to active plasmin in a reaction that is catalyzed by PA (uPA or tPA) (11). It has been reported that the proteolytic activity of plasmin contributes to angiogenesis (1, 12) and invasion/metastasis (13-15). Reports also suggest that the morbidity and mortality of mice with Lewis lung carcinoma are due to excessive plasmin generation (16). Receptors that regulate the proteolytic cascade may be attractive targets for blocking these processes.

Extensive evidence supports the notion that the components of the plasminogen/plasmin system, such as uPA, including its receptor, the urokinase-type plasminogen receptor, and the plasminogen activator inhibitor 1, contribute to tumorigenesis in a variety of tissue types(17) including breast tissue (18). Consistent with this evidence, inhibition of uPA activity decreases breast cancer growth and metastasis (18). Chernicky et al. recently showed that tPA is associated with the invasion of highly aggressive MDA-MB435s breast cancer cells (19). These reports further document the importance of tPA- or uPA-dependent generation of plasmin in breast cancer. Despite the established role of the plasminogen/plasmin system in breast cancer, the receptor(s) that regulate plasminogen conversion to plasmin in breast cancer are poorly understood. Annexin II is one of the most studied receptors for plasminogen and tPA (8, 20). A number of prior articles have described the fact that cell surface annexin II regulates plasmin generation (11, 20), which in turn facilitates ECM degradation and cell invasion (10, 11, 21) and migration (22), which leads to the formation of new blood vessels (neoangiogenesis) (12). Tumor cells must acquire these biological activities in order to switch to the angiogenic

phenotype, which is characterized by exponential tumor growth and metastasis. Consistent with results reported for most types of tumors, numerous studies suggest that breast cancer growth and metastasis are also dependent on angiogenesis (6). We recently showed that angiostatin, which inhibits breast cancer growth and metastasis, binds to cell surface annexin II, suggesting involvement of annexin II in breast cancer (23). To understand the molecular mechanisms, we proposed in this grant the silencing of the annexin II gene.

BODY: In task 1 of our statement of work (SOW), we proposed silencing annexin II in MDA-MB231 cells. We also planned to characterize the biological properties of these cells.
We have successfully completed the portion of task 1 described in the SOW. The results of the experiments for task 1 are summarized below.

Task 1: To delineate the molecular mechanism by which breast cancer cell surface annexin II generates plasmin, which in turn facilitates ECM degradation and cellular invasion and migration, leading to tumor growth and metastasis.

a) Silence the annexin II gene in mDA-MB231 cells using polymerase chain reaction-based short hairpin RNA (1–7 months)
b) Characterize the proliferative, invasive, and migratory properties of the annexin II-silenced cells (7–12 months)

RESULTS:

Annexin II Gene Silencing:

We reported previously that annexin II is overexpressed in highly invasive MDA-MB231cells. We proposed suppressing annexin II expression in MDA-MB231 cells. We characterized the proliferative and migratory capacities of the annexin II-suppressed cells.

Methods:

We used antisense RNA technology to silence the annexin II gene in MDA-MB231 cells according to methods described by Li et al. (24). Briefly, three different diothionated antisense nucleotides (ODN) were synthesized. Optimum time for cellular ODN uptake was determined using fluorescein isothiocyanate(FITC)-labeled ODN.
MDA-MB231 cells were cultured in 96-well plates, and FITC-ODN was incubated in cell cultures for various periods of time. Cells were fixed and visualized under a fluorescence microscope to determine cellular uptake.

Results presented in Fig.1A suggest that the optimum time for ODN uptake is about 48 hours. In subsequent studies, we used 48 hours for cellular uptake to suppress annexin II. Cells were transfected with antisense ODNs for 48 hours and lysed. Annexin II expression was measured by immunoblot analysis. Immunoblot analysis (Fig.1B) indicated that two antisense ODNs were highly effective in silencing the annexin II gene. We quantified the band density to determine the level of suppression. Band density analysis indicated that both antisense ODNs almost completely suppressed (95%) annexin II protein synthesis (Fig.1C). We also used β-actin as a loading control. Scrambled antisense ODNs were used as controls in all transfection studies. The data presented in Fig.1B suggest selective silencing of the annexin II gene in MDA-MB231 cells.

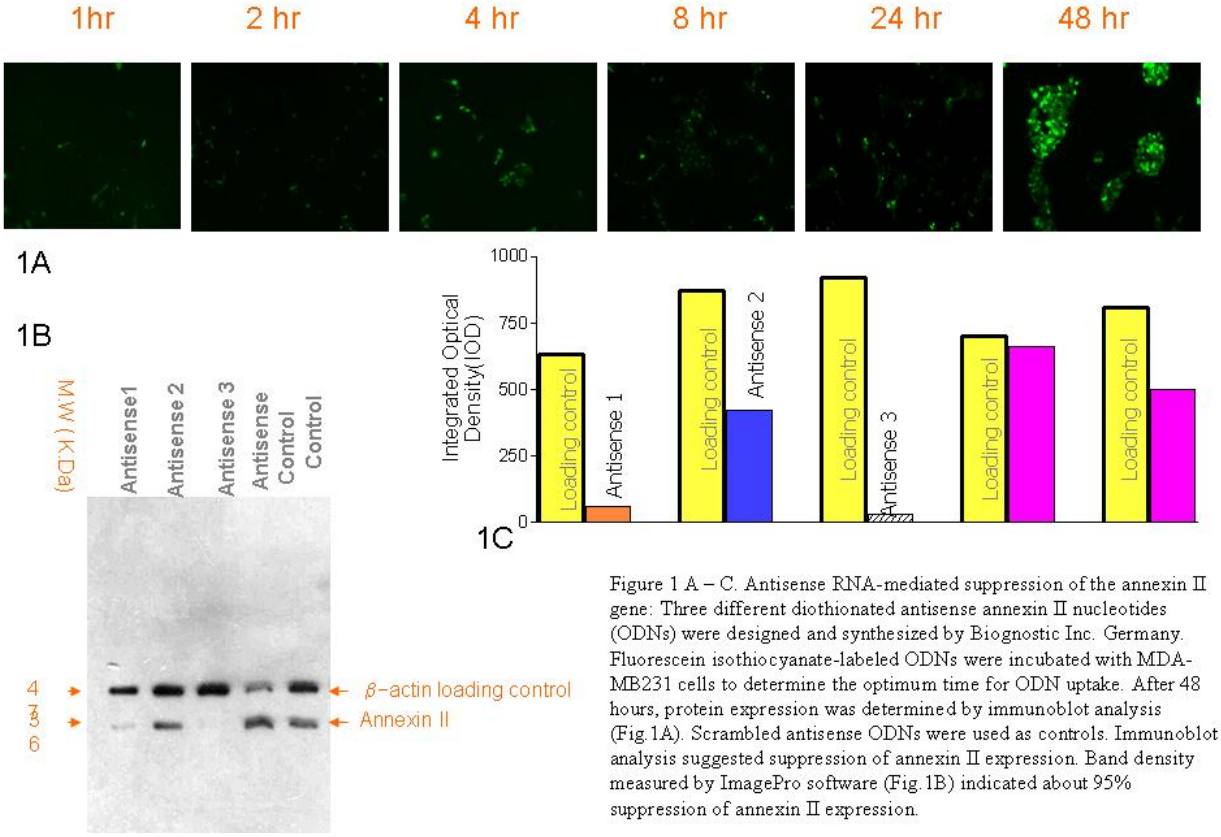

Figure 1 A – C. Antisense RNA-mediated suppression of the annexin II gene: Three different diothionated antisense annexin II nucleotides (ODNs) were designed and synthesized by Biognostic Inc. Germany. Fluorescein isothiocyanate-labeled ODNs were incubated with MDA-MB231 cells to determine the optimum time for ODN uptake. After 48 hours, protein expression was determined by immunoblot analysis (Fig.1A). Scrambled antisense ODNs were used as controls. Immunoblot analysis suggested suppression of annexin II expression. Band density measured by ImagePro software (Fig.1B) indicated about 95% suppression of annexin II expression.

Characterization of Annexin II-silenced MDA-MB231 Cells for Proliferative and Migratory/Invasive Activity.

The annexin II knockout cells were characterized for biological activities such as proliferation and migration.

Cell Proliferation: Cell proliferation was assayed according to our published protocol (25). Briefly, equal numbers (5000) of annexin II-silenced and wild-type cells were seeded in 96-well plates. After 72 hours, proliferation was measured using a cell proliferation assay reagent from Promega. Results indicate that annexin II gene silencing does not affect MDA-MB 231 cell proliferation (Fig.2).

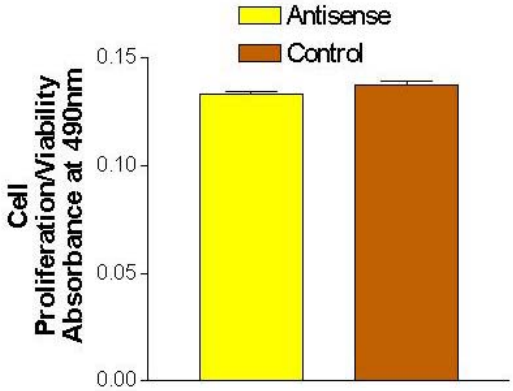

Figure 2. Cell Proliferation: Annexin II-silenced cells were seeded (about 5000 cells) in 96-well plates in triplicate. Proliferation was measured using a cell proliferation assay kit from Promega. Statistical analysis was performed using GraphPad Prism software.

Cell Migration/Invasion: A number of published articles have shown that plasmin degrades ECM, which in turn facilitates cellular migration and plays a key role in the deterioration of additional biological functions that cause tumors to become angiogenic and invasive phenotype. To test the role of annexin II, we investigated the migratory/invasive behavior of annexin II-

suppressed cells. Cell migration was measured *in vitro* using a scratch-wound healing assay as described by Yarrow et al.(25). Monolayer cells were grown in 96-well plates; wounds were created by scratching the cells with a sterile pipette tip. Cells were allowed to grow for 24 hours. Cells were fixed and stained with Giemsa stain. Cell migration was evaluated by counting the cells that migrated from the wound edge. The experiment was repeated three times. Representative pictures of cell migration are shown in Fig. 3A. Data were analyzed for statistical significance using GraphPad Prism software.

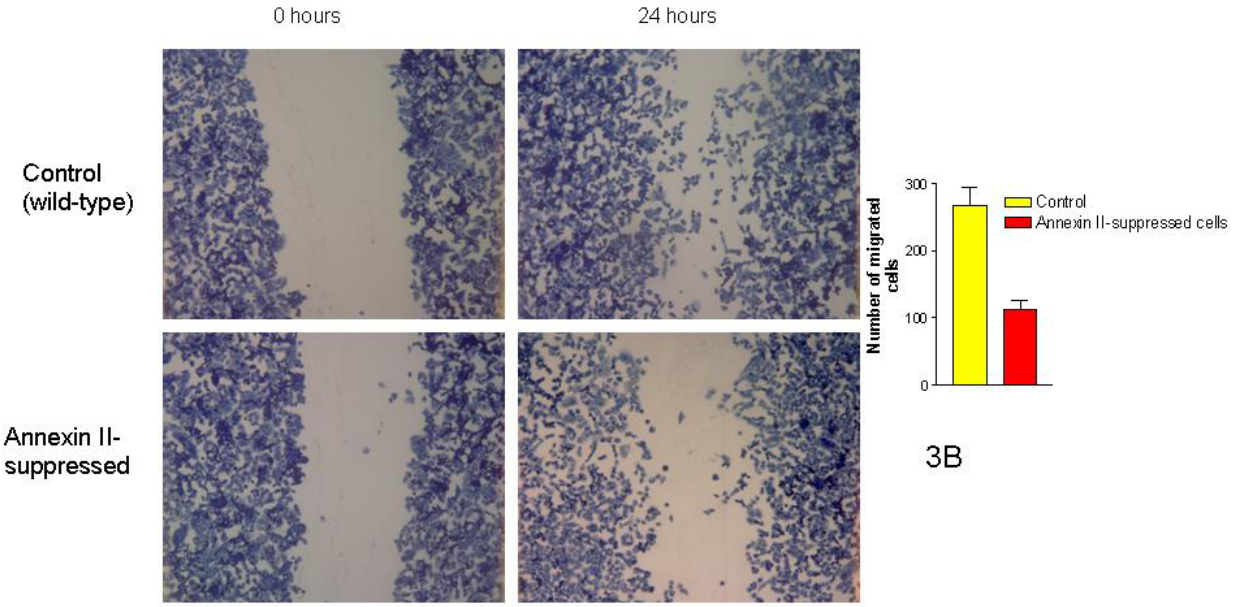

Figure 3A, B. Cell Migration/Invasion: Annexin II-silenced cells were seeded (about 70% confluency) in 96-well plates in triplicate. The next day, a wound was created using a pipette tip. After 24 hours, the cells were fixed and stained with Giemsa stain and photographed (20X) (Fig.3A). Migratory cells were counted in three different fields (Fig.3B). Statistical analysis was performed using GraphPad Prism software.

Key Achievements: We have successfully completed the task described in the SOW. Following are our key achievements:

1. The annexin II gene was almost completely silenced in human breast cell line MDA-MB 231.
2. We showed that annexin II-suppressed cells have no effect on cell proliferation.
3. We showed that annexin II-suppressed cells show significantly inhibited cell migration.

Reportable Outcomes: Results obtained from this study will be presented at the 5th Era of Hope meeting in Baltimore (June 25-28, 2008) sponsored by the Department of Defense. A copy of the abstract will be forwarded to your office after the presentation at the meeting.

Conclusions: Based on our experiments, we concluded that 1) annexin II does not affect MDA-MD231 cell proliferation; 2) annexin II is required for MDA-MB231 cell migration/invasion.

References:

1. Pepper, M. S. Extracellular proteolysis and angiogenesis. Thromb Haemost, *86:* 346-355, 2001.
2. Pepper, M. S., Montesano, R., Mandriota, S. J., Orci, L., and Vassalli, J. D. Angiogenesis: a paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. Enzyme Protein, *49:* 138-162, 1996.
3. Stephens, R. W., Brunner, N., Janicke, F., and Schmitt, M. The urokinase plasminogen activator system as a target for prognostic studies in breast cancer. Breast Cancer Res Treat, *52:* 99-111, 1998.
4. Janicke, F., Schmitt, M., Ulm, K., Gossner, W., and Graeff, H. Urokinase-type plasminogen activator antigen and early relapse in breast cancer. Lancet, *2:* 1049, 1989.
5. Look, M. P., van Putten, W. L., Duffy, M. J., Harbeck, N., Christensen, I. J., Thomssen, C., Kates, R., Spyratos, F., Ferno, M., Eppenberger-Castori, S., Sweep, C. G., Ulm, K., Peyrat, J. P., Martin, P. M., Magdelenat, H., Brunner, N., Duggan, C., Lisboa, B. W., Bendahl, P. O., Quillien, V., Daver, A., Ricolleau, G., Meijer-van Gelder, M. E., Manders, P., Fiets, W. E., Blankenstein, M. A., Broet, P., Romain, S., Daxenbichler, G., Windbichler, G., Cufer, T., Borstnar, S., Kueng, W., Beex, L. V., Klijn, J. G., O'Higgins, N., Eppenberger, U., Janicke, F., Schmitt, M., and Foekens, J. A. Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients. J Natl Cancer Inst, *94:* 116-128, 2002.
6. Weidner, N., Folkman, J., Pozza, F., Bevilacqua, P., Allred, E. N., Moore, D. H., Meli, S., and Gasparini, G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst, *84:* 1875-1887, 1992.
7. Blasi, F. Urokinase and urokinase receptor: a paracrine/autocrine system regulating cell migration and invasiveness. Bioessays, *15:* 105-111, 1993.
8. Cesarman, G. M., Guevara, C. A., and Hajjar, K. A. An endothelial cell receptor for plasminogen/tissue plasminogen activator (t-PA). II. Annexin II-mediated enhancement of t-PA-dependent plasminogen activation. J Biol Chem, *269:* 21198-21203, 1994.
9. McColl, B. K., Baldwin, M. E., Roufail, S., Freeman, C., Moritz, R. L., Simpson, R. J., Alitalo, K., Stacker, S. A., and Achen, M. G. Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. J Exp Med, *198:* 863-868, 2003.
10. Brownstein, C., Deora, A. B., Jacovina, A. T., Weintraub, R., Gertler, M., Khan, K. M., Falcone, D. J., and Hajjar, K. A. Annexin II mediates plasminogen-dependent matrix invasion by human monocytes: enhanced expression by macrophages. Blood, *103:* 317-324, 2004.
11. Diaz, V. M., Hurtado, M., Thomson, T. M., Reventos, J., and Paciucci, R. Specific interaction of tissue-type plasminogen activator (t-PA) with annexin II on the membrane of pancreatic cancer cells activates plasminogen and promotes invasion in vitro. Gut, *53:* 993-1000, 2004.
12. Ling, Q., Jacovina, A. T., Deora, A., Febbraio, M., Simantov, R., Silverstein, R. L., Hempstead, B., Mark, W. H., and Hajjar, K. A. Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo. J Clin Invest, *113:* 38-48, 2004.
13. Bajou, K., Masson, V., Gerard, R. D., Schmitt, P. M., Albert, V., Praus, M., Lund, L. R., Frandsen, T. L., Brunner, N., Dano, K., Fusenig, N. E., Weidle, U., Carmeliet, G.,

Loskutoff, D., Collen, D., Carmeliet, P., Foidart, J. M., and Noel, A. The plasminogen activator inhibitor PAI-1 controls in vivo tumor vascularization by interaction with proteases, not vitronectin. Implications for antiangiogenic strategies. J Cell Biol, *152:* 777-784, 2001.

14. Kobayashi, H., Shinohara, H., Fujie, M., Gotoh, J., Itoh, M., Takeuchi, K., and Terao, T. Inhibition of metastasis of Lewis lung carcinoma by urinary trypsin inhibitor in experimental and spontaneous metastasis models. Int J Cancer, *63:* 455-462, 1995.
15. Tanaka, N., Ogawa, H., Kinjo, M., Kohga, S., and Tanaka, K. Ultrastructural study of the effects of tranexamic acid and urokinase on metastasis of Lewis lung carcinoma. Br J Cancer, *46:* 428-435, 1982.
16. Bugge, T. H., Kombrinck, K. W., Xiao, Q., Holmback, K., Daugherty, C. C., Witte, D. P., and Degen, J. L. Growth and dissemination of Lewis lung carcinoma in plasminogen-deficient mice. Blood, *90:* 4522-4531, 1997.
17. Ossowski, L. and Aguirre-Ghiso, J. A. Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. Curr Opin Cell Biol, *12:* 613-620, 2000.
18. Rabbani, S. A. and Gladu, J. Urokinase receptor antibody can reduce tumor volume and detect the presence of occult tumor metastases in vivo. Cancer Res, *62:* 2390-2397, 2002.
19. Chernicky, C. L., Yi, L., Tan, H., and Ilan, J. Tissue-type plasminogen activator is upregulated in metastatic breast cancer cells exposed to insulin-like growth factor-I. Clin Breast Cancer, *6:* 340-348, 2005.
20. Hajjar, K. A., Jacovina, A. T., and Chacko, J. An endothelial cell receptor for plasminogen/tissue plasminogen activator. I. Identity with annexin II. J Biol Chem, *269:* 21191-21197, 1994.
21. Mignatti, P. and Rifkin, D. B. Biology and biochemistry of proteinases in tumor invasion. Physiol Rev, *73:* 161-195, 1993.
22. Tarui, T., Majumdar, M., Miles, L. A., Ruf, W., and Takada, Y. Plasmin-induced migration of endothelial cells. A potential target for the anti-angiogenic action of angiostatin. J Biol Chem, *277:* 33564-33570, 2002.
23. Tuszynski, G. P., Sharma, M., Rothman, V. L., and Sharma, M. C. Angiostatin binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. Microvasc Res, *64:* 448-462, 2002.
24. Li, D. and Mehta, J. L. Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. Circulation, *101:* 2889-2895, 2000.
25. Yarrow, C., Benoit, G., and Klein, M. C. Outcomes after vacuum-assisted deliveries. Births attended by community family practitioners. Can Fam Physician, *50:* 1109-1114, 2004.