AD_____

Award Number: DAMD17-02-1-0541

TITLE: Metastasis Genes in Breast Cancer Metastasis to Bone

PRINCIPAL INVESTIGATOR: Danny R. Welch, Ph.D.

CONTRACTING ORGANIZATION: The Pennsylvania State University
Hershey, Pennsylvania 17033-2390

REPORT DATE: June 2003

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. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual (31 May 2002 - 30 May 2003)

4. TITLE AND SUBTITLE
Metastasis Genes in Breast Cancer Metastasis to Bone

5. FUNDING NUMBERS
DAMD17-02-1-0541

6. AUTHOR(S)
Danny R. Welch, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Pennsylvania State University
Hershey, Pennsylvania 17033-2390

E-Mail: dwelch@path.uab.edu

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. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

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

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
The most prevalent site of breast cancer metastasis is bone. We will begin to elucidate the molecular mechanisms involved in bone metastasis. We propose to develop and utilize green (GFP) and/or red (dsRed) fluorescent protein-tagged breast carcinoma xenografts to measure bone metastasis following intracardiac injection. Cell lines developed will be used to test whether a metastasis suppressor (BRMS1) and a gene it down-regulates (osteopontin) alter the efficiency of bone colonization. Concomitantly, we will test the impact of changed gene expression on the ability of tumor cells to adhere to human osteoblast cell cultures or human bone marrow endothelium.

14. SUBJECT TERMS
Metastasis, bone metastasis, BRMS1, osteopontin

15. NUMBER OF PAGES
57

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

The overall objective of the proposed research is to test the roles of BRMS1 in the ability of breast xenografts to metastasize to bone following intracardiac injection. A corollary is that BRMS1-regulated genes, especially osteopontin (OPN) will influence metastasis. Briefly, OPN is thought to be a metastasis promoting gene, while BRMS1 is a metastasis suppressor.

Knowing that BRMS1 suppresses metastasis from an orthotopic site to lung and regional lymph nodes (1-3), it is not known whether metastasis is suppressed all sites. Since breast cancer spreads most commonly to bone, we will test whether BRMS1 blocks bone metastasis.

BRMS1 is part of a histone deacetylase complex (4), it follows that it might be regulating effector molecules. In a single microarray study, one of the most prominent changes was down-regulation of osteopontin, a molecule known to promote metastasis (5-7). The question to be addressed in this grant is whether OPN is a downstream regulator of metastasis. Originally, we planned to over-express OPN; however, because of technical issues (see below), we have modified the approach and will use small interfering RNAs (RNAi) (8;9) to decrease OPN expression specifically.

In order to accomplish the above experiments, better bone metastasis models for breast cancer were needed. At the time of the original submission, we had tagged some melanoma cells with green fluorescent protein and showed increased ability to detect lesions at multiple sites, including bone. Subsequently, we tagged MDA-MB-435 human breast carcinoma cells with GFP and recently published the utilization of those cells for assessing bone metastasis (10).

Summary of Progress

We moved from Penn State to UAB in November 2002. Unfortunately, the grant has not yet transferred to UAB (as of July 15, 2003) and we have not yet been able to make progress in the time frame proposed. As this report is being written, the *final* paperwork is being prepared and we hope to hire the personnel and continue work on this project. The program administrators have agreed to extend the term of the contract one year to accommodate the delays.

We have made some progress toward the aims nonetheless. Specific progress will be listed along with the statement of work to assist review of our progress thus far.

Original Statement of Work

Task 1: Develop stable fluorescent cell lines
- Transfect 435BRMS1 and 231BRMS cells with GFP and dsRED
- Transfect neo11/435 cells with GFP and dsRED
- Select highly fluorescent subpopulations by fluorescence activated cell sorting

Task 2: Restore OPN expression in poorly metastatic cells
- Transfect 435BRMS1^{GFP}, 231BRMS1^{GFP} and neo11/435^{GFP} with OPN
- Select low, medium and high expressing clones

Task 3: Test metastatic potential of transfectants (injections and histological examination)

Task 4: Test tumor cell - osteoblast (hFOB) interactions

Task 5: Test tumor cells - sinusoidal endothelium (HBME) interactions

Key Research Accomplishments

- In a replicate experiment, with BRMS1-transfected MDA-MB-435 cells, cells were still metastatic to bone, suggesting that the metastasis suppressor may have organ-specific effects. While we would like to repeat this experiment, the histology and labor intensive nature of the experiment are cost prohibitive. Hence, we have decided to utilize GFP-tagged cells exclusively. The conclusion that the suppressor is not inhibiting bone metastasis cannot be made because the sensitivity of detection and the baseline frequency are relatively low to start.
- During the initial reporting period, we concluded experiments with the GFP-tagged 435 cells and published those findings(10). This manuscript establishes the baseline model and demonstrates the power of the GFP technology for the purposes of monitoring bone metastasis in breast cancer.
- We also developed a method whereby we could decalcify bone while retaining green fluorescence (11). This relatively simple technique will allow us to perform larger experiments (i.e., increased *n*) to improve statistical power since we can maintain fluorescence for longer times, thereby allowing more flexibility for quantifying the metastases.
- Initial experiments with GFP- and BRMS1-transfected 435 cells have been frustrating since stability is low (i.e., cells lose GFP fluorescence or BRMS1 expression or both). Loss of expression occurs even in the presence of selective pressures (i.e., grown in antibiotic-containing media). Generally, GFP expression is lost. Although we have not exhaustively studied all of the cells, it appears that the transgene is still present, but silenced. Therefore, we just began to prepare plasmids with internal ribosome entry sites (IRES). Bicistronic vectors offer the advantage of coupling transgene expression with GFP expression. Moreover, since original submission, Dr. T.C. He at the University of Chicago developed a tetracycline inducible IRES system that will allow more powerful testing of the roles of the metastasis suppressor genes since we will be able to turn the genes on-off using this inducible system. We have obtained the vector and preparation of the constructs is underway already. Dr. He's expression system also incorporates some histone deacetylase binding sites that reduce "leakiness" of the vector in the absence of doxycycline. Transfections will commence once the sequence has been verified.
- Once we get reproducible results with GFP, we will begin work with dsRed. However, since there have been unexpected complications, we will focus on one construct at this time.
- Restoration of OPN expression has not been successful. While we could get expression, the levels were not even close to those in the parental cell line. We discussed this with Dr. Ann Chambers, who has experienced similar difficulties. Therefore, we have opted for our contingency strategy. We have designed RNAi to decrease OPN expression in parental cells. In general, we believe that this approach is better because it more closely recapitulates what is occurring when BRMS1 is re-expressed (i.e., BRMS1 levels will decrease).
- An alternative approach was proposed in the grant application. With primary funding from the company and supplemental funding from this contract and a SPORE grant, we have made progress on that objective. Briefly, Pharmacia Corporation (now Pfizer) was testing small

molecule inhibitors of the alpha-v, beta-3 integrin for their impact on osteoporosis. I approached them regarding testing of these compounds in breast cancer to bone because alpha-v, beta-3 is the primary receptor for osteopontin. Mice were treated continuously with an inhibitor (designated S247) at three doses on two different schedules. In short, the results showed that presence of S247 prior to tumor cell colonization of bone would inhibit establishment of metastasis. S247 did not appear to diminish proliferation of tumor cells once they got to bone, however. A manuscript is in preparation and undergoing legal review. Therefore, data is not provided in this report. A reprint of the publication will be provided in a subsequent report.

Reportable outcomes:

Publications in peer-reviewed journals

Harms J.F., Budgeon L.R., Christensen N.D., Welch D.R. Maintaining GFP tissue fluorescence through bone decalcification and long-term storage. Biotechniques 2002; 33(6): 1197-1200.

Harms, J.F. and Welch, D.R. (2003) MDA-MB-435 human breast carcinoma metastasis to bone. *Clinical and Experimental Metastasis* 19: 327-334.

Shevde-Samant, L.A. and Welch, D.R. (2003) Metastasis suppressor pathways – an evolving paradigm. *Cancer Letters* (In press).

Welch, D.R., Harms, J.F., Mastro, A.M., Gay, C.V., Donahue, H.J. (2003) Breast cancer metastasis to bone: Research challenges and opportunities. *Journal of Musculoskeletal and Neuronal Interactions*. 3: 30-38.

Hunter, K.W., Welch, D.R. and Liu, E.T. (2003) Genetic Background is a Major Determinant of Metastatic Potential. *Nature Genetics* 34: 23-24.

Abstracts

Campo, D.A., Sosnoski, D.M., Mastro, A.M., Welch, D.R. and Gay, C.V. Differences between osteoblast-secreted and breast cancer-secreted osteonectin: N-linked glycosylation may be key in chemoattraction. *Oncology*. (2003) 17: 20

Donahue, H.J., Kapoor, P, Li, Z., Welch, D.R. and Zhou, Z. Connexin 43 and breast cancer metastasis to bone. *Oncology*. (2003) 17: 19-20.

Welch, D.R., Harms, J.F., Samant, R.S., Babu, G.R., Gay, C.V., Mastro, A.M., Donahue, H.J., Griggs, D.W., Kotyk, J.J., Pagel, M.D., Rader, R.K., Westlin, W.F., The small molecule $\alpha v\beta 3$ antagonist (S247) inhibits MDA-MB-435 breast cancer metastasis to bone. 3rd North American Symposium on Skeletal Complications of Malignancy. Oncology (2003) 17:18

Mercer, R.R., Gay, C.V., Welch, D.R., and Mastro, A.M., Breast cancer cells down-regulate alkaline phosphatase production in osteoblasts. Oncology. (2003) 17: 54.

Mercer, R.R., Gay, C.V., **Welch, D.R.**, and Mastro, A.M. Breast cancer skeletal metastases induce osteoblast apoptosis. Proceedings of the American Association for Cancer Research (2003).

Welch, D.R. Mechanisms of breast cancer metastasis suppression by BRMS1. 2nd BACT International Symposium (2003) 2: 5-7.

Welch, D.R., Samant, R.S. and Meehan, W.J. A novel mechanism of metastasis suppression by the BRMS1 metastasis suppressor gene. 21st COE Symposium at the University of Tokyo – Future cancer therapy through understanding metastasis. pp. 5-9.

Presentations

Cancer metastasis: What is the next generation of clinical targets?, North Dakota State University COBRE Symposium on Proteinases and Proteinase Inhibitors. Fargo, ND (6/1/03)

BRMS1: Illuminating a surprising regulatory point for breast cancer metastasis? National Cancer Institute, Metastasis: Prevention or Therapy, Bethesda, MD (5/20/03)

Metastasis suppressor genes in human cancer, 18th Annual Symposium on the Biological Approaches to Cancer Treatment, Nagoya Japan (5/17/03)

A novel mechanism of metastasis regulation by the BRMS1 metastasis suppressor gene. University of Tokyo Symposium on Cancer Metastasis – Future Cancer therapy through understanding metastasis (5/16/03)

Genetics of breast cancer metastasis. Plenary Lecture, Era of Hope DOD Breast Cancer Research Program Meeting, Orlando, FL (9/26/02)

Do single cells constitute a metastatic lesion? Interactive Session – How can we keep metastatic lesions dormant?, Era of Hope DOD Breast Cancer Research Program Meeting, Orlando, FL (9/26/02)

Metastasis suppressor genes in human cancer: from discovery to mechanism of action to the clinic, MedImmune Inc. (7/17/03)

BRMS1: Biochemical advances, Lankaneau Research Institute Seminar (6/19/03)

Use of metastasis suppressor genes to prevent and treat metastasis. Eli Lilly Corporation (5/29/03)

A surprising mechanism for breast cancer metastasis suppression by BRMS1, Laboratory of Population Genetics, Center for Cancer Research (5/19/03)

Metastasis suppressor genes in human breast cancer. Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy (1/15/03)

Metastasis suppressor genes in human breast cancer. Penn State College of Medicine, Department of Pharmacology (11/18/03)

Metastasis suppressor genes: from discovery to mechanisms of action. M.D. Anderson Cancer Center, Division of Gastroenterology Seminar Series (10/24/02)

Metastasis suppressor genes: from discovery to mechanisms of action. Lombardi Cancer Center Tumor Biology Seminar Series (10/4/02)

Degrees obtained that were supported, in part, by this award

John F. Harms, Ph.D., degree granted May 2003

Opportunities applied for and/or received based upon experience supported by this award

Since moving to UAB, I was asked to participate in the Breast SPORE grant based upon our experience with this DOD award. We are preparing a proposal that extends and complements the ongoing studies by looking at other metastasis suppressor genes and model development.

Conclusions

Delay in the transfer of the grant to UAB has delayed progress some; however, we made significant progress prior to the move on the specific aims. Preliminary data suggested that stability of the double transfectants would be an issue complicating interpretation of *in vivo* data. Therefore, we have modified our cloning strategy to incorporate IRES vectors. Likewise, we opted for one of our alternative strategies related to the OPN experiments, RNAi. Constructs have been prepared and are currently being screened for activity.

References

1. **Seraj MJ, Samant RS, Verderame MF, Welch DR.** Functional evidence for a novel human breast carcinoma metastasis suppressor, *BRMS1*, encoded at chromosome 11q13. *Cancer Res.* 2000; 60: 2764-9.

2. **Samant RS, Seraj MJ, Saunders MM et al.** Analysis of mechanisms underlying *BRMS1* suppression of metastasis. *Clin.Exptl.Metastasis* 2001; 18: 683-93.

3. **Shevde LA, Samant RS, Goldberg SF et al.** Suppression of human melanoma metastasis by the metastasis suppressor gene, BRMS1. *Exp.Cell Res.* 2002; 273: 229-39.

4. **Shevde LA, Welch DR.** Metastasis suppressor pathways - an evolving paradigm. *Cancer Lett.* 2003; (In press).

5. **Oates AJ, Barraclough R, Rudland PS.** The role of osteopontin in tumorigenesis and metastasis. *Invasion Metastasis* 1997; 17: 1-15.

6. **Debies MT, Welch DR.** Genetic basis of human breast cancer metastasis. *J.Mamm.Gland Biol.Neopl.* 2001; 6: 441-51.

7. **Yeatman TJ, Chambers AF.** Osteopontin and colon cancer progression. *Clin.Exptl.Metastasis* 2003; 20: 85-90.

8. **McManus MT, Sharp PA.** Gene silencing in mammals by small interfering RNAs. *Nature Rev.Genet.* 2002; 3: 737-47.

9. **Ramaswamy G, Slack FJ.** siRNA. A guide for RNA silencing. *Chem.Biol.* 2002; 9: 1053-5.

10. **Harms JF, Welch DR.** MDA-MB-435 human breast carcinoma metastasis to bone. *Clin.Exptl.Metastasis* 2003; 20: 327-34.

11. **Harms JF, Budgeon LR, Christensen ND, Welch DR.** Maintaining green fluorescent protein tissue fluorescence through bone decalcification and long-term storage. *Biotechniques* 2002; 33: 1197-200.

APPENDICES

D.R. Welch

Journal of Musculoskeletal & Neuronal Interactions

J Musculoskel Neuron Interact 2003; 3(1):30-38

Hylonome

Review Article

Breast cancer metastasis to bone: Evolving models and research challenges

D.R. Welch[1-5], J.F. Harms[5], A.M. Mastro[4,7], C.V. Gay[4,7], H.J. Donahue[4,6]

[1]Department of Pathology, [2]Comprehensive Cancer Center, [3]Center for Metabolic Bone Diseases,
[4]National Foundation for Cancer Research Center for Metastasis Research,
The University of Alabama at Birmingham, Birmingham, AL, [5]Jake Gittlen Cancer Research Institute,
[6]Department of Orthopaedics and Rehabilitation, The Pennsylvania State University College of Medicine, Hershey, PA,
[7]Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA, USA

Keywords: Adhesion, Osteopontin, Osteonectin, Organotropism, Osteoblast, Osteoclast, PTHrP, RANK, RANK-L, Osteoprotegrin, Chemokine, Green Fluorescent Protein, MDA-MB-435, MDA-MB-231, 4T1, Osteolysis

Overview of the clinical problem

When cancer is confined to breast, long-term survival rates are high. But, when cells metastasize, cure rates drop significantly (90% vs. 20% 5-year survival). Quality of life for patients with metastatic disease is also significantly worse than for patients with local carcinoma[1,2]. Thus, improvements in long-term survival will be most helped by better understanding of the metastatic process.

Skeletal metastases are common, particularly from breast, prostate and myeloma tumors. In many cases, the frequency of metastasis to bone is greater than metastases elsewhere. Whereas 73% of women develop bone metastases, only 33% develop lung and/or liver metastases. While patients can survive a relatively long time with bone lesions, their quality of life is miserable due to intractable pain, fractures, spinal cord compression and metabolic complications[3-6]. Besides the human cost, bone metastasis imposes a significant economic cost (2/3 of the costs of breast cancer treatment are due to bone metastasis[5]; ~$3 billion/yr[7]). The disparity between the clinical and economic importance of the problem and our knowledge of the underlying mechanisms responsible is staggering.

Nonetheless, there have been gains in knowledge regarding the mechanisms involved in breast cancer induction of osteolysis. This has led to improvements in treatment with drugs (e.g., bisphosphonates) designed to reduce loss of bone. Unfortunately, patients treated with these drugs seldom replace lost bone even when tumor cells are removed. Likewise, antecedent steps are largely understudied. In this review, we will focus on current knowledge about the earliest steps in breast cancer metastasis to bone. We will also present an evolving model for early steps of breast carcinoma metastasis to bone based upon currently available data and highlight some of the reasons for the relative sparsity of information about metastasis to bone.

Corresponding author: Danny R. Welch, Ph.D., Department of Pathology, University of Alabama at Birmingham, 1670 University Blvd. VH-G038, Birmingham, AL 35294-0019, USA
E-mail: dwelch@path.uab.edu

Accepted 26 August 2002

The metastatic cascade

Cancers derived from bone cells (e.g., osteosarcomas) are distinct from tumor cells that have immigrated to bone. Unfortunately, many lay people and even some physicians/researchers assume that bone-derived tumors are equivalent to bone-colonizing tumors. The reality is that the cell origins are different; the basal gene expression patterns are different and the underlying oncogenesis is different.

Metastasis is defined as the spread of tumor cells to establish a discontinuous secondary tumor mass. Tumor cells can get to other tissues by direct extension (not defined as a metastasis since the secondary lesion is not discontinuous from the primary tumor) or transport via blood vessels, lymphatics or in epithelial cavities. The predominance of metastatic spread to bone is thought to be via the hematogenous route.

Large numbers of tumor cells (in some cases $>10^7$ cells/day) enter the bloodstream daily, but fortunately establishment of secondary lesions is a rare event (i.e., $<<0.1\%$). In order to successfully form a metastatic colony, a specialized subset of tumor cells must possess all of the properties that give it selective survival and proliferative advantages over normal cells plus additional properties that confer the ability to spread and colonize secondary sites.

In the first step of metastasis, tumor cells must migrate away from the primary tumor and enter a circulatory compartment. Upon penetrating the basement membrane and endothelial barrier, tumor cells must evade innate immune surveillance and sheer mechanical forces associated with turbulent blood flow. At the secondary site, tumor cells either arrest because they are larger than the capillary diameter or they arrest because of tumor cell – endothelial recognition. After they have stopped moving, the cells must then divide in situ or extravasate. Extravasation requires the tumor cells to penetrate the intimal layer using a variety of motility and proteolytic mechanisms. Finally, tumor cells must proliferate in response to local growth factors and must be resistant to local growth inhibitors.

Development of metastasis contains stochastic elements as well as selection pressures. It is striking that breast cancer, prostate cancer and myeloma cells metastasize to bone 70-80% of the time[6]. The explanation for organotropism was first formally articulated by Sir Stephen Paget in his seminal paper in 1889[8]. In that work, Paget recognized that tumor cell <seed> and host <soil> properties worked in concert to determine success of metastasis. Rather than a comprehensive review of the literature, we will focus on the extravasation steps and terminal tumor cell – bone cell interactions that determine the osteolytic process.

Besides predisposition of cancer cells to colonize bone, it is crucial to understand that not all bones are equally involved. The predominance of osseous metastases occur in the long bones, ribs or vertebrae[6]. Furthermore, the metastases tend to occur at the ends of the bones, near the trabecular metaphyses. Therefore, it is essential to understand what is special about the trabecular bone structure and environment that make it amenable to frequent colonization.

Properties of the bone microenvironment that contribute to metastasis

The metaphyseal region is characterized by a meshwork of trabecular bone, rich blood flow and red bone marrow. Interdigitating the trabecular tongues are bone marrow in close proximity to the vascular sinusoids. The vascular and marrow compartments are separated by a trilamellar structure composed of endothelium, basement membrane and supportive adventitial cells[9]. Trabecular bone is covered by osteoblasts and bone lining cells; the latter are believed to differentiate into osteoblasts. Bone lining cells and osteoblasts have many properties in common, including alkaline phosphatase and Type I collagen expression[10].

Metastatic breast carcinoma cells that arrive in the metaphyses first interact with sinusoidal endothelial cells that line the vascular system. Binding probably occurs in a manner similar to leukocyte homing[11]. Compared to other tissue sites, it is less likely that tumor cell arrest in bone is non-specific. Rather than a network of small diameter (e.g., 5-10 μm) capillaries in the lungs or sinusoids of the liver (~30 μm), the diameters of the sinusoidal lumens can be several hundred microns in diameter.

Blood flow in sinusoids is also amenable to tumor cell arrest. Blood flow in sinusoids is sluggish compared to capillaries and post-capillary venules[12,13]. In murine calvaria, where blood cells can be readily visualized, blood flow in the venous sinusoids is ~30-fold lower than the arterial rate[12]. Schnitzer et al. measured blood flow using microsphere distribution in canine long bones and found that flow in metaphyseal and marrow cavities was 7-14 ml/min/100 gm tissue, compared to ~200 ml/min/100 gm tissue in post-prandial intestine[14].

Taken together, these properties suggest that more specific recognition properties are involved in tumor cell homing to bone. Among the more appealing hypotheses related to bone organotropism are the endothelial "addresses". A growing body of evidence suggests that lymphocytes and tumor cells can recognize unique macromolecules or combinations or surface molecules on bone endothelium[15,16].

In contrast to vascular endothelium elsewhere in the body, bone endothelial cells simultaneously and constitutively express the tethering molecules, p-selectin and e-selectin, and vascular cell adhesion molecules, VCAM-1 and ICAM-1[12,17,18]. In other cells, expression is transient in response to inflammatory stimuli[11,19]. In light of findings that metastases are more frequent at sites of inflammation[20-22], it is intriguing to speculate that tumor cells bind well to sinusoidal endothelium because those cells have similar surface markers as cells at an inflammatory site. The hypothesis gains credence because many breast carcinoma cells express the counter-receptors for these ligands[23-25].

Histological examination of bone metastases shows tumor cells in intimate contact with bony surfaces. It follows, then, that tumor cells penetrate the endothelial barrier or extravasate. Cancer cells in close proximity to vascular endothelial surfaces have been shown to stimulate endothelial cell retraction[26]. For example, osteonectin secretion by breast cancer cells has been reported to stimulate flux of macromolecules and pulmonary endothelial cell rounding[27]. HER2/neu over-expressing MCF-7 cells have been shown to stimulate vascular endothelial cell retraction[28].

Extravasation is, by definition, a directional movement. Therefore, it follows that tumor cells may be responding to bone-derived chemotactic gradients. Several examples consistent with this hypothesis have been observed. Three molecules that are highly expressed in bone – osteonectin, osteopontin, bone sialoprotein, collagen – have been shown to be chemoattractants for some tumor cells[29-32].

Osteonectin, which is produced by osteoblasts, has recently been shown to be a powerful chemoattractant for several prostate cancer cell lines and one breast cancer cell line[29,33]. Moreover, osteonectin can increase endothelial monolayer permeability[27] and has been shown to induce matrix metalloproteinase-2 secretion by MDA-MB-231 breast carcinoma cells[34,35].

Osteopontin is produced by many cell types, including osteoblasts, breast epithelium, breast and other types of can-

cer cells. In bone, osteopontin is deposited in matrix, binds to hydroxyapatite and serves as an anchor for osteoclast binding via the avb3 integrin[36]. Breast carcinoma cells also frequently express the high affinity avb3 integrin. As bone resorption occurs, Ca^{++}, PO_4 ions and matrix proteins are released. It is possible that intact and fragmented forms of osteopontin serve as diffusible chemotactic factors for breast cancer cells. In breast cancer, osteopontin is secreted in a soluble form[37]. Metastatic MDA-MB-435 cells have been shown to migrate toward soluble osteopontin fragments[30]. In addition to this limited list, osteopontin has been shown to be a promoter of metastasis in a variety of other systems (reviewed in[38,39]).

Bone sialoprotein is secreted primarily by osteoblasts[40,41] fosters chemotactic migration via an RGD-dependent binding to avb3 integrin[31]. Like the other matrix-derived proteins described above, it has multiple roles in both normal bone tissue and in the development of skeletal malignancies.

Chemokines are a family of small, cytokine-like peptides that induce cytoskeletal rearrangement, adhesion to endothelial cells and directed cell migration[42-44] and are therefore ideal for serving in the metastatic process. This notion was recently elegantly confirmed by Taichman et al.[45] who, considering the fact that hematopoietic cells use osteoblast-derived CXCL12/SDF-1 to home to bone normally, examined this factor in prostate cancers. They found that all bone metastases from prostate cancers expressed the CXCR4 receptor for SDF-1 and that SDF-1 increased prostate cancer cell migration and adherence *in vivo*. Muller et al.[46] cataloged expression of known chemokine receptors and found that breast cancer cell lines express abundant CXCR4 and/or CXCR7. This finding was particularly enlightening since the ligands for CXCR4 and CXCR7 are CXCL12/SDF-1 and CXCL21/6Ckine, respectively. The ligand expression is most abundant in tissues to which breast cancers most frequently metastasize (bone marrow, lymph node, lung and liver) and less abundant in less frequently involved tissues (intestine, kidney, skin, brain, skeletal muscle). They hypothesized that a combination of chemotactic factors present in bone matrix (e.g., CXCL12, osteonectin, osteopontin and others) could interact with a repertoire of receptors on breast cancer cells that confer the high specificity of these cancers for the skeleton.

Finally, once breast carcinoma cells have made their way into bone, many find the growth environment particularly hospitable. The precise molecular basis for breast cancer growth in bone is not known, but it is easy to speculate that the microenvironment is rich in growth factors based upon the normal function of bone marrow for sustaining stem cells and hematopoiesis. Indeed, the milieu of the bone marrow is ideal for many proliferating cells. Additionally, the continuous remodeling of the bone matrix would contribute to the growth potentiating surroundings by release of matrix-bound factors.

Thus, metaphyseal bone appears to have a unique combination of properties that renders it highly attractive to certain cancer cells. These properties include: a) slowed blood flow which may allow time for cell–cell interactions to occur; b) large lumenal diameters which would reduce sheer; c) constitutively expressed array of vascular surface proteins that may contribute to initial cancer cells binding; d) expression of matrix-associated molecules and chemokines which could serve as potent chemoattractants for tumor cells; and e) a milieu of growth factors which would provide a rich environment for tumor cell proliferation.

Entry of tumor cells into the bone microenvironment disrupts homeostasis

Bone matrix is constantly undergoing reorganization, based upon an intricate ballet of matrix-depositing cells (osteoblasts) and matrix–degrading cells (osteoclasts). When tumor cells enter the trabecular-marrow space, the balance is disrupted. In most breast cancers, the balance is shifted toward net bone degradation. It is beyond the scope of this review to discuss the many mechanisms involved in bone turnover and readers are referred to several outstanding reviews on this topic[47-50].

While many factors regulate bone turnover, members of the tumor necrosis family (TNF) and TNF receptor families appear to be essential. RANK-Ligand (receptor activator of nuclear factor kappa B, NFkB, ligand) is a TNF family member expressed by stromal cells and osteoblasts while RANK is expressed by osteoclasts; however, it was not detected in breast cancer cells[51]. *In vivo* and *in vitro* evidence indicates that interaction of these two molecules is essential for osteoclastogenesis. Other factors (e.g., glucocorticoids, vitamin D3, IL-1, IL-6, IL-11, IL-17, TNF-α, PGE2, PTH, and PTHrP) may modulate expression levels.

Osteoprotegerin (OPG, also known as osteoclastogenesis inhibiting factor) is another osteoblast-derived product that counters bone loss caused by RANK-L/RANK interactions[48,49]. OPG can serve as a decoy receptor for RANK-L. Interestingly OPG can also bind and inactivate TRAIL (TNF-regulated apoptosis-inducing ligand) and prevent TRAIL-initiated osteoblast apoptosis[52]. Under normal conditions OPG balances bone loss by competing with RANK-L for RANK on osteoclasts. However, OPG expression is down-regulated by breast cancer cells[53].

The RANK-L/RANK/OPG system may also explain how chronic inflammation and autoimmune diseases can cause bone loss. Activated T cells express RANK-L and also produce pro-inflammatory cytokines, e.g., TNF-α, IL-1, IL-11, IL-6 which up-regulate RANK or Fas or other death molecules in osteoblasts[54]. T cells also produce IFN- (which suppresses bone loss). In addition, activated macrophages secrete many of the same pro-inflammatory cytokines as the stromal cells. Thus, the inflammation associated with the presence of metastatic tumor cells favors bone loss. A current model in the literature presents these three molecules, RANK-L, RANK and OPG, as the basic factors controlling normal skeletal remodeling[47]. Other factors modulate the

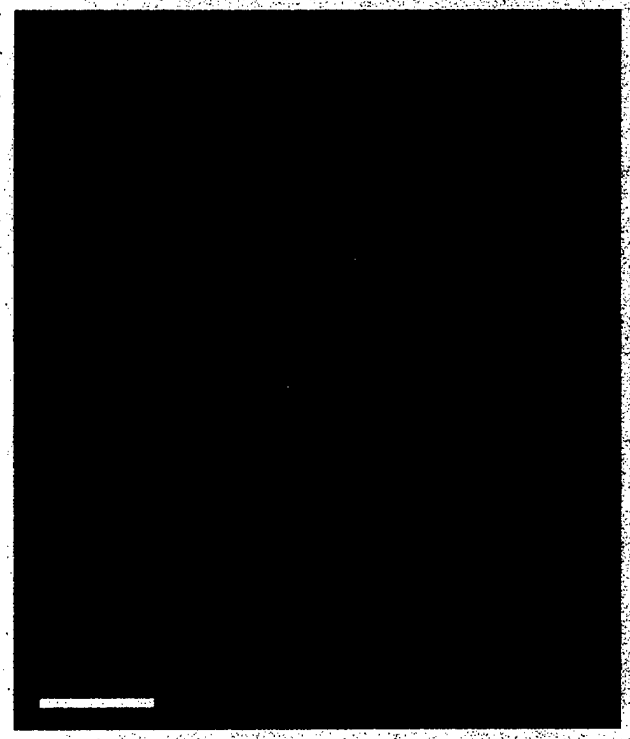


Figure 1. Representative image of whole bone with GFP-tagged tumor cells. Three separate lesions are visualized using GFP. The uppermost lesion contains elements that are brighter than the majority of cells. Frequently, this is indicative of full or partial penetration of tumor cells penetration through the bone. Bar = 1 mm.

system indirectly by up-regulating or down-regulating RANK-L, RANK and OPG. One of these regulatory molecules is PTHrP.

PTHrP (parathyroid hormone related peptide) is produced in excess by many metastatic cancer cells. Its effects were known long before the molecule was identified. Early in the twentieth century a connection was made between hypercalcemia and neoplastic diseases. The next 70 or so years were spent trying to explain this association and to discover how hypercalcemia associated with metastasis was different from that seen in hyperparathyroidism. It is now known that the molecule critical in metastatic hypercalcemia is PTHrP. The N-terminus of PTHrP is structurally homologous to parathyroid hormone (PTH) and has PTH-like activity although it is a product of a different gene. PTHrP binds to a G-protein-coupled receptor on osteoblasts[55]. Thus, PTHrP acts on osteoblasts to indirectly cause bone resorption mediated by osteoclasts. PTHrP produced locally in excess by metastatic tumor cells can bind to PTH/PTHrP receptors on osteoblasts and cause them to up-regulate RANK-L and down-regulate OPG[48,53]. The result is the differentiation of preosteoclasts and the activation of mature osteoclasts to become fully bone resorbing cells. This activity can be further enhanced by TGF-β which is released as the bone matrix is resorbed. While TGF-β has normally been shown to down-regulate RANK-L expression by osteoblasts and thus decrease resorption[56], many metastatic breast cancer cells express TGF-β receptors. TGF-β binding to the receptor induces PTHrP production[57]. Thus, a so-called "vicious cycle" is established in which osteolytic metastasis indirectly enhances osteoclastogenesis[47] and provides a positive feedback loop. Recent reports by Gay et al.[58] and Faucheux[59] and earlier reports (reviewed by Gay and Weber[60]) show that osteoclasts also have PTHrP receptors, suggesting a direct action of PTHrP on osteoclasts even if osteoblasts are absent.

In short, tumor cells manipulate the bone microenvironment upon entering the metaphyseal region. While tumor cells themselves can cause bone matrix resorption[61,62], the predominant mechanism is usurping the mechanisms used in normal bone physiology. As noted above, the predominance of research into the mechanisms of breast cancer-induced osteolysis have focused on activation of the osteoclast. However, another mechanism could also be operative, inactivation or elimination of the osteoblast.

Normally, osteoclasts remain viable for 2-3 weeks, whereas osteoblasts exist for 2-3 months or more[63]. If the lifespan of osteoclasts were increased or the lifespan of osteoblasts decreased, the net effect would be bone loss because the basic bone unit (osteoblast: osteoclast ratio) would be out of balance. Detailed studies of proliferation and apoptosis in these cells has not been extensively studied; however, we have obtained evidence that osteolysis-inducing breast tumor cells can increase apoptosis of osteoblasts[64]. This observation is consistent with the clinical observations that osteolytic lesions often have fewer osteoblasts and that patients treated with osteoclast-inhibiting bisphosphonates do not normally repair the bone defects (i.e., because they no longer have sufficient viable osteoblasts in the region)[62,65]. Clearly, additional studies are needed in this area.

Models to study skeletal metastasis in breast cancer

Although metastasis to bone is a common and serious problem, it has historically been extremely difficult to study. In large part, this is due to the near-complete lack of experimental models that recapitulate the metastatic process. An ideal model would replicate the entire metastatic cascade (i.e., growth of a primary tumor to metastasis). However, there are currently no human cancer cell lines that reproducibly metastasize to the bone from an orthotopic site, (i.e., mammary gland)[66]. There is only one rodent model that spreads from an orthotopic site to bone (4T1[67]). While 4T1 is an important model, worldwide experience with it has not been sufficient to ascertain whether it is predictive of biology in humans. Recently, several transgenic mouse models have been developed which exhibit metastatic capacity[68-75]. However, to the best of our knowledge, none of them metastasize to bone.

An alternative methodology for studying bone metastasis

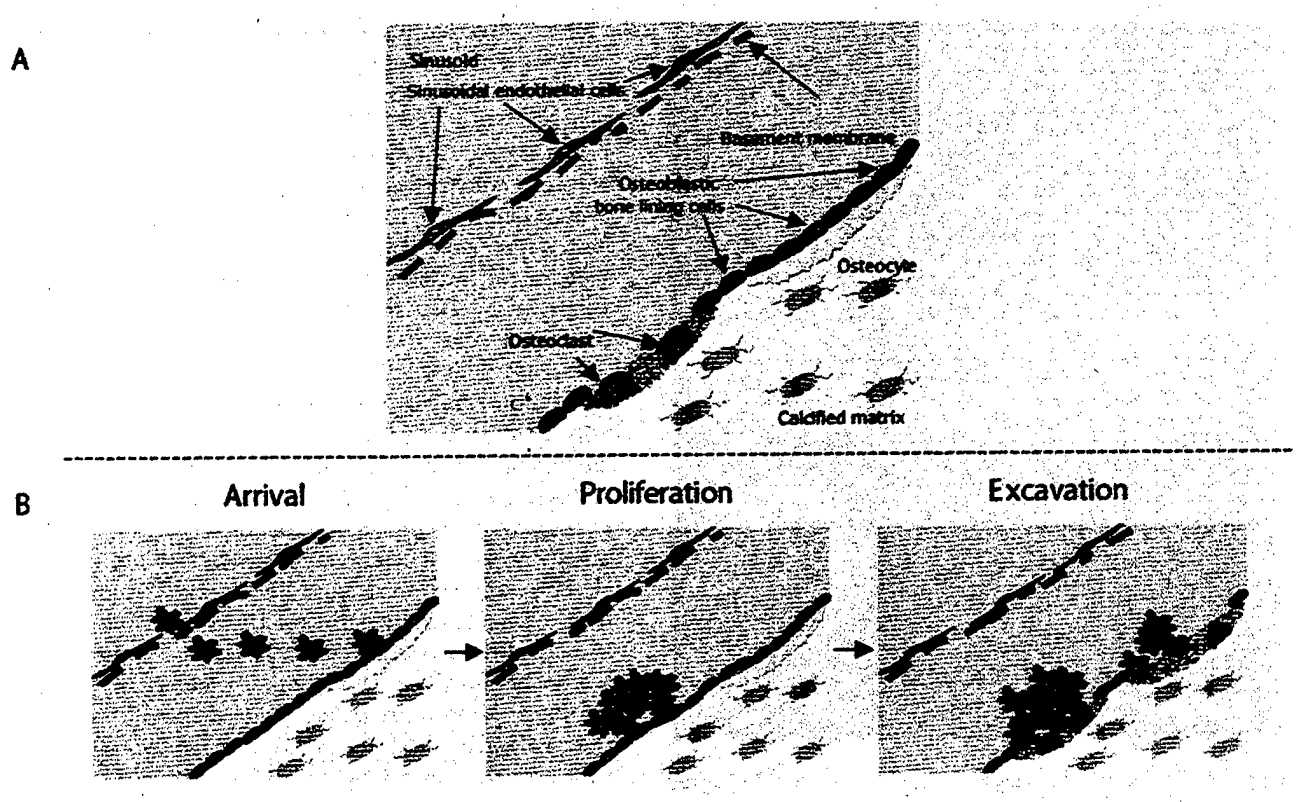


Figure 2. Schematic diagram of trabecular bone with the major cell types highlighted (A). Panel B represents the three major steps of bone metastasis formation. Tumor cells arrive in the bone via the vascular sinusoids and bind to the specialized endothelium. After the tumor cells pass through the endothelial barrier and extravasate through the underlying basement membrane, they migrate toward the trabecular bone surface which is lined by osteoblastic bone lining cells. Tumor cells then proliferate in response to local growth factors. Breast cancer cells that enter the bone disrupt the balance between osteoblast and osteoclast activities, resulting in a net bone loss. Osteolysis (excavation) can be accomplished by tumor cell: (i) activation of osteoclasts; (ii) inactivation of osteoblasts; (iii) a combination of osteoclast activation and osteoblast inactivation; or (iv) direct tumor cell degradation of bone matrix.

was pioneered by Arguello[76], who injected melanoma cells into the left ventricle of the heart. Yoneda and colleagues adapted this procedure using MDA-MB-231 human breast cancer cells and showed reliable colonization of bone with subsequent osteolysis[77,78]. The bulwark of the field and the vast majority of experimental data in the breast field with regard to bone metastasis have been collected using this cell line. We recently showed that another human breast carcinoma cell line, MDA-MB-435 could also form osteolytic lesions following intracardiac injection[79]. Yoneda, Guise and colleagues have shown that MCF7 and T47D variants can form osteoblastic metastases following intracardiac injection as well[51].

Besides the inherent limitation of extrapolating findings using limited numbers of cell lines, the experiments with bone metastasis were limited by technology as well. Basically, the standard method for detecting bone lesions – radiography – requires ≥50% bone degradation to be detectable. This means that only the latest stages of bone colonization and osteolysis can be studied. Histological examination is arduous and time-consuming. Serial sectioning of bone is technically challenging; so, step sections are more commonplace. As a result, small lesions can be easily missed. Again, studying early steps of bone colonization are not well-served by this technique.

To alleviate some of these limitations, we engineered MDA-MB-435 and MDA-MB-231 cells to constitutively express enhanced green fluorescent protein (GFP). This modification has increased detection sensitivity tremendously[79]. Representative images are depicted in Figure 1. GFP-expressing cancer cells can be detected through the intact bone even when radiographic evidence of tumor involvement is not apparent. We have even been able to detect single GFP-tagged cancer cells in bone. Furthermore, GFP allows three-dimensional examination and the ability to distinguish foci visually. This technique offers the capability of studying metastasis early in the process, before major bone degradation has occurred. The stages beginning with microscopic metastasis and latency, and ending in aggressive bone degradation can now be separated. Moreover, the response of the bone cells including osteoblasts, ranging from bone lining to fully differentiated cells, as well as osteoclasts can

be examined before they are destroyed as part of metastatic tumor growth.

The genetics of cancer cell metastasis to bone

We have been interested in determining the underlying genetic defects responsible for cancer metastasis. Specifically, our laboratory has identified metastasis suppressor genes for human breast carcinoma[80-83] and melanoma[84-87]. Data with the metastasis suppressor for melanoma is instructive to the discussion of organotropism.

Late-stage melanomas have losses or rearrangements of the long-arm of chromosome 6 in 66-75% of cases. Since losses occurred concomitant with acquisition of metastatic potential, we hypothesized that a metastasis suppressor gene was encoded on 6q. To test this, we introduced an intact copy of chromosome 6 into a metastatic human melanoma cell line[87]. The resulting hybrids were completely suppressed for metastasis while primary tumor growth still occurred. Subsequent experiments showed that the chromosome 6–melanoma cell hybrids were able to complete every step of the metastatic cascade, except proliferation at the secondary site[88]. Recovery of single cells in lung followed by injection into the skin (i.e., the orthotopic site) showed that the cells grew well[88], suggesting that the metastasis suppressor gene(s) were organ specific. To evaluate this possibility, we injected chromosome 6–melanoma hybrids into the left ventricle of the heart and monitored metastasis to all organs (J.F. Harms and D.R. Welch, manuscript in preparation). Metastasis was suppressed to all organs except bone.

While our results are striking, they are not completely unprecedented. Rinker-Schaeffer[89-91] and Steeg[92] have shown that the metastasis suppressor genes MKK4 and Nm23 also inhibit at late stages of the metastatic cascade. Additionally, using intravital microscopy, Chambers, Groom and colleagues have described frequent arrest and extravasation of tumor cells without subsequent proliferation at the secondary site[93,94]. Our results extend those findings to demonstrate (we believe for the first time) organ-specific metastasis suppression. The implication is that there will be classes of genes that determine organotropism of metastasis. On a theoretical level, this is not surprising. However, while the seed and soil hypothesis has been around for over a century, this is among the first molecular footholds into understanding the mechanism(s) responsible.

Working model for the earliest steps of bone metastasis

The simplest model for bone metastasis formation involves three steps. **Arrival:** Tumor cells enter bone through the vasculature, adhering strongly and preferentially to metaphyseal region sinusoidal endothelium and/or basement membrane. **Proliferation:** Tumor cells then migrate into the bone marrow space and eventually proliferate to form macroscopic lesions. [Note: the mere presence of single tumor cells does not constitute a metastasis which, by definition, is a tumor mass.] It is not entirely clear whether proliferation precedes osteolysis since the latter may release growth stimulatory signals from the matrix. **Excavation/Osteolysis:** Tumor cells interact with trabecular, osteoblast-like bone-lining cells, osteoblasts and osteoclasts to initiate the cascade of events leading to matrix dissolution.

Each of the steps of bone metastasis involves the interplay between breast carcinoma cells and bone cells. Understanding how the bone cells and tumor cells communicate will be essential to controlling metastasis to bone. Recently, we found human breast carcinoma cells that were suppressed by transfection of the metastasis suppressor gene BRMS1 exhibited restored homotypic gap junctional intercellular communication[95,96]. Studies are underway to explore whether there are differences between metastasis-competent and metastasis-suppressed cells with regard to heterotypic communication.

Conclusions

Metastasis to bone is an important clinical problem that has been relatively understudied. Recent development of models has provided, for the first time, the opportunity to study the earliest steps of the process of bone colonization. Careful utilization of the new models and expansion of the number of available models will provide new insights into the initial events taking place during bone colonization.

Acknowledgments

The experimental studies discussed herein were supported by the following agencies: Penn State University Life Sciences Consortium Innovation Grant Program, National Institutes of Health (CA89019, CA88728, CA90991, AG13527, and DE09459), U.S. Army Medical Research and Material Command (DAMD17-02-1-0541, DAMD17-00-1-0646 and DAMD17-00-1-0647) and the National Foundation for Cancer Research Center for Metastasis Research.

References

1. Hortobagyi GN, Piccart-Gebhart MJ. Current management of advanced breast cancer. Sem Oncol 1996; 23:1-5.
2. Fremgen AM, Bland KI, McGinnis LS, Eyre HJ, McDonald CJ, Menck HR, Murphy GP. Clinical highlights from the National Cancer Data Base, 1999. CA Cancer J Clin 1999; 49:145-158.
3. Coleman RE. Skeletal complications of malignancy. Cancer 1997; 80:1588-1594.
4. Yoneda T. Cellular and molecular mechanisms of breast and prostate cancer metastasis to bone. Eur J Cancer 1998; 34:240-245.
5. Guise TA, Mundy GR. Cancer and bone. Endocr Rev 1998; 19:18-54.
6. Rubens RD, Mundy GR. Cancer and the skeleton. London, Martin Dunitz, London; 2000.
7. Mundy GR. Mechanisms of bone metastasis. Cancer 1997;

80:1546-1556.
8. Paget S. The distribution of secondary growths in cancer of the breast. Lancet 1889; 1:571-573.
9. Sasaki A, Boyce BF, Story B, Wright KR, Chapman M, Boyce R, Mundy GR, Yoneda T. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res 1995; 55:3551-3557.
10. Everts V, Delaisse JM, Korper W, Jansen DC, Tigchelaar-Gutter W, Saftig P, Beertsen W. The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. J Bone Miner Res 2002; 17:77-90.
11. Kubes P, Kerfoot SM. Leukocyte recruitment in the microcirculation: the rolling paradigm revisited. News Physiol Sci 2001; 16:76-80.
12. Mazo IB, Von Andrian UH. Adhesion and homing of blood-borne cells in bone marrow microvessels. J Leukoc Biol 1999; 66:25-32.
13. Orr FW, Wang HH, Lafrenie RM, Scherbarth S, Nance D. Interactions between cancer cells and the endothelium in metastasis. J Pathol 2000; 190:310-329.
14. Schnitzer JE, McKinstry P, Light TR, Ogden JA. Quantitation of regional chondro-osseous circulation in canine tibia and femur. Am J Physiol 1982; 242:H365-H375.
15. Pasqualini R, Ruoslahti E. Organ targeting *in vivo* using phage display peptide libraries. Nature 1996; 380:364-366.
16. Ruoslahti E, Rajotte D. An address system in the vasculature of normal tissues and tumors. Ann Rev Immunol 2000; 18:813-827.
17. Lee AV, Hilsenbeck SG, Yee D. IGF system components as prognostic markers in breast cancer. Breast Cancer Res Treat 1998; 47:295-302.
18. Mazo IB, Gutierrez-Ramos JC, Frenette PS, Hynes RO, Wagner DD, Von Andrian UH. Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. J Exp Med 1998; 188:465-474.
19. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: a multistep paradigm. Cell 1994; 76:301-314.
20. Murthy MS, Scanlon EF, Jelachich ML, Klipstein S, Goldschmidt RA. Growth and metastasis of human breast cancers in athymic nude mice. Clin Exp Metastasis 1995; 13:3-15.
21. Orr FW, Adamson IYR, Young L. Promotion of pulmonary metastases in mice by bleomycin-induced endothelial injury. Cancer Res 1986; 46:891-897.
22. Warren BA. The microinjury hypothesis and metastasis. Dev Oncol 1984; 22:56-61.
23. Ali S, Kaur J, Patel KD. Intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and regulated on activation normal T cell expressed and secreted are expressed by human breast carcinoma cells and support eosinophil adhesion and activation. Am J Pathol 2000; 157:313-321.
24. Kam JL, Regimbald LH, Hilgers JHM, Hoffman P, Krantz MJ, Longenecker BM, Hugh JC. MUC1 synthetic peptide inhibition of intercellular adhesion molecule-1 and MUC1 binding requires six tandem repeats. Cancer Res 1998; 58:5577-5581.
25. Regimbald LH, Pilarski LM, Longenecker BM, Reddish MA, Zimmermann G, Hugh JC. The breast mucin MUC1 as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer. Cancer Res 1996; 56:4244-4249.
26. Kramer RH, Nicolson GL. Interactions of tumor cells with vascular endothelial cell monolayers: a model for metastatic invasion. Proc Natl Acad Sci USA 1979; 76:5704-5708.
27. Goldblum SE, Ding X, Funk SE, Sage EH. SPARC (secreted protein acidic and rich in cysteine) regulates endothelial cell shape and barrier function. Proc Natl Acad Sci USA 1994; 91:3448-3452.
28. Carter WB, Hoying JB, Boswell C, Williams SK. HER2/neu over-expression induces endothelial cell retraction. Int J Cancer 2001; 91:295-299.
29. Jacob K, Webber M, Benayahu D, Kleinman HK. Osteonectin promotes prostate cancer cell migration and invasion: a possible mechanism for metastasis to bone. Cancer Res 1999; 59:4453-4457.
30. Senger DR, Perruzzi CA. Cell migration promoted by a potent GRGDS-containing thrombin-cleavage fragment of osteopontin. Biochim Biophys Acta 1996; 1314:13-24.
31. Sung V, Stubbs JT III, Fisher L, Aaron AD, Thompson EW. Bone sialoprotein supports breast cancer cell adhesion proliferation and migration through differential usage of the alpha(v)beta3 and alpha(v)beta5 integrins. J Cell Physiol 1998; 176:482-494.
32. Orr W, Varani J, Gondex MK, Ward PA, Mundy GR. Chemotactic responses of tumor cells to products of resorbing bone. Science 1979; 203:176-179.
33. Gay CV, Mastro AM, Welch DR. Scanning electron microscopy reveals directional responses of breast cancer cells to osteonectin. J Bone Miner Res 2001; 16:S333.
34. Gilles C, Bassuk JA, Pulyaeva H, Sage EH, Foidart JM, Thompson EW. SPARC/osteonectin induces matrix metalloproteinase 2 activation in human breast cancer cell lines. Cancer Res 1998; 58:5529-5536.
35. Shankavaram UT, DeWitt DL, Funk SE, Sage EH, Wahl LM. Regulation of human monocyte matrix metalloproteinases by SPARC. J Cell Physiol 1997; 173:327-334.
36. Denhardt DT, Guo X. Osteopontin: a protein with diverse functions. FASEB J 1993; 7:1475-1482.
37. Rittling SR, Chen Y, Feng F, Wu Y. Tumor-derived osteopontin is soluble, not matrix associated. J Biol Chem 2002; 277:9175-9182.
38. Weber GF. The metastasis gene osteopontin: a candidate target for cancer therapy. Biochem Biophys Acta 2001; 1552:61-85.
39. Tuck AB, Chambers AF. The role of osteopontin in breast cancer: clinical and experimental studies. J Mammary Gland Biol Neoplasia 2001; 6:419-429.
40. Fisher LW, Whitson SW, Avioli LV, Termine JD. Matrix sialoprotein of developing bone. J Biol Chem 1983; 258:12723-12727.
41. Bianco P, Fisher LW, Young MF, Termine JD, Robey PG. Expression of bone sialoprotein (BSP) in developing human tissues. Calcif Tissue Int 1991; 49:421-426.
42. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. Immunity 2000; 12:121-127.
43. Campbell JJ, Butcher EC. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. Curr Opin Immunol 2000; 12:336-341.
44. Butcher EC, Williams M, Youngman K, Rott L, Briskin M. Lymphocyte trafficking and regional immunity. Adv Immunol 1999; 72:209-253.
45. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the stromal cell-derived factor-

1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res 2002; 62:1832-1837.
46. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. Nature 2001; 410:50-56.
47. Guise TA. Molecular mechanisms of osteolytic bone metastases. Cancer 2000; 88:2892-2898.
48. Hofbauer LC, Heufelder AE. Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. J Mol Med 2001; 79:243-253.
49. Aubin JE, Bonnelye E. Osteoprotegerin and its ligand: a new paradigm for regulation of osteoclastogenesis and bone resorption. Osteoporos Int 2000; 11:905-913.
50. Theill LE, Boyle WJ, Penninger JM. RANK-L and RANK: T cells, bone loss, and mammalian evolution. Annu Rev Immunol 2002; 20:795-823.
51. Thomas RJ, Guise TA, Yin JJ, Elliot J, Horwood NJ, Martin TJ, Gillespie MT. Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology 1999; 140:4451-4458.
52. Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dul E, Appelbaum ER, Eichman C, DiPrinzio R, Dodds RA, James IE, Rosenberg M, Lee JC, Young PR. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem 1998; 273:14363-14367.
53. Hofbauer LC, Neubauer A, Heufelder AE. Receptor activator of nuclear factor-kappaB ligand and osteoprotegerin: potential implications for the pathogenesis and treatment of malignant bone diseases. Cancer 2001; 92:460-470.
54. Tsuboi M, Kawakami A, Nakashima T, Matsuoka N, Urayama S, Kawabe Y, Fujiyama K, Kiriyama T, Aoyagi T, Maeda K, Eguchi K. Tumor necrosis factor-alpha and interleukin-1 beta increase the Fas-mediated apoptosis of human osteoblasts. J Lab Clin Med 1999; 134:222-231.
55. Rouleau MF, Mitchell J, Goltzman D. *In vivo* distribution of parathyroid hormone receptors in bone: evidence that a predominant osseous target cell is not the mature osteoblast. Endocrinology 1988; 123:187-191.
56. Borton AJ, Frederick JP, Datto MB, Wang XF, Weinstein RS. The loss of Smad3 results in a lower rate of bone formation and osteopenia through dysregulation of osteoblast differentiation and apoptosis. J Bone Miner Res 2001; 16:1754-1764.
57. Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massague J, Mundy GR, Guise TA. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J Clin Invest 1999; 103:197-206.
58. Gay CV, Zheng BZ, Gilman VR, Mastro AM. Immunolocalization of PTH receptors in osteoclasts of rat metaphyses. J Bone Miner Res 2001; 16:S425.
59. Faucheux C, Horton MA, Price JS. Nuclear localization of type I parathyroid hormone/parathyroid hormone-related protein receptors in deer antler osteoclasts: evidence for parathyroid hormone-related protein and receptor activator of NF-kappaB-dependent effects on osteoclast formation in regenerating mammalian bone. J Bone Miner Res 2002; 17:455-464.
60. Gay CV, Weber JA. Regulation of differentiated osteoclasts. Crit Rev Eukaryot Gene Expr 2000; 10:213-230.
61. Sanchez-Sweatman OH, Orr FW, Singh G. Human metastatic prostate PC3 cell lines degrade bone using matrix metalloproteinases. Invasion Metastasis 1998; 18:297-305.
62. Sanchez-Sweatman OH, Lee J, Orr FW, Singh G. Direct osteolysis induced by metastatic murine melanoma cells: role of matrix metalloproteinases. Eur J Cancer 1997; 33:918-925.
63. Manolagas SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev 2000; 21:115-137.
64. Mastro AM, Gay CV, Welch DR, Donahue HJ, Jewell J. A role for osteoblast apoptosis in breast cancer osteolytic metastasis? Proc Amer Assoc for Cancer Res 2002; 43:1570.
65. Stewart AF, Vignery A, Silverglate A, Ravin ND, LiVolsi V, Broadus AE, Baron R. Quantitative bone histomorphometry in humoral hypercalcemia of malignancy: uncoupling of bone cell activity. J Clin Endocrinol Metab 1982; 55:219-227.
66. Welch DR. Technical considerations for studying cancer metastasis *in vivo*. Clin Exptl Metastasis 1997; 15:272-306.
67. Lelekakis M, Moseley JM, Martin TJ, Hards D, Williams E, Ho P, Lowen D, Javni J, Miller FR, Slavin J, Anderson RL. A novel orthotopic model of breast cancer metastasis to bone. Clin Exptl Metastasis 1999; 17:163-170.
68. Clarke R. Animal models of breast cancer: experimental design and their use in nutrition and psychosocial research. Breast Cancer Res Treat 1997; 46:117-133.
69. Davies MPA, Rudland PS, Robertson L, Parry EW, Jolicoeur P, Barraclough R. Expression of the calcium-binding protein S100A4 (p9Ka) in MMTV- neu transgenic mice induces metastasis of mammary tumours. Oncogene 1996; 13:1631-1637.
70. Granovsky M, Fata J, Pawling J, Muller WJ, Khokha R, Dennis JW. Suppression of tumor growth and metastasis in Mgat5-deficient mice. Nature Med 2000; 6:306-312.
71. Jeffers M, Fiscella M, Webb CP, Anver M, Koochekpour S, Vande Woude GF. The mutationally activated Met receptor mediates motility and metastasis. Proc Natl Acad Sci USA 1998; 95:14417-14422.
72. LeVoyer T, Lifsted T, Williams M, Hunter K. Identification and mapping of a mammary tumor metastasis susceptibility gene. U.S. Army Med Res & Material Command Era of Hope Meeting 2, 625; 2000.
73. Li Y, Hively WP, Varmus HE. Use of MMTV-Wnt-1 transgenic mice for studying the genetic basis of breast cancer. Oncogene 2000; 19:1002-1009.
74. Maglione JE, Moghanaki D, Young LJT, Manner CK, Ellies LG, Joseph SO, Nicholson B, Cardiff RD, MacLeod CL. Transgenic polyoma middle-T mice model premalignant mammary disease. Cancer Res 2001; 61:8298-8305.
75. Zhang M, Shi Y, Magit D, Furth PA, Sager R. Reduced mammary tumor progression in WAP-TAg/WAP-maspin bitransgenic mice. Oncogene 2000; 19:6053-6058.
76. Arguello F, Baggs RB, Frantz CN. A murine model of experimental metastasis to bone and bone marrow. Cancer Res 1988; 48:6876-6881.
77. Yoneda T, Williams PJ, Hiraga T, Niewolna M, Nishimura R. A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone *in vivo* and *in vitro*. J Bone Miner Res 2001; 16:1486-1495.
78. Yoneda T, Sasaki A, Mundy GR. Osteolytic bone metastasis in breast cancer. Breast Cancer Res Treat 1994; 32:73-84.
79. Harms JF, Welch DR. MDA-MB-435 human breast carcinoma metastasis to bone. Clin Exp Metastasis 2003; (in press).

80. Seraj MJ, Samant RS, Verderame MF, Welch DR. Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. Cancer Res 2000; 60:2764-2769.
81. Samant RS, Seraj MJ, Welch DR. Breast carcinoma metastasis suppressor, BRMS1. Cancer Research Alert 2000; 2:57-59.
82. Samant RS, Debies MT, Shevde LA, Welch DR. Identification and characterization of mouse homolog (Brms1) of the breast cancer metastasis suppressor BRMS1. Proc Amer Assoc for Cancer Res 2001; 42:2808.
83. Shevde LA, Samant RS, Welch DR. Suppression of human melanoma metastasis by breast metastasis suppressor [BRMS1]. Proc Amer Assoc for Cancer Res 2001; 42:646.
84. Lee J-H, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, Welch DR. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst 1996; 88:1731-1737.
85. Lee J-H, Welch DR. Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1. Cancer Res 1997; 57:2384-2387.
86. Miele ME, Gresham VC, Stanbridge EJ, Weissman BE, Welch DR. Metastasis, but not tumorigenicity, is suppressed and nm23 levels are increased by introduction of chromosome 6 into human malignant melanoma cell line C8161. Proc Amer Assoc for Cancer Res 1994; 35:325.
87. Welch DR, Chen P, Miele ME, McGary CT, Bower JM, Weissman BE, Stanbridge EJ. Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity. Oncogene 1994; 9:255-262.
88. Goldberg SF, Harms JF, Quon K, Welch DR. Metastasis-suppressed C8161 melanoma cells arrest in lung but fail to proliferate. Clin Exp Metastasis 1999; 17:601-607.
89. Yoshida BA, Dubauskas Z, Chekmareva MA, Zaucha MM, Christiano TR, Christiano AP, Stadler WM, Rinker-Schaeffer CW. Identification and characterization of candidate prostate cancer metastasis-suppressor genes encoded on human chromosome 17. Cancer Res 1999; 59:5483-5487.
90. Rinker-Schaeffer CW, Welch DR, Sokoloff M. Defining the biologic role of genes that regulate prostate cancer metastasis. Curr Opin Urol 2001; 10:397-401.
91. Yoshida BA, Sokoloff M, Welch DR, Rinker-Schaeffer CW. Metastasis-suppressor genes: a review and perspective on an emerging field. J Natl Cancer Inst 2000; 92:1717-1730.
92. Freije JM, MacDonald NJ, Steeg PS. Nm23 and tumour metastasis: basic and translational advances. Biochem Soc Symp 1998; 63:261-271.
93. Chambers AF, MacDonald IC, Schmidt EE, Koop S, Morris VL, Khokha R, Groom AC. Steps in tumor metastasis: New concepts from intravital videomicroscopy. Cancer Metastasis Rev 1995; 14:279-301.
94. Koop S, MacDonald IC, Luzzi K, Schmidt EE, Morris VL, Grattan M, Khokha R, Chambers AF, Groom AC. Fate of melanoma cells entering the microcirculation: Over 80% survive and extravasate. Cancer Res 1995; 55:2520-2523.
95. Saunders MM, Seraj MJ, Li ZY, Zhou ZY, Winter CR, Welch DR, Donahue HJ. Breast cancer metastatic potential correlates with a breakdown in homospecific and heterospecific gap junctional intercellular communication. Cancer Res 2001; 61:1765-1767.
96. Shevde LA, Samant RS, Goldberg SF, Sikaneta T, Alessandrini A, Donahue HJ, Mauger DT, Welch DR. Suppression of human melanoma metastasis by the metastasis suppressor gene, BRMS1. Exp Cell Res 2002; 273:229-239.

Clinical & Experimental Metastasis **20:** 327–334, 2003.

MDA-MB-435 human breast carcinoma metastasis to bone

John F. Harms & Danny R. Welch
Jake Gittlen Cancer Research Institute, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania, USA

Received 21 August 2002; accepted in revised form 3 December 2002

Key words: bone, breast cancer, green fluorescent protein, MDA-MB-435, metastasis

Abstract

Breast cancer metastasizes to bone with high frequency and incidence. However, studies of breast cancer metastasis to bone have been limited by two factors. First, the number of models that colonize bone are limited. Second, detection of bone metastases is too insensitive or too laborious for routine, large-scale studies or for studying the earliest steps in bone colonization. To partially alleviate these problems, the highly metastatic MDA-MB-435 (435) human breast carcinoma cell line was engineered to constitutively express enhanced green fluorescent protein (GFP). While 435^{GFP} cells did not form femoral metastases following orthotopic or intravenous injections, they produced widespread osteolytic skeletal metastases following injection into the left ventricle of the heart. All mice developed at least one femur metastasis as well as a mandibular metastasis. As in humans, osseous metastases localized predominantly to trabecular regions, especially proximal and distal femur, proximal tibia, proximal humerus and lumbar vertebrae. 435^{GFP} cells also developed metastases in adrenal glands, brain and ovary following intracardiac injection, suggesting that this model may also be useful for studying organotropism to other tissues as well. Additionally, GFP-tagging permitted detection of single cells and microscopic metastases in bone at early time points following arrival and at stages of proliferation prior to coalescence of individual metastases.

Abbreviations: 231 – MDA-MB-231; 435 – MDA-MB-435; CMF-DPBS – calcium- and magnesium-free Dulbecco's phosphate-buffered saline solution; FACS – fluorescence activated cell sorting; GFP – enhanced green fluorescent protein

Introduction

Breast cancer directly affects one in eight women [1]. Of women who develop breast cancer, as many as 85% will develop metastases in bone [2]. Skeletal colonization by breast cancer cells most frequently causes osteolytic lesions with corresponding sequelae – pathological fractures, spinal chord compression, pain and hypercalcemia. Despite its prevalence, studies of breast cancer metastasis to bone are infrequent, limited by a paucity of models and the technical challenges associated with detection of osseous metastases. Thus far, research of breast cancer metastasis to bone has been predominated by a single human cell line (MDA-MB-231 [3–7]) and recently, a murine cell line (4T1 [8, 9]). In most cases, studies have focused on late stages of bone metastases (i.e., osteolysis) because analysis of early steps (e.g., tumor cell arrival and colonization) has been infeasible.

Despite being uniformly derived from metastases, surprisingly few human breast carcinoma cell lines retain the capacity for metastasis in immune-compromised mice. Even fewer metastasize efficiently from the orthotopic site [10, 11]. Research of breast cancer metastasis has been dominated by two human breast carcinoma cell lines, MDA-MB-231 (231) and MDA-MB-435 (435), but recently, additional lines are being developed [12, 13]. Bone metastasis research has hinged almost exclusively upon 231 [4, 5, 7, 14, 15], with isolated studies using other cell lines [12, 13, 16, 17]. Although there are sporadic claims to the contrary [18], colonization of bone by 231 cells requires injection into the left ventricle of the heart. And while 435 cells can grow in bone if directly injected [16], the ability to colonize bone has heretofore not been systematically examined.

Studying metastasis to bone requires methods to routinely detect bone lesions. Because they are located in a solid matrix, bone metastases are readily visible only when considerable red marrow is displaced. In the absence of a pigment, colorimetric marker or bioluminescent tag, identification of skeletal metastases depends upon laborious histological sectioning or radiographic detection. Radiography requires sufficient (e.g., > 50% [19]) osteolytic reduction in bone mass; so, microscopic metastases confined within the marrow are overlooked entirely. Detection of microscopic metastases by histology is technically feasible but tedious and impractical for large-scale studies. These limitations have been partly alleviated by detection of B16 melanoma metastases in bone because of endogenous melanin production [20]. Others have used cells tagged with β-galactosidase

Correspondence to: Danny R. Welch, PhD, Department of Pathology, University of Alabama – Birmingham, 1670 University Blvd, Volker Hall, Rm G038, Birmingham, AL 35294-0019; USA. Tel: +1-205-934-2956; Fax: +1-205-934-1775; E-mail: dwelch@path.uab.edu

(lacZ) [21–23] or luciferase [24]. Unfortunately, additional cofactors are necessary to detect these reporters. In contrast, the convenience and utility of fluorescent molecules, such as enhanced green fluorescent protein (GFP), for the detection of metastases has been clearly demonstrated in many sites [25–27], including bone [18, 28, 29].

In this report, we compare 435 metastasis to bone following orthotopic, intravenous and intracardiac injection. In addition, we take advantage of the increased sensitivity of GFP detection to map the distribution of microscopic and macroscopic skeletal metastases.

Materials and methods

Cell lines and culture

Metastatic human breast carcinoma cell line, MDA-MB-435 (435) was a generous gift from Dr Janet E. Price (University of Texas–M.D. Anderson Cancer Center, Houston) and was stably transfected with pEGFP-N1 (BD Biosciences Clontech, Palo Alto, California) by electroporation (Bio-Rad Model GenePulser™, Hercules, California; 220 V, 960 μFd, $\infty\Omega$). Neomycin resistant cells were selected for growth in, and maintained in, a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F-12; Invitrogen, Gaithersburg, Maryland), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.02 mM non-essential amino acids, 5% fetal bovine serum (Atlanta Biologicals, Norcross, Georgia) and 500 μg/ml Geneticin (G418; Invitrogen). The brightest 25% of fluorescing cells were sorted using a Coulter EPICS V cell sorter (Beckman-Coulter, Fullerton, California). All cultures were confirmed negative for *Mycoplasma* spp. infection using a PCR-based test (TaKaRa, Shiga, Japan).

In vivo *metastasis assays*

Immediately prior to injection, cells at 80–90% confluence were detached from 100-mm cell culture plates (Corning, Acton, Massachusetts) with 2 mM EDTA and 0.125% trypsin in calcium- and magnesium-free Dulbecco's phosphate-buffered saline solution (CMF-DPBS). Cells were counted using a hemacytometer, and resuspended in Hank's balanced salt solution to the appropriate final concentration. For spontaneous metastasis assays, cells (1×10^6 in 0.1 ml) were injected into the right subaxillary mammary fat pad of anesthetized (ketamine-HCl 129 mg/kg, xylazine 4 mg/kg) 5–6 week-old female athymic mice (Harlan Sprague-Dawley, Indianapolis). Food and water were provided *ad libitum*. Resulting tumors were removed at a group mean tumor diameter [11] of 12 mm and mice were necropsied four weeks later. Lungs and femurs were removed and viewed by fluorescence microscopy (see below) prior to fixation. Macroscopic lung metastases, were also quantified as described [11].

For intravenous (i.v.) and intracardiac (i.c.) injections, cells (2×10^5 in 0.2 ml) were injected into 4–5-week-old female athymic mice via the lateral tail vein or left ventricle of the heart, respectively, using a 27 gg needle and 1 ml tuberculin syringe. Intracardially injected mice were fully anesthetized. Immediately preceding and subsequent to inoculation, drawback of bright red arterial blood into the syringe was used as an indication of arterial administration, as opposed to darker, burgundy colored blood. Mice were necropsied four or five weeks post-injection. Distribution of bone metastases was mapped following examination of all thoracic and abdominal organs. Bones were dissected free of musculature and soft tissues using a #21 scalpel blade and gauze or squares of paper towel to grip and remove remnants. Where possible, bones were left connected (e.g., femur–tibia–fibula, scapula–humerus–radius–ulna, ribcage–vertebrae) to facilitate orientation. Following external fluorescence examination of the dissected skull for bone and brain metastases, a sagittal bisection of the skull was performed to expose the brain interior.

Animals were maintained under the guidelines of the National Institute of Health and the Pennsylvania State University College of Medicine. All protocols were approved and monitored by the Institutional Animal Care and Use Committee.

Fluorescence microscopy

To visualize metastases derived from the GFP-tagged cell line, intact viscera and whole bones (dissected free of soft tissue), were placed into petri dishes containing CMF-DPBS and examined by fluorescence microscopy utilizing a Leica MZFLIII dissecting microscope with 0.5× and PlanApo 1.6× objectives and GFP fluorescence filters ($\lambda_{excitation}$ = 480 ± 20 nm, $\lambda_{emission}$, 510 nm barrier) (Leica, Deerfield, Illinois). Photomicrographs were collected using a MagnaFire™ digital camera (Optronics, Goleta, California), and ImagePro Plus software (Media Cybernetics, Silver Spring, Maryland).

Faxitron X-ray analysis

Dissected bones were X-rayed using a Hewlett-Packard Faxitron model 43855B and Kodak X-Omat TL film (Kodak, Rochester, New York). Tube voltage was set at either 19 kVp or 59 kVp, and exposure time was determined automatically.

Bone decalcification and storage

Intact, dissected bones from individual mice were placed in 25-ml glass scintillation vials and fixed in freshly prepared 4% paraformaldehyde in CMF-DPBS at 4 °C for 24–48 h. Bones destined for histological sectioning were subsequently removed and decalcified in 0.5 M EDTA in CMF-DPBS for 18–24 h before paraffin embedding. Non-embedded bones could be stored long-term (months) at 4 °C with retention of fluorescence if the solution was replaced at 1–5 days with 0.5 M EDTA in CMF-DPBS or 1% paraformadehyde in CMF-DPBS. Fluorescence was typically lost if tissues were stored in 4% paraformaldehyde or ethanol solutions.

Mapping of bone metastases

During fluorescence microscopy, skeletal metastases were drawn on diagrams of murine bones (adapted from [30]). A custom computer program was written using Visual Basic 6 (Microsoft Corp., Redmond, Washington) in which the same diagrams were overlaid with a grid of squares ($\sim$0.30 mm^2). Metastases drawn for each mouse bone were transferred to the computerized grid. The program then calculated the percentage of mice in which tumor encompassed each square in the grid and depicted a composite image using color or grayscale. Composite images were then smoothed in Photoshop 6.0 (Adobe, San Jose, California) to reduce granularity.

Results

Skeletal metastases obtained via intracardiac injection

MDA-MB-435 cells were transfected with a plasmid conveying enhanced GFP under a cytomegalovirus constitutive promoter. The resulting mixed population of neomycin resistant cells contained both fluorescing and non-fluorescing clones. Cells comprising the highest 25% of fluorescence intensity were selected using a fluorescence activated cell sorter. Cells (1×10^6) were injected into the mammary fat pad of female athymic mice. Tumorigenicity and *in vivo* growth rates of the resulting 435^{GFP} tumors were indistinguishable from the parental line (data not shown). Pulmonary metastatic potentials were likewise not significantly different. Only a small fraction of 435^{GFP} cells lost or had decreased fluorescence when continuously cultured. Nonetheless, to validate fluorescence as a method to quantify metastases, lungs were fixed in Bouin's solution following fluorescence microscopy and macroscopic metastases recounted. The number of lung metastases using fluorescence and traditional methods was nearly identical in most cases ($n = 11$), differing by only 1 to 3 metastases. In only one mouse were counts significantly greater following Bouin's staining (36 vs.15 metastases), suggesting outgrowth of non-fluorescing clones. Thus, the number of metastases numerated under fluorescence would represent, at worst, an under-estimation. In subsequent fluorescent analyses, steps were taken to monitor for non-fluorescent skeletal metastases.

Metastatic potential of 435^{GFP} cells was assessed following orthotopic, i.v., or i.c. injection in a pilot experiment. The objective was primarily to evaluate bone metastasis formation. While it has been previously shown that 435 cells infrequently establish pulmonary metastases following i.v. injection [31], bone colonization following this route had not been reported. To minimize first-pass clearance of cells in the lung microvasculature (the first capillary bed encountered by cells entering the venous circulation), 435^{GFP} cells were injected i.c. Four weeks following tumor removal or vascular injection, mice were necropsied; both femurs removed, dissected free of soft tissue and scrutinized by fluorescence microscopy. Femoral lesions did not develop following injection into the mammary fat pad or i.v. inoculation (Figure 1). Green fluorescent foci were observed in the femurs of intracardially injected mice with high frequency. Moreover, the metastases were osteolytic (Figure 2A). In mice necropsied following longer durations, osteolytic lesions were apparent by radiography (Figures 2B, C).

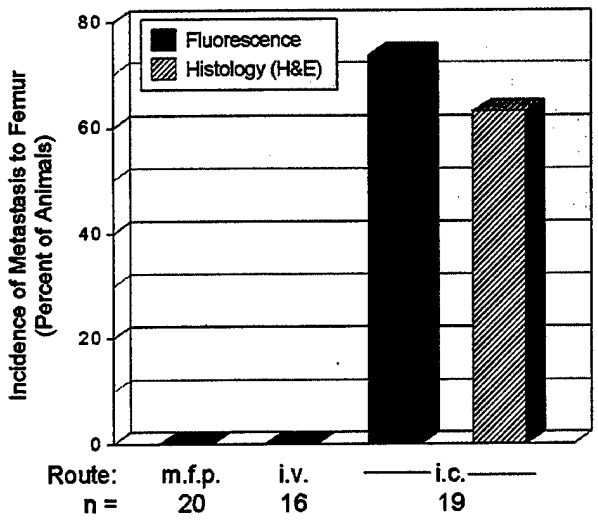


Figure 1. Skeletal metastases are obtained following intracardiac injection of 435^{GFP}, but not following orthotopic (mammary fat pad) or intravenous injection. The number of detectable metastases to bone is increased by GFP-tagging (compared to step section analysis of bones), although the increase is not significant. Cells (2×10^5) were introduced into 4–6-week-old female athymic mice by intracardiac injection. Mice were necropsied at four weeks and femoral bones dissected free of soft tissues. Femurs were first examined by fluorescence and then using H&E-stained sections at five levels.

GFP-tagging allows detection of bone metastases

To determine whether the convenience of GFP detection translates to increased detection of macroscopic metastases in bone, femurs of intracardially injected mice were fixed in 10% neutral buffered formalin, decalcified and embedded in paraffin for standard histology. Longitudinal sections representing five levels through approximately two-thirds of the bone were stained with hematoxylin and eosin. Tumor in histological sections corresponded to green fluorescence observed in 435^{GFP} injected mice. However, fluorescent foci were detected in two mice that were undetected in the limited number of sections evaluated. Incidence of 435^{GFP} bone metastases by histology from 63% (1.7 ± 0.37; mean $\pm$ SEM), compared to 74% (2.4 ± 0.46) by fluorescence (Figure 1). The difference was not statistically significant and could be explained by sampling error in the histology. Additionally, in this pilot analysis, mice with bone metastases had multiple lesions in each femur. This made us question whether mice with no metastases were successfully injected in the left ventricle. For subsequent studies, the color of blood drawn into the syringe was assessed prior to, and after, injection. When arterial injection was verified in this manner, incidence of bone metastasis increased to 100% of femurs.

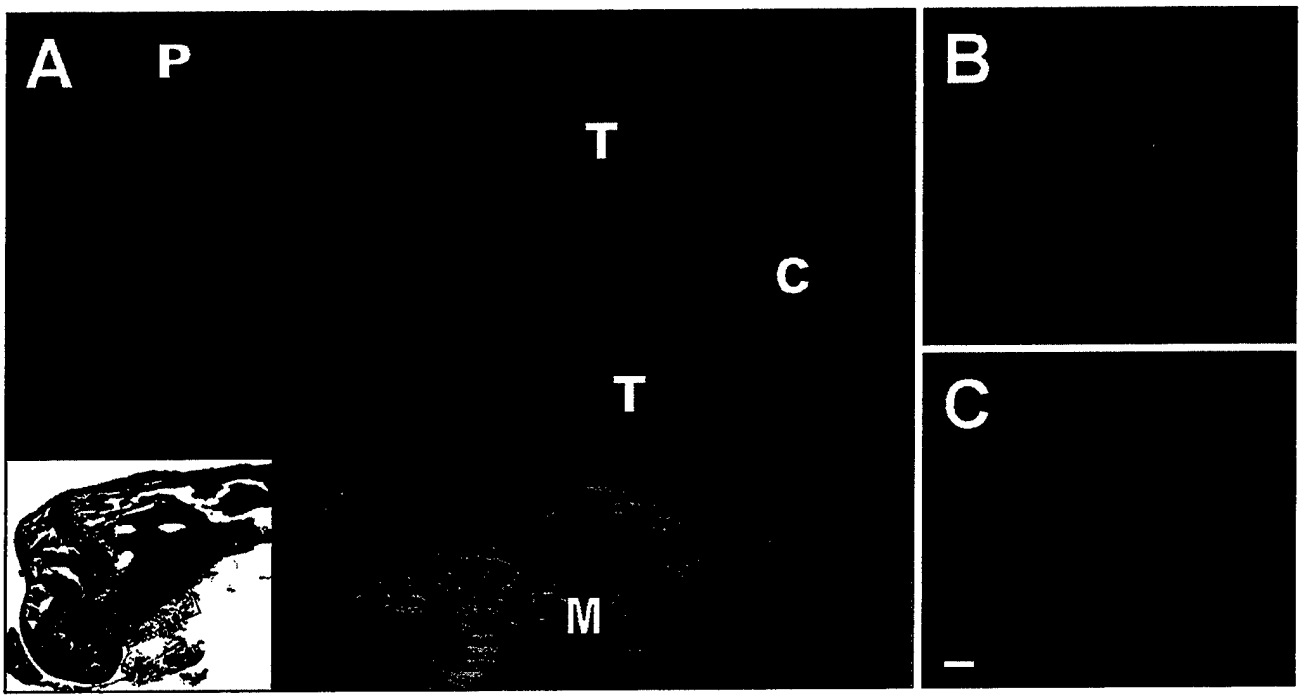


Figure 2. 435^{GFP} skeletal metastases are osteolytic. A. A metastasis is shown in distal femur four weeks following intracardiac injection. Tumor within the medullary cavity has invaded through the cortical bone to the exterior of the shaft. Cortical bone (C), tumor (T), distal epiphyseal growth plate (P), skeletal muscle (M). B. X-ray (19 kVp) 7.5 weeks following i.c. injection shows significant osteolysis in proximal tibia corresponding to a fluorescing lesion (Figure C). Bar = 1 mm.

To determine whether skeletal metastases were randomly distributed, 435^{GFP} cells were injected i.c. and all bones (femur, tibia, fibula, scapula, humerus, radius, ulna, pelvis, skull, mandible, ribcage, vertebrae) were examined five weeks later by fluorescence. Bones were examined following removal of soft tissues. While not essential for detecting large metastases in the vertebral column or exposed joints such as the knee, detection of microscopic lesions and deep joints (e.g., proximal femur) required dissection of musculature. 435^{GFP} produced skeletal metastases with highest incidence in femur and mandible (Figure 3A). While 100% of mice ($n = 16$) had at least one femoral metastasis, 56% of mice had involvement of both femurs. Overall, 78% of femurs had at least one metastasis. Mandible metastases were found in all mice (94% of bones, 2 dentary bones per mandible). Sixty-three percent of mice developed vertebral metastases, accounting for 13% of all cervical, thoracic, lumbar and sacral vertebrae examined. Skull, pelvis, humerus and tibia were also involved in $\geq 50\%$ of animals. Except for the vertebral column, which yielded a mean of 4 ± 1.6 (mean $\pm$ SEM) metastases per mouse, the greatest number of metastases per mouse were in femur (2 ± 0.3) and mandible (2 ± 0.2) (Figure 3C).

The location and size of fluorescent metastases were graphed and the distribution of metastases was evaluated using custom software. As in humans, metastases localized predominantly to trabeculae in appendicular bones (proximal and distal femur, proximal tibia, and proximal humerus (Figure 4A)). Within the vertebral column, the lumbar and sacral vertebrae were involved with higher incidence than cervical or thoracic vertebrae.

Metastasis to non-osseous sites

Viscera and other organs were also evaluated for metastases. Except for brain, in which a sagittal bisection was performed, fluorescent metastases were quantified in intact tissues (Figures 4B–D). Using a relatively simple setup allows visualization of metastases within most tissues [32]. The most frequent sites of non-skeletal metastasis included adrenal glands (11/16 mice), brain (8/16) and ovary (7/16). Few macroscopic pulmonary metastases were observed (3/16), limited to only 1–3 macroscopic metastases per mouse. Numerous microscopic metastases (1–10 cells) were present in a total of 8 mice. Metastases also developed in the pancreas, kidney, liver, and eye in three to five mice. Rare metastases were encountered in stomach, uterus, bladder and spleen. Mice (4/16) also had metastases in mesenteric lymph nodes, but the number involved per mouse ranged from 11 to > 70.

GFP allows assessment of early time points in bone colonization

Tracking the arrival of metastasizing cells and subsequent proliferation at the secondary site has revealed key information regarding the role of the microenvironment in metastasis [25, 33, 34]. To assess the effectiveness of GFP tagging in the detection of metastases in bone at early time points, mice were necropsied following intracardiac injection of 435^{GFP} cells, beginning at 10 min. Single fluorescing cells were seldom detectable in intact femur; however, longitudinal bisection revealed single cells in the bone interior (Figure 4B). At two weeks, microscopic metastases and single cells were

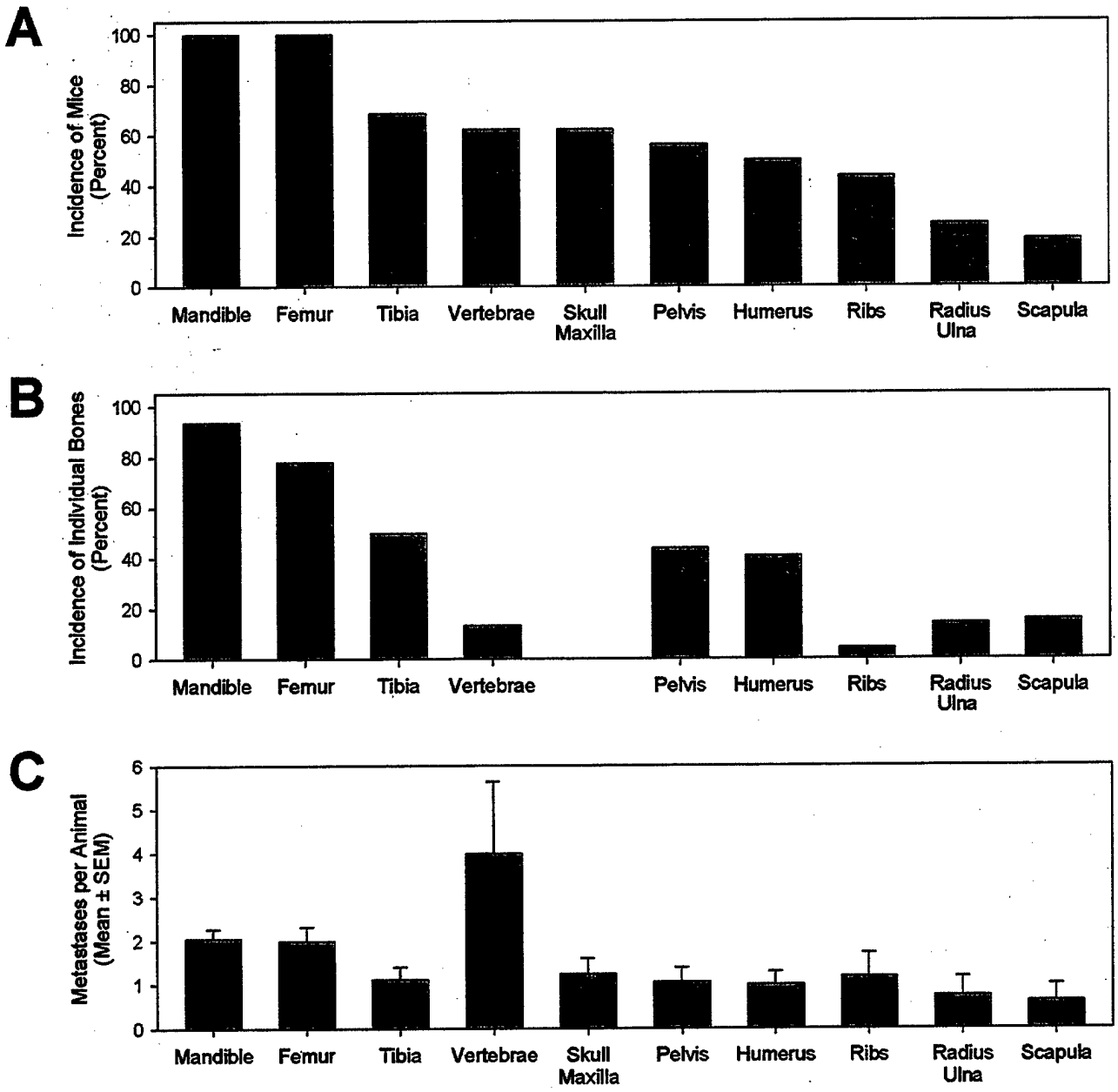


Figure 3. Distribution of skeletal metastases. Mice necropsied 5 weeks after injection of 2×10^5 435^{GFP} cells into the left ventricle. Intact bones were dissected free of soft tissue and examined by fluorescence microscopy. A. Percent of mice with at least one metastasis in specified bones (skull and facial bones are grouped) ($n = 16$). B. Percent of individual bones with at least one metastasis. (mandible considered as 2 bones; pelvis, 2; ribs, 26; vertebrae, 30). C. Number (mean ± SEM) of metastases per mouse within specified bones ($n = 16$).

observed through uncut bone (Figures 4D, E). Observation of GFP through intact bone permitted convenient three-dimensional examination of lesions because bones could be fully rotated and manipulated. Adjacent, but separate foci could be distinguished prior to coalescing (Figure 4). In addition, macroscopic metastases were readily detected prior to radiographic evidence of osteolysis (Figure 5).

Discussion

The human breast carcinoma cell line, MDA-MB-435, has been widely used in the study of human breast cancer, both *in vivo* and *in vitro*. It has been extremely useful because it is one of the few breast cancer cell lines that metastasizes. However, its propensity to colonize bone, the most common site of breast cancer metastasis, has not been thoroughly examined. Previously, a single study directly injected 435 cells into bone [16]; however, lesions formed by this method cannot be construed as metastases.

To assess whether 435 might be a useful model for bone metastasis, we stably transfected cells with enhanced green fluorescent protein. The resulting cells behaved as parental cells in tumorigenicity and spontaneous metastasis assays (i.e., following orthotopic injection). As we have observed previously, GFP does not appear to adversely affect tumor cell behavior. GFP transfection was performed in

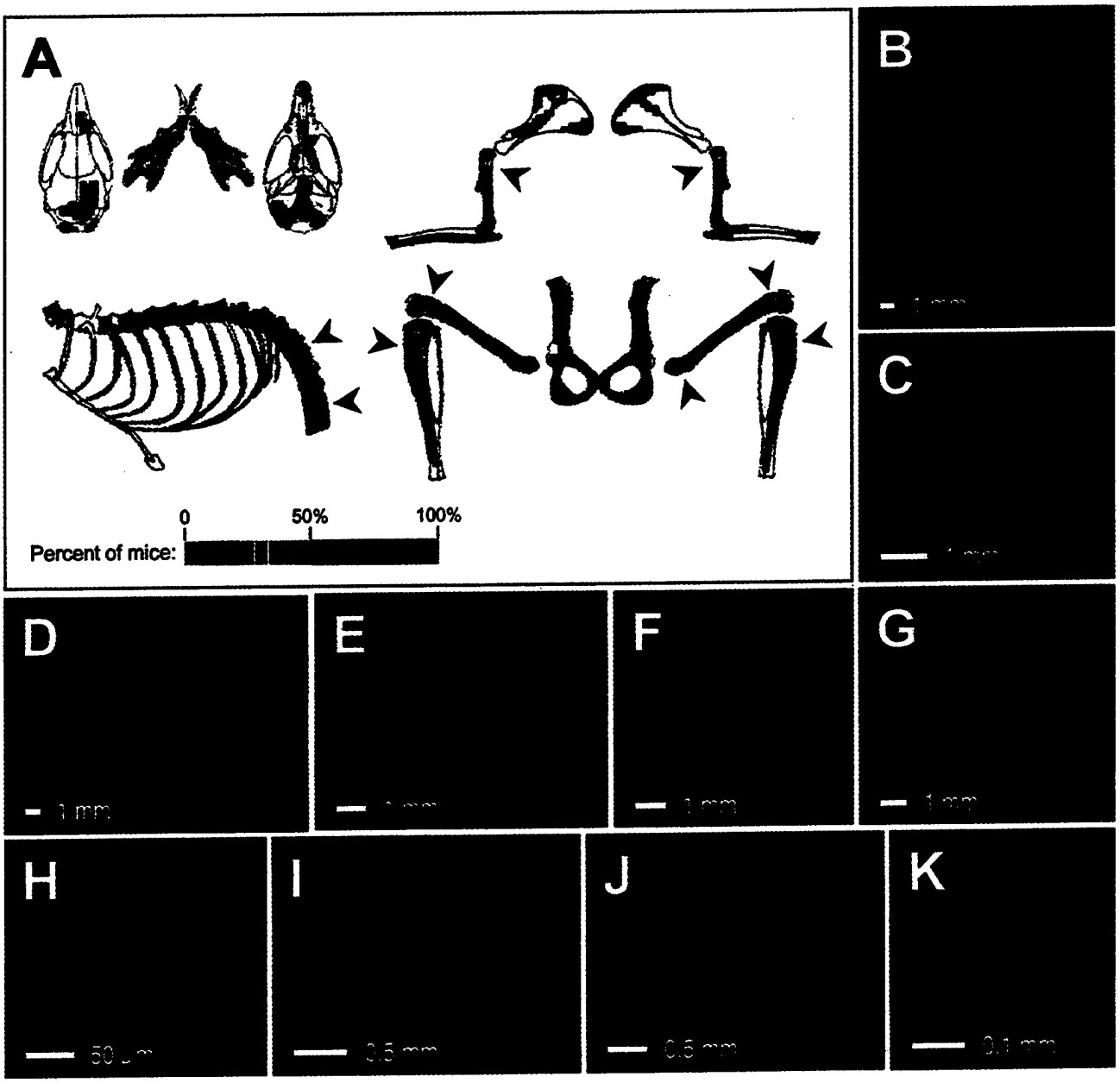


Figure 4. A. Compilation of 435^{GFP} skeletal metastases in 16 mice. Metastases (five weeks) localize predominantly to trabecular regions in femurs, proximal tibia, proximal humerus and vertebrae. Mandibular metastases are frequent. Arrowheads highlight regions of the skeleton with the highest incidence of bone metastases. B. Sagittal bisection of the skull and brain reveals a fairly large number of 435^{GFP} brain metastases 5 weeks following i.c. injection. C. Metastases (5–6) in adrenal gland. D. Rare 435^{GFP} metastases to liver. E. Two involved lumbar vertebrae at four weeks. F. The metastasis in the left most vertebrae of (E) is localized to the centrum. G. A scapular metastasis. H. Single tumor cells and microscopic metastases are detectable by fluorescence microscopy. Longitudinal bisection revealing single 435^{GFP} cell in distal femur 10 min following intracardiac injection. Note, the cell is already forming pseudopodial processes. I. Left tibia two weeks post-intracardiac injection. Cluster of three to five cells visible from the exterior of intact bone. J. Whole, distal left femur two weeks post-intracardiac injection. Multiple adjacent, but separate, foci are easily distinguished. By four to five weeks, such lesions would most likely have coalesced. K. Three 435^{GFP} cells visible at the lung surface two weeks following intracardiac injection.

order to enhance the detection of tumor cells in the bone. Our data show that fluorescent detection was greater than radiographic methods or step-sections through bone. Bone metastases formed at sites similar to those colonized in breast cancer patients (proximal appendicular long bones, vertebrae, pelvis) [35, 36]. In patients, 80–90% of skeletal metastases occur in the axial skeleton [35, 37], whereas we observed 62% of metastases are within axial bones in our model. The pattern and frequency of metastasis following intracardiac injection of 435^{GFP} cells was similar to those for the widely used 231 cells [3, 5, 14, 18, 38–41]. Additionally, Sasaki et al. [41, 42] have used 231 to study maxillofacial bone metastases, which typically comprise approximately 1% of oral malignancies. So, the high frequency of 435 mandibular involvement suggests this would be a suitable model for this metastasis site as well. Further supporting the quality of the 435^{GFP} model, metastases were predominantly osteolytic, as in the majority of human breast cancers. Also, the majority of bone lesions occurred in metaphyseal tra-

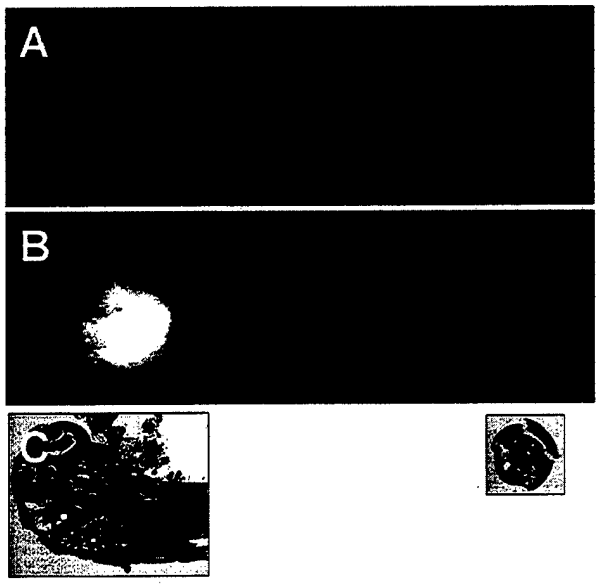


Figure 5. Metastases, visible by fluorescence microscopy (B) 4 weeks following i.c. injection, are not detectable by radiography (59 kVp) (A). H&E stained histology confirms the presence of tumor (C).

beculae, sites most commonly colonized in human cancer metastasis to bone.

In addition to osseous metastases, 435^{GFP} cells colonize several other organs that are frequent sites of breast cancer metastases – adrenal gland, brain and ovary. Rare lesions were found in lung, pancreas, kidney, liver and eye. Thus, the intracardiac injection model using 435^{GFP} affords opportunities to study metastases of human breast carcinoma to other relevant sites in a xenograft model.

From a technical perspective, this report highlights several issues. First, extrapulmonary metastases are infrequent unless 435^{GFP} cells are injected into arterial circulation. Proper injection into the left ventricle of the heart could be routinely validated by careful examination of blood color prior to and after tumor cell inoculation. The additional manipulation did not appear to have any adverse effect on the mice. Viability and complete recovery within 30 min were routine. Second, GFP allowed detection of metastases in intact bones. Building on the pioneering work of Hoffman and colleagues, who examined melanoma, prostate and lung cancer metastases to bone [28, 29, 32, 43], we developed the 435^{GFP} breast carcinoma model. During the course of these studies, Peyuchaud et al. reported development of 231^{GFP} variants [18]. GFP-tagging allowed detection of lesions one week prior to radiographic detection. We were similarly able to detect single cells or microscopic foci within two weeks, almost two to four weeks prior to radiographic evidence of osteolytic metastases. The ability to detect metastases before severe osteolysis provides a powerful tool for studying the earliest stages of bone colonization. In addition, the potential to minimize pain and suffering associated with more extensive bone involvement (e.g., paralysis or fracture) provides significant ethical improvement. Additionally, obviating the need for histology to observe bone metastases is a major savings in time and resources. Third, the sensitivity of GFP detection permits imaging of single cells. While we had previously used fluorescently labeled tumor cells to quantify single cells in lung, the capacity to detect microscopic, single cell foci within intact bone or in a bone which had been bisected was a fortuitous finding. Coupled with newly developed techniques that allow decalcification and sectioning, while maintaining fluorescence [44], we believe that it is now possible to study the earliest steps of tumor cell arrival and movement within the bone micro-environment. Fourth, we were able to store tissues for long periods (several months) while maintaining fluorescence. This ability provides investigators with adequate time to thoroughly examine tissues in large scale experiments involving multiple experimental groups.

In conclusion, we have added another human breast carcinoma cell line to the armamentarium for studies of metastasis to bone. By incorporating improved detection due to fluorescent tagging, a model is now available for studying the earliest steps in osseous metastasis and for large scale experiments where significant osteolysis is not desirable.

Acknowledgements

This research was support by grants from the National Institutes of Health (CA87728 and CA89019) and the National Foundation for Cancer Research Center for Metastasis Research. We are indebted to Dr Janet E. Price (University of Texas–M.D. Anderson Cancer Center) for providing the MDA-MB-435 cells and Lynn R. Budgeon for histological sectioning. We also acknowledge Drs Henry Donahue, Carol Gay and Andrea Mastro for their advice and helpful discussions.

References

1. Ries LAG, Eisner MP, Kosary CL et al. (eds). SEER Cancer Statistics Review, 1973–1999. Bethesda, Maryland: National Cancer Institute 2002.
2. Body JJ. Metastatic bone disease: Clinical and therapeutic aspects. Bone 1992; 13: S57–S62.
3. Duivenvoorden WC, Popovic SV, Lhotak S et al. Doxycycline decreases tumor burden in a bone metastasis model of human breast cancer. Cancer Res 2002; 62: 1588–91.
4. Guise TA. Molecular mechanisms of osteolytic bone metastases. Cancer 2000; 88(12 Suppl): 2892–8.
5. Sasaki A, Boyce BF, Story B et al. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res 1995; 55: 3551–7.
6. Yoneda T, Williams PJ, Hiraga T et al. A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone *in vivo* and *in vitro*. J Bone Miner Res 2001; 16: 1486–95.
7. Yoneda T, Sasaki A, Dunstan C et al. Inhibition of osteolytic bone metastasis of breast cancer by combined treatment with the bisphosphonate ibandronate and tissue inhibitor of the matrix metalloproteinase-2. J Clin Invest 1997; 99: 2509–17.
8. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 1992; 52: 1399–405.
9. Yoneda T, Michigami T, Yi B et al. Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma. Cancer 2000; 88: 2979–88.
10. Price JE, Zhang RD. Studies of human breast cancer metastasis using nude mice. Cancer Metastasis Rev 1990; 8: 285–97.

11. Welch DR. Technical considerations for studying cancer metastasis *in vivo*. Clin Exp Metastasis 1997; 15: 272–306.
12. Miller C, Kiriakova G, Su Kim L et al. Characterization of a novel breast cancer cell line established from a bone metastasis. Proc Am Assoc Cancer Res 2002; 43: 316.
13. Thompson EW, Sung V, Lavigne M et al. LCC15-MB: A vimentin-positive human breast cancer cell line from a femoral bone metastasis. Clin Exp Metastasis 1999; 17: 193–204.
14. Sasaki A, Kitamura K, Alcalde RE et al. Effect of a newly developed bisphosphonate, YH529, on osteolytic bone metastases in nude mice. Int J Cancer 1998; 77: 279–85.
15. Weber MH, Lee J, Orr FW. The effect of Neovastat (AE-941) on an experimental metastatic bone tumor model. Int J Oncol 2002; 20: 299–303.
16. Wang CY, Chang YW. A model for osseous metastasis of human breast cancer established by intrafemur injection of the MDA-MB-435 cells in nude mice. Anticancer Res 1997; 17: 2471–4.
17. Thomas RJ, Guise TA, Yin JJ et al. Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology 1999; 140: 4451–8.
18. Peyruchaud O, Winding B, Pecheur I et al. Early detection of bone metastases in a murine model using fluorescent human breast cancer cells: Application to the use of the bisphosphonate zoledronic acid in the treatment of osteolytic lesions. J Bone Miner Res 2001; 16: 2027–34.
19. Averbuch SD. New bisphosphonates in the treatment of bone metastases. Cancer 1993; 72: 3443–52.
20. Arguello F, Baggs RB, Frantz CN. A murine model of experimental metastasis to bone and bone marrow. Cancer Res 1988; 48: 6876–81.
21. Holleran JL, Miller CJ, Edgehouse NL et al. Differential experimental micrometastasis to lung, liver, and bone with lacZ-tagged CWR22R prostate carcinoma cells. Clin Exp Metastasis 2002; 19: 17–24.
22. Lin WC, Pretlow TP, Pretlow TG et al. Bacterial lacZ gene as a highly sensitive marker to detect micrometastasis formation during tumor progression. Cancer Res 1990; 50: 2808–17.
23. Culp LA, Lin WC, Kleinman NR. Tagged tumor cells reveal regulatory steps during earliest stages of tumor progression and micrometastasis. Histol Histopathol 1999; 14: 879–86.
24. Wetterwald A, van der PG, Que I et al. Optical imaging of cancer metastasis to bone marrow: A mouse model of minimal residual disease. Am J Pathol 2002; 160: 1143–53.
25. Goldberg SF, Harms JF, Quon K et al. Metastasis-suppressed C8161 melanoma cells arrest in lung but fail to proliferate. Clin Exp Metastasis 1999; 17: 601–7.
26. Hoffman RM. Orthotopic transplant mouse models with green fluorescent protein-expressing cancer cells to visualize metastasis and angiogenesis. Cancer Metastasis Rev 1998; 17: 271–7.
27. Naumov GN, Wilson SM, MacDonald IC et al. Cellular expression of green fluorescent protein, coupled with high-resolution *in vivo* videomicroscopy, to monitor steps in tumor metastasis. J Cell Sci 1999; 112: 1835–42.
28. Yang M, Jiang P, An Z et al. Genetically fluorescent melanoma bone and organ metastasis models. Clin Cancer Res 1999; 5: 3549–59.
29. Yang M, Jiang P, Sun FX et al. A fluorescent orthotopic bone metastasis model of human prostate cancer. Cancer Res 1999; 59: 781–6.
30. Cook MJ. The Anatomy of the Laboratory Mouse. New York: Academic Press 1965.
31. Price JE, Polyzos A, Zhang RD et al. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Res 1990; 50: 717–21.
32. Hoffman RM. Visualization of GFP-expressing tumors and metastasis *in vivo*. Biotechniques 2001; 30: 1016–26.
33. Al Mehdi AB, Tozawa K, Fisher AB et al. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: A new model for metastasis. Nat Med 2000; 6: 100–2.
34. Naumov GN, MacDonald IC, Weinmeister PM et al. Persistence of solitary mammary carcinoma cells in a secondary site: A possible contributor to dormancy. Cancer Res 2002; 62: 2162–8.
35. Lote K, Walloe A, Bjersand A. Bone metastasis. Prognosis, diagnosis and treatment. Acta Radiol Oncol 1986; 25: 227–32.
36. Miller F, Whitehill R. Carcinoma of the breast metastatic to the skeleton. Clin Orthop 1984; 184: 121–7.
37. Nielsen OS, Munro AJ, Tannock IF. Bone metastases: Pathophysiology and management policy. J Clin Oncol 1991; 9: 509–24.
38. Mbalaviele G, Dunstan CR, Sasaki A et al. E-cadherin expression in human breast cancer cells suppresses the development of osteolytic bone metastases in an experimental metastasis model. Cancer Res 1996; 56: 4063–70.
39. Sasaki A, Alcalde RE, Nishiyama A et al. Angiogenesis inhibitor TNP-470 inhibits human breast cancer osteolytic bone metastasis in nude mice through the reduction of bone resorption. Cancer Res 1998; 58: 462–7.
40. Zhang RD, Fidler IJ, Price JE. Relative malignant potential of human breast carcinoma cell lines established from pleural effusions and a brain metastasis. Invasion Metastasis 1991; 11: 204–15.
41. Sasaki A, Yoneda T, Terakado N et al. Experimental bone metastasis model of the oral and maxillofacial region. Anticancer Res 1998; 18: 1579–84.
42. Sasaki A, Nishiyama A, Alcalde RE et al. Effects of bisphosphonate on experimental jaw metastasis model in nude mice. Oral Oncol 1999; 35: 523–9.
43. Yang M, Hasegawa S, Jiang P et al. Widespread skeletal metastatic potential of human lung cancer revealed by green fluorescent protein expression. Cancer Res 1998; 58: 4217–21.
44. Harms JF, Budgeon LR, Christensen ND et al. Maintaining GFP tissue fluorescence through bone decalcification and long-term storage. Biotechniques 2002; 33: 1197–200.

This result allowed us to conclude that arsenite-induced apoptosis of this cell line was through the activation of caspase-3, although it remains unclear what reaction component(s) were modified by boiling. The mechanism of increasing sensitivity by the boiling method needs to be studied further.

REFERENCES

1. **Ackermann, E.J., J.K. Taylor, R. Narayana, and C.F. Bennett.** 1999. The role of antiapoptotic Bcl-2 family members in endothelial apoptosis elucidated with antisense oligonucleotides. J. Biol. Chem. *274*:11245-11252.
2. **Drake, R.R., T.N. Wilbert, T.A. Hinds, and K.M. Gilbert.** 1999. Differential ganciclovir-mediated cell killing by glutamine 125 mutants of herpes simplex virus type 1 thymidine kinase. J. Biol. Chem. *274*:37186-37192.
3. **Fonteh, A.N., T. LaPorte, D. Swan, and M.A. McAlexander.** 2001. A decrease in remodeling accounts for the accumulation of arachidonic acid in murine mast cells undergoing apoptosis. J. Biol. Chem. *276*:1439-1449.
4. **Janicke, R.U., P. Ng, M.L. Sprengart, and A.G. Porter.** 1998. Caspase-3 is required for α-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. J. Biol. Chem. *273*:15540-15545.
5. **Marches, R., R. Hsueh, and J.W. Uhr.** 1999. Cancer dormancy and cell signaling: Induction of $p21^{waf1}$ initiated by membrane IgM engagement increases survival of B lymphoma cells. Proc. Natl. Acad. Sci. USA *96*:8711-8715.
6. **Wang, S., J.A. Vrana, T.M. Bartimole, A.J. Freemerman, W.D. Jarvis, L.B. Kramer, G. Krystal, P. Dent, and S. Grant.** 1997. Agents that down-regulate or inhibit protein kinase C circumvent resistance to 1-β-D-arabinofuranosylcytosine-induced apoptosis in human leukemia cells that overexpress Bcl-2. Mol. Pharmacol. *52*:1000-1009.

Address correspondence to Dr. Lizhi Zeng, H.L. Snyder Medical Research Institute, 1407 Wheat Road, Winfield, KS 67156, USA. e-mail: lzeng@snyderrf.org

Received 17 June 2002; accepted 16 August 2002.

Lizhi Zeng and Larry D. Smith
H.L. Snyder Medical Research Institute
Winfield, KS, USA

Maintaining GFP Tissue Fluorescence through Bone Decalcification and Long-Term Storage

BioTechniques 33:1197-1200 (December 2002)

Decalcification of bone is required for frozen or standard histological sectioning; however, acidic decalcification solutions abrogate the fluorescence of tissues expressing enhanced GFP. In addition, long-term storage of fluorescing tissues from in vivo studies necessitates maintaining GFP fluorescence in a solution that does not compromise tissue and cellular integrity.

The spread of metastatic cancer to skeletal sites is a grim complication frequent in breast, prostate, and lung cancers. In particular, the incidence of breast cancer metastasis to bone has been estimated to be as high as 85% (2), causing osteolytic lesions that result in pathological fractures, spinal cord compression, and hypercalcemia. Why

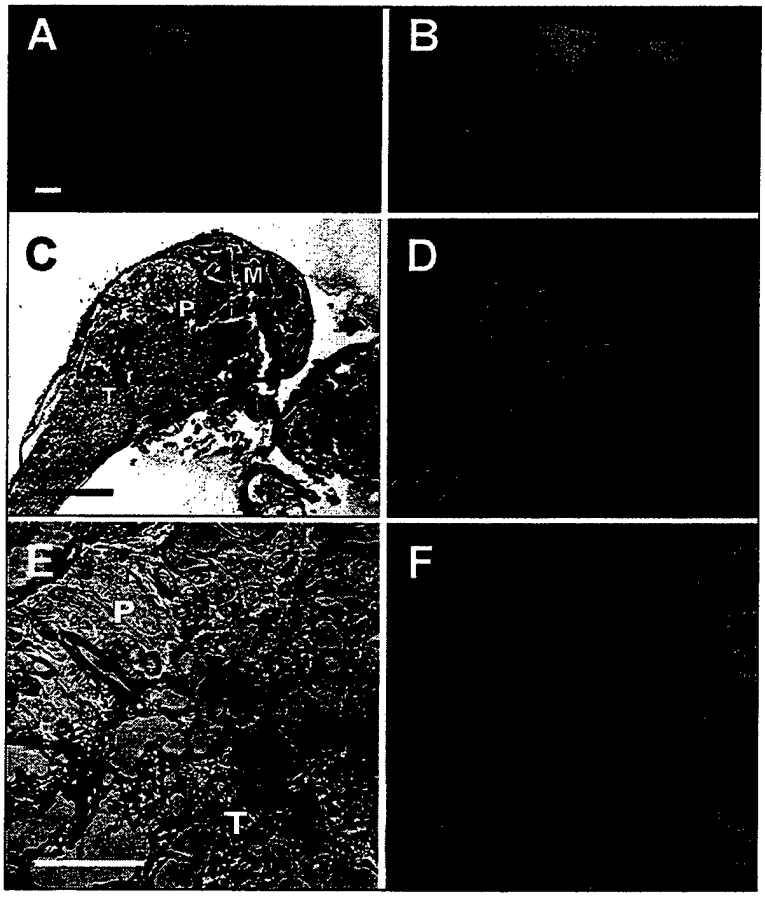


Figure 1. Fluorescence of GFP-tagged breast cancer metastases is maintained through decalcification and frozen sectioning of murine hind limb bones. (A) Fluorescence microscopy of whole femur and proximal tibia following 4% paraformaldehyde fixation. Bar = 1 mm. (B) Fluorescence following 14 h incubation in 0.5 M EDTA in CMF-PBS, immediately preceding frozen sectioning. (C and E) Bright-field photomicrographs of frozen sections. Tumor (T) has filled medullary canal but has not crossed the epiphyseal growth plate (P) into distal normal marrow (M). Since the epiphyseal growth plate is normal murine tissue, it does not fluoresce. Normal murine tissue also exists between tumor cells, and some spaces are the result of tissue sectioning artifact. (C, Bar = 1 mm; E, Bar = 0.1 mm). (D and F) Corresponding fluorescence microscopy reveals fluorescing tumor tissue replacing marrow of the medullary canal.

breast cancer exhibits significant predilection for bone is unknown. To model skeletal metastasis in vivo, we engineered metastatic human breast carcinoma cell lines (MDA-MB-435 and MDA-MB-231) to constitutively express GFP (4). Intracardiac injection of cells into the left ventricle of female athymic mice produces widespread skeletal metastases, localized predominantly to the trabecular regions of bones including femur, proximal tibia, proximal humerus, and lumbar vertebrae.

The utility of GFP-tagging for the detection of metastases and tracking of single cells in vivo has been clearly demonstrated in several models (3,5,7) including bone metastasis (6,8–11). Bone metastases may be easily identified in whole bone without laborious sectioning or radiographic detection that customarily requires degradation of at least 50% of the mineralized bone (1). However, histological sectioning is required to determine the position of metastases at the microscopic level. Paraffin or frozen sectioning of bone without fixation and decalcification often causes shattering of the calcified tissue and contributes to significant wear or chipping of blades. While 4% paraformaldehyde fixation of GFP tissues maintains fluorescence, decalcification methods employing acidic solutions quickly abrogate fluorescence.

Metastatic MDA-MB-435 cells were transfected with pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA) by electroporation (GenePulser™, Bio-Rad, Laboratories, Hercules, CA, USA; 220 V, 960 µFd, ∞Ω). The brightest 25% of the neomycin-resistant fluorescing cells were sorted using a Coulter EPICS™ V cell sorter (Beckman Coulter, Fullerton, CA, USA). Cells were introduced into athymic mice by either mammary fat pad or intracardiac injection. Fresh primary tumors fluoresced and continued to fluoresce following fixation in freshly prepared 4% paraformaldehyde (4°C) for 24–48 h. Tissues were examined using a Leica MZFLIII dissection microscope, equipped with GFP2 filter set (Leica, Deerfield, IL, USA). Samples were subsequently exposed to common decalcification solutions including CalEX® (Fisher Scientific, Pittsburgh, PA, USA), 10% sodium citrate/22.5% formic acid, and 0.5 M EDTA in calcium and magnesium-free Dulbecco's PBS (CMF-PBS) (pH 7.8, 4°C). While fluorescence was eliminated following incubation in the acidic solutions (CalEX and sodium citrate/formic acid), 0.5 M EDTA maintained tissue fluorescence (Figure 1, A and B). Next, to determine the minimum incubation time sufficient for decalcification, two hind limbs, dissected free of soft tissue, were incubated in 10 mL 0.5 M EDTA (4°C) and removed at various time points including 6, 12, 18, 24, 36 and 48 h. Bones were then mounted in O.C.T. compound (Tissue-Tek, Elkhart, IN, USA) and frozen-sectioned. Bones decalcified for 18 h contained limited calcified deposits, as determined by blade sound and feel during frozen sectioning, while 24-h treatment achieved complete decalcification and eliminated blade wear. Fluorescing skeletal metastases were readily visible in frozen sections by fluorescence microscopy (Figure 1, C–F).

Historically, extended archiving of fluorescently tagged tissues, including GFP-labeled cells, has also been problematic. Freezing of tissues may protect fluorescence but can introduce freezing artifacts. While 4% paraformaldehyde maintains fluorescence during fixation, long incubations can deteriorate fluorescence, as can extended storage in 70% ethanol following fixation. Nevertheless, retention of paraformaldehyde-fixed tissues (24-h fixation) in only CMF-PBS (4°C) is incapable of preventing tissue autolysis and cellular degradation. Having observed that tissue still fluoresced after several weeks in 0.5 M EDTA at 4°C and that no tissue autolysis was apparent, we tested whether this was a possible long-term storage medium. We have also tested two dilute solutions of paraformaldehyde (0.5% and 1% paraformaldehyde in CMF-PBS) in parallel with 4% paraformaldehyde. Whole murine bones (including skull, mandible, ribcage, vertebral column, pelvis, and limbs) were dissected free of soft tissues, and all bones derived from an individual mouse were combined in a single 25-mL vial. Vials were filled with 4% paraformaldehyde (approximately 18 mL) for

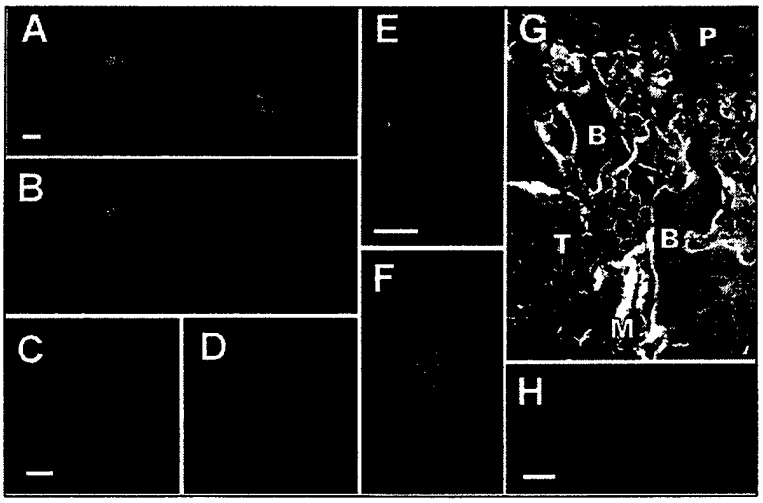


Figure 2. Storage of fluorescent tissues in 0.5 M EDTA or 1% paraformaldehyde solutions maintains both tissue fluorescence and morphology. (A and B) Two brain metastases visible in a sagittal section of the skull at two months (A) and 11 months (B). Photomicrographs with identical exposure settings were collected using a MagnaFire™ digital camera (Optronics, Goleta, CA, USA), and ImagePro Plus software (Media Cybernetics, Silver Spring, MD, USA). (C and D) While there is an overall decrease in tumor cell fluorescence and a slight increase in background autofluorescence, GFP-tagged cells are still apparent. Two vertebral metastases stored for 11 months (D, 10-s exposure) compared to an image captured of the specimen when fresh and unfixed (C, 5-s exposure). (E–G) Bright-field (E) and fluorescence (F) images of proximal humerus immediately before histological sectioning at 11 months (G). H&E staining shows tumor (T) infiltrating through normal marrow (M) and trabecular bone (B) toward the epiphyseal growth plate (P). (H) Metastasis in proximal right femur fluoresces following 19 months in 1% paraformaldehyde. Bars = 1 mm.

Benchmarks

24–48 h, and the solution was then replaced with 0.5 M EDTA, 0.5% paraformaldehyde, or 1% paraformaldehyde, each in CMF-PBS. Vials were stored at 4°C at all times. We can report that 11 months storage in 0.5 M ETDA, and 19 months in 0.5% and 1% paraformaldehyde, both soft-tissue and skeletal metastases still fluoresce (Figure 2, A and B). Under these conditions, a few metastases lost considerable fluorescence and were only slightly visible above background. By contrast, the majority of samples stored concurrently in 4% paraformaldehyde no longer fluoresced.

Background auto-fluorescence commonly increases following fixation, and the intensity of GFP fluorescence is sometimes reduced compared to fresh tissue (Figure 2, C and D). Nonetheless, maintenance of fluorescence, along with relatively good preservation of cell morphology when tissues are routinely sectioned, renders this inconvenience acceptable for most uses (Figure 2, E–G). This technique now provides investigators adequate time to thoroughly examine tissues in large-scale experiments involving several replicates in multiple experimental groups. The safety of 0.5 M EDTA for both decalcification and storage also eliminates the need for additional solution changes and enables immediate histological processing of archived samples, including use for standard histological staining.

In conclusion, 0.5 M EDTA in CMF-PBS is capable of decalcifying murine bones in at least 24 h without harming GFP fluorescence and can be utilized for long-term archival of fluorescent specimens. Additionally, extended storage in 0.5%–1% paraformaldehyde maintains tissue fluorescence without concomitant decalcification.

REFERENCES

1. **Averbuch, S.D.** 1993. New bisphosphonates in the treatment of bone metastases. Cancer *72*:3443-3452.
2. **Body, J.J.** 1992. Metastatic bone disease: clinical and therapeutic aspects. Bone *13(Suppl1)*:S57-S62.
3. **Goldberg, S.F., J.F. Harms, K. Quon, and D.R. Welch.** 1999. Metastasis-suppressed C8161 melanoma cells arrest in lung but fail to proliferate. Clin. Exp. Metastasis *17*:601-607.
4. **Harms, J.F. and D.R. Welch.** Human MDA-MB-435 human breast carcinoma metastasis to bone. Clin. Exp. Metastasis (In Press.)
5. **Hoffman, R.M.** 2001. Visualization of GFP-expressing tumors and metastasis in vivo. BioTechniques *30*:1016.
6. **Li, Y., M. Tondravi, J. Liu, E. Smith, C.C. Haudenschild, M. Kaczmarek, and X. Zhan.** 2001. Cortactin potentiates bone metastasis of breast cancer cells. Cancer Res. *61*:6906-6911.
7. **Naumov, G.N., S.M. Wilson, I.C. MacDonald, E.E. Schmidt, V.L. Morris, A.C. Groom, R.M. Hoffman, and A.F. Chambers.** 1999. Cellular expression of green fluorescent protein, coupled with high-resolution in vivo videomicroscopy, to monitor steps in tumor metastasis. J. Cell Sci. *112(Pt 12)*:1835-1842.
8. **Peyruchaud, O., B. Winding, I. Pecheur, C.M. Serre, P. Delmas, and P.Clezardin.** 2001. Early detection of bone metastases in a murine model using fluorescent human breast cancer cells: application to the use of the bisphosphonate zoledronic acid in the treatment of osteolytic lesions. J. Bone Miner. Res. *16*:2027-2034.
9. **Yang, M., S. Hasegawa, P. Jiang, X. Wang, Y. Tan, T. Chishima, H. Shimada, A.R. Moossa, and R.M. Hoffman.** 1998. Widespread skeletal metastatic potential of human lung cancer revealed by green fluorescent protein expression. Cancer Res. *58*:4217-4221.
10. **Yang, M., P. Jiang, Z. An, E. Baranov, L. Li, S. Hasegawa, M. Al Tuwaijri, T. Chishima, et al.** 1999. Genetically fluorescent melanoma bone and organ metastasis models. Clin. Cancer Res. *5*:3549-3559.
11. **Yang, M., P. Jiang, F.X. Sun, S. Hasegawa, E. Baranov, T. Chishima, H. Shimada, A.R. Moossa, and R.M. Hoffman.** 1999. A fluorescent orthotopic bone metastasis model of human prostate cancer. Cancer Res. *59*:781-786.

This research was supported by grants from the National Institutes of Health (CA87728) and the Penn State-National Foundation for Cancer Research Center for Metastasis Research. We are indebted to Dr. Janet E. Price (University of Texas-M.D. Anderson Cancer Center) for providing the MDA-MB-435 cells. Address correspondence to Dr. Danny R. Welch, Dept. of Pathology, University of Alabama at Birmingham, 1670 University Blvd., Birmingham, AL 35294-0019, USA. e-mail: dwelch@path.uab.edu

Received 21 August 2002; accepted 17 September 2002.

John F. Harms, Lynn R. Budgeon, Neil D. Christensen, and Danny R. Welch
Pennsylvania State University College of Medicine
Hershey, PA, USA

Vital Stain to Study Cell Invasion in Modified Boyden Chamber Assay

BioTechniques 33:1200-1204 (December 2002)

The capacity of cancer cells to invade basement membrane is a hallmark of metastasis. The modified Boyden chamber assay is often used to analyze and quantify the migratory and invasive potential of cells (5,8). It has a microporous membrane, which separates the two chambers, and is coated with Matrigel™ (BD Biosciences, San Jose, CA, USA) or any other extracellular matrix proteins like fibronectin, laminin, or collagen. Invasive cells seeded in the upper chamber respond to the chemoattractant in the lower chamber, invade the gel, and migrate to the lower surface of the membrane, whereas noninvasive cells remain in the upper chamber. The chambers are fixed, and cells on the upper side of the filter are removed with a cotton swab. Cells that have migrated to the lower side of the filter are stained [e.g., Diff Quick (9)] and counted. Quantitation of the results is usually tedious, as cells may not be clearly visible. Also, it is not possible to assess invasion during the incubation period and count cells that have started invading the gel but have not reached the lower surface of the membrane. Numerous technical papers have addressed these difficulties (2,7,9).

We have used a supra vital dye, Hoechst 33342 (Sigma, St. Louis, MO, USA) to stain cells during the invasion assay. This is a vital fluorescent stain that binds specifically to AT-rich nuclear DNA (1,3,10). It is excited by UV rays and emits blue fluorescence.

In the preliminary experiments, cervical carcinoma (SiHa) cells pulse-labeled for 20 min with Hoechst 33342 at 2 µg/mL showed bright nuclear fluorescence and did not exhibit any toxicity on viability, proliferation, and motility. These observations were comparable to earlier reports (6). The same concentration was used in the subsequent experiments, performed in triplicate. The methodology used was as described previously (4), using 6.5-mm transwell chambers with 8 µm pore size (Corning Costar, Acton, MA, USA).

Genomic analysis of primary tumors does not address the prevalence of metastatic cells in the population

Ramaswamy *et al.*[1] compared gene expression profiles of adenocarcinoma metastases to unmatched primary adenocarcinomas. They found an expression pattern that distinguished primary tumors from metastases but also reported that a subset of primary tumors had the expression pattern of metastases. This finding led them to challenge "the notion that metastases arise from rare cells within the primary tumor"[1]. In fact, their finding provides no evidence to contradict this notion.

To produce a metastasis, a tumor cell must complete a series of sequential steps, including detachment, invasion, survival in the circulation, attachment, extravasation, proliferation, induction of neovasculature and evasion of host defenses[2]. Because metastases are largely clonal in origin[3–5], the successful metastatic cell must have a set of characteristics that enable it to complete each step in the sequence. Lack of any single characteristic derails the process and prevents the cell from developing into a metastasis. Thus, the successful metastatic cell has been likened to a decathlon champion, who must be proficient in all ten events, not just a few, to be successful[2]. A primary tumor may contain many different cells, each of which can complete some of the steps in the metastatic process but not all. In aggregate, all of the steps may be represented among cells of the primary tumor, but it may still be the rare cell that can complete all the steps and thus give rise to a metastasis. The study by Ramaswamy *et al.*[1] looked at primary tumors in aggregate and, therefore, cannot rule out this possibility. The authors seem to have overlooked the large body of evidence indicating that primary tumors are heterogeneous with respect to many characteristics, including those associated with metastasis[2,6,7]. One example came from our work in which we found, by cloning, that unselected tumor cell lines with low metastatic potential contained a small number of cells with high metastatic potential, as well as many non-metastatic cells[3]. More recently, *in situ* hybridization was used to detect the expression of genes associated with the metastatic phenotype, specifically, those encoding MMP-2, MMP-9 and E-cadherin[8–10]. This approach allows not only the detection of gene expression but also its visualization in the tumor. These studies showed that expression of these three genes varied independently between the peripheral and central zones of the tumor and among other regions in a single section of the tumor. It stands to reason that the more cells express such genes, the higher the likelihood will be that the tumor will eventually give rise to metastases, a correlation substantiated in retrospective studies[9,10]. The findings of Ramaswamy *et al.*[1] using a genomics approach are consistent with those using *in situ* hybridization but have the added advantage of being able to identify previously unknown genes involved in the metastatic process.

Much evidence supports the view that progression from a benign to a malignant tumor is associated with acquisition of a set of genetic and epigenetic alterations that provide the phenotypic characteristics of malignancy[11–13]. These changes accumulate at different rates in different tumors and are reflected, albeit imperfectly, in the pathologist's classification of tumor stages. The stage I and II lung adenocarcinomas and early breast cancers studied by Ramaswamy *et al.*[1] generally expressed the non-metastatic pattern of genes, and only a few expressed the metastatic pattern. This probably reflects the fact that some of these primary tumors have indeed generated unique cells with full metastatic capabilities, as indicated by the patient survival data. The true significance of the study of Ramaswamy *et al.*[1] is not that it runs contrary to popular dogma, which, in our opinion, it does not, but that it may enable the identification of the small subset of tumors designated as early stage by pathologic criteria that nonetheless have already released a few metastatic cells. Thus, the study constitutes an important step in the quest to predict the behavior of tumors detected at an early stage, even though it does not address the prevalence of fully metastatic cells in primary tumors.

Isaiah J. Fidler[1] & Margaret L. Kripke[2]

[1]Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA. [2]Office of the Executive Vice President and Chief Academic Officer, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA. Correspondence should be addressed to I.J.F. (e-mail: ifidler@mdanderson.org).

1. Ramaswamy, S., Ross, K.N., Lander, E.S. & Golub, T.R. *Nat. Genet.* 33, 49–54 (2002).
2. Fidler, I.J. *Cancer Res.* 50, 6130–6138 (1990).
3. Fidler, I.J. & Kripke, M.L. *Science* 33, 893–895 (1977).
4. Talmadge, J.E., Wolman, S.R. & Fidler, I.J. *Science* 217, 361–363 (1982).
5. Fidler, I.J. & Talmadge, J.E. *Cancer Res.* 46, 5167–5171 (1986).
6. Waghorne, C. *et al. Cancer Res.* 48, 6109–6114 (1988).
7. Fidler, I.J. *et al. Lancet Oncol.* 3, 53–57 (2002).
8. Kitadai, Y. *et al. Am. J. Pathol.* 149, 1541–1551 (1996).
9. Kuniyasu, H. *et al. Clin. Cancer Res.* 6, 2295–2308 (2000).
10. Herbst, R.S. *et al. Clin. Caner Res.* 6, 790–797 (2000).
11. Nowell, P.C. *Science* 194, 23–28 (1976).
12. Fearon, E.R., Hamilton, S.R. & Vogelstein, B. *Science* 238, 193–197 (1987).
13. Vogelstein, B. *et al. Science* 224, 207–211 (1989).

Genetic background is an important determinant of metastatic potential

Recently there has been some debate about the etiology of cancer metastatic potential. Using microarray gene expression patterns of breast carcinomas, van't Veer *et al.*[1] reported that a set of 117 genes predicted metastatic potential. More recently, a small set of 17 genes was reported to predict metastatic potential for a variety of solid tumors[2]. These findings suggest that most primary tumor cells express a 'metastasis signature', in contrast to the classic model, which predicts that only a rare subpopulation of primary tumor cells have accumulated the numerous alterations required for metastasis. Based on this evidence, Bernards and Weinberg[3] recently posited that combinations of early oncogenic alterations, not specific events that promote metastasis, determine metastatic potential. This hypothesis might explain why metastasis occurs in some individuals with small, localized tumors (that is, tumors whose

cell number is too small to have statistical likelihood of accumulating adequate numbers of mutations proposed in the conventional model).

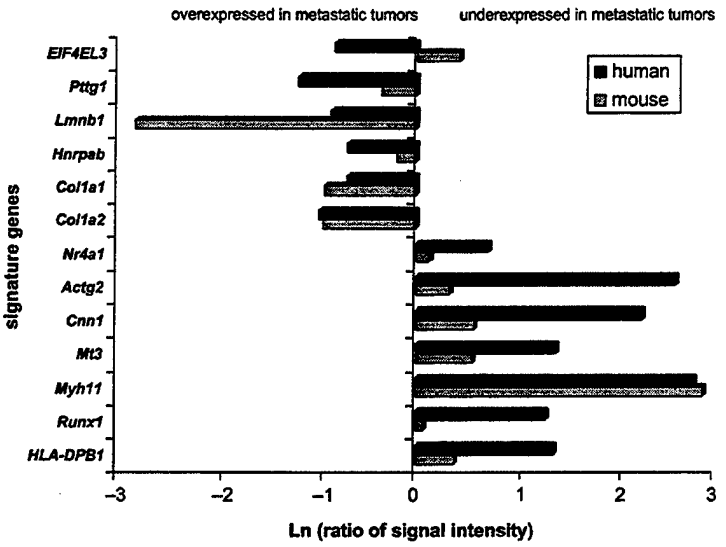
In contrast, there is persuasive evidence for the existence of mutations that promote metastasis. For example, metastasis-specific loss of heterozygosity has been associated with many solid tumors. Based on the tumor-suppressor paradigm, several laboratories have cloned genes that, when reintroduced into tumor cells, suppress the formation of secondary tumors without altering primary tumor initiation or kinetics. So far, eight metastasis-suppressor genes have been described (reviewed in ref. 4).

Thus, compelling evidence for both models exists. How, then, can these seemingly conflicting hypotheses be reconciled? One possibility, based on our studies, would be the contribution of genetic background. Using a transgene-induced mouse tumor model and a breeding strategy to vary genetic background, we found significant differences in metastatic efficiency (as much as 10-fold) between the original FVB/NJ mice and F1 hybrids without altering tumor initiation or growth kinetics[5,6]. We recently examined microarray data from our high-efficiency and low-efficiency metastatic genotypes for the set of 17 genes that comprise the metastasis signature[2]. Of these 17 genes, 13 were represented on the mouse chip. The expression of 12 of these changed in the same direction as in the human set (see figure).

Because all tumors were initiated by the same oncogenic event, differences in the metastasis microarray signature and metastatic potential are probably due to genetic background effects rather than different combinations of oncogenic mutations. Consistent with our observations in metastasis, several laboratories have shown similar strain differences with regard to oncogenesis, aging and fertility in transgenic mouse models[7–9]. Data on both primary tumors and metastases reinforce the notion that tumorigenesis and metastasis are complex phenotypes involving both inherent genetic components and cellular responses to extrinsic stimuli.

Thus, although our expression data is preliminary and additional studies are required to confirm these results, the cumulative data suggest that differential gene expression patterns may reflect individual genetic profiles that, in turn, are important determinants of metastatic potential. Unlike highly penetrant cancer susceptibility genes, metastasis susceptibility is probably due to complex allelic combinations. Work in our laboratories has shown that multiple genes probably affect the efficiency of this process[6].

The metastatic paradox may, therefore, by resolved by combining the two hypotheses: metastatic potential is determined early in oncogenesis but primarily by host genetic background (rather than oncogenic mutations), on which specific mutations that promote metastasis then occur. The theory also suggests that some families may be more susceptible to metastasis. If this were carried to its logical extension, the data imply that it might be possible to define metastasis susceptibility based on gene expression in readily accessible tissues (for example, blood) rather than from tumor. This would be a less costly and less invasive method to predict metastatic propensity.



Comparison of gene expression profiles in the mouse and human metastasis signature sets. Gene expression is represented as natural log of the signal intensity ratio either of human primary to human secondary metastases or of mouse low-efficiency to high-efficiency metastatic genotypes. Genes overexpressed in metastatic tumors fall to the left of the center line; those underexpressed fall to the right.

Kent Hunter[1], Danny R. Welch[2] & Edison T. Liu[3]

[1]Laboratory of Population Genetics, Center for Cancer Research, National Cancer Institute, US National Institutes of Health, Building 41, Room D702, 41 Library Drive, Bethesda, Maryland 20892-5060, USA. [2]Department of Pathology and Comprehensive Cancer Center, University of Alabama at Birmingham, 1670 University Blvd., Volker Hall G-038, Birmingham, Alabama 35294-0019, USA. [3]Genome Institute of Singapore, 1 Science Park Road, The Capricorn #05-01, Science Park II, 117528, Singapore. Correspondence should be addressed to K.H. (e-mail: hunterk@mail.nih.gov).

1. Van't Veer, L.J. *et al. Nature* **415**, 530–536 (2002).
2. Ramaswamy, S., Ross, K.N., Lander, E.S. & Golub, T.R. *Nat. Genet.* **33**, 49–54 (2003).
3. Bernards, R. & Weinberg, R.A. *Nature* **418**, 823 (2002).
4. Steeg, P.S. *Nat. Rev. Cancer* **3**, 55–63 (2003).
5. Lifsted, T. *et al. Int. J. Cancer* **77**, 640–644 (1998).
6. Hunter, K.W. *et al. Cancer Res.* **61**, 8866–8872 (2001).
7. Herzig, M. & Christofori, G. *Biochim. Biophys. Acta* **1602**, 97–113 (2002).
8. Ingram, D.K. & Jucker, M. *Neurobiol. Aging* **20**, 137–145 (1999).
9. Raineri, I. *et al. Free Radic. Biol. Med.* **31**, 1018–1030 (2001).

The Small Molecule $\alpha_v\beta_3$ Antagonist (S247) Inhibits MDA-MB-435 Breast Cancer Metastasis to Bone

D. R. Welch, J. F. Harms, R. S. Samant, G. R. Babu, C. V. Gay, A. M. Mastro, H. J. Donahue, D. W. Griggs, J. J. Kotyk, M. D. Pagel, R. K. Rader, W. F. Westlin

Jack Gittlen Cancer Research Institute, Department of Orthopaedics and Rehabilitation, Penn State University College of Medicine, Hershey, Pennsylvania; Department of Biochemistry and Molecular Biology, Penn State University, University Park, Pennsylvania; Discovery Oncology Pharmacology and Analytical Sciences Center, Pharmacia Corporation, St. Louis, Missouri

We developed green fluorescent protein (GFP)-tagged variants of MDA-MB-435 breast carcinoma cell line that, upon injection into the left ventricle of the heart (2×10^5 cells/0.2 mL), form progressively growing, osteolytic bone metastases. Since osteoclast formation of a lytic zone and tumor cell adhesion to matrix could involve the $\alpha_v\beta_3$ integrin, we hypothesized that antagonist of $\alpha_v\beta_3$-vitronectin interactions may block metastasis to bone. S247 is a potent antagonist of purified $\alpha_v\beta_3$ in a solid-phase receptor assay in vitro with an IC_{50} of 0.2 nM for purified $\alpha_v\beta_3$ and is selective against the related $\alpha_{IIb}\beta_3$ integrin (IC_{50} 244 nM). S247 is also a potent antagonist in cell-based assays including adhesion of human $\alpha_v\beta_3$-transfected 293 cells on vitronectin and osteoclast adhesion and actin-ring formation in vitro.

Athymic mice were divided into eight experimental groups: (1) no treatment; (2) vehicle (saline); (3)–(5) treatment with S247 (1, 10, or 100 mg/kg/d using subcutaneous implanted osmotic pumps) beginning 1 week prior; and (6)–(8) treatment with S247 beginning 1 week after tumor cell injection. Mice were euthanized 36 days after tumor cell inoculation. Presence and size of green fluorescent metastatic lesions in bones and viscera was recorded using a stereomicroscope. Femurs and tibia lesions were quantitatively evaluated using new multiple-slice ex vivo MRI methods developed to examine formalin-fixed samples. Relaxation-weighted parameters for these studies were optimized using 7T Varian INOCA microimaging system (~ 80 G/cm gradient insert; 38-mm inner-diameter imaging).

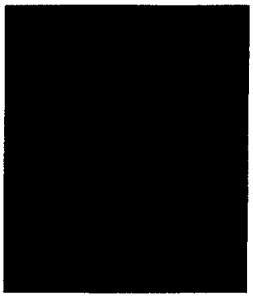
Incidence of femur and tibia metastases was 100% for control groups. For mice treated with S247 prior to tumor cell inoculation, incidence was 67%, 30%, and 27% for the 1, 10, and 100 mg/kg/d groups. For mice treated post tumor cell inoculation, incidence was 75%, 80%, and 75%, respectively.

CONCLUSION: Selective antagonists of o the potential to decrease the incidence of met bone. Since the inhibition was greatest wh was present prior to tumor cell inoculation, in is likely at an early step in bone colonizat adhesion or arrest) rather than at a later s proliferation). Additional studies are underwa derstand the efficacy and to determine whether of bone lesions (ie, lacunae size) is inhibited b These studies suggest that $\alpha_v\beta_3$ antagonists useful in prevention of the formation of bone m as occurs often in cancers of the breast and pr

Expression of RANK and RANKL i Altered in Invasive Carcinoma an Bone Metastasis of Breast Cancer

P. Bhatia, M. Sanders, M. F. Hansen

Molecular Medicine, Department of Pathology, t ty of Connecticut Health Center, Farmington, Conn



P. Bhatia

Bone is the most c site of metastases by breast cancer. Mos cancers form osteoly tastases, in contrast tc such as prostate can form osteosclerotic m es. Although some e suggests that forma bone metastases by cancer cells is medi the increased osteoclastogenesis at the targe clear controversy exists whether formation metastases is mediated by breast cancer cells or by stimulated osteoclasts.

We have therefore examined the expres RANK and RANKL, two proteins importar bone remodeling signaling pathway, in invasi noma of the breast and bone metastases of the br observed that both RANK and RANKL were up ed in these breast tumors and metastases. Furthe tumor cells were directly in contact with t without any osteoclasts in the vicinity.

CONCLUSION: We suggest that overexp of RANK and RANKL in breast cancer cells p a growth advantage to the breast tumor cells, the tumor cells appear to be directly respons the degradation of bone.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Cancer Letters xx (0000) xxx–xxx
CANCER Letters
www.elsevier.com/locate/canlet

Mini Review

Metastasis suppressor pathways—an evolving paradigm

Lalita A. Shevde[a], Danny R. Welch[a,b,*]

[a]*Department of Pathology, 1670 University Boulevard, Volker Hall - G-038, The University of Alabama at Birmingham, Birmingham, AL 35294-0019, USA*
[b]*UAB-Comprehensive Cancer Center, 1670 University Boulevard, Volker Hall - G-038, The University of Alabama at Birmingham, Birmingham, AL 35294-0019, USA*

Received 5 April 2003; accepted 16 April 2003

Abstract

A greater understanding of the processes of tumor invasion and metastasis, the principal cause of death in cancer patients, is essential to determine newer therapeutic targets. Metastasis suppressor genes, by definition, suppress metastasis without affecting tumorigenicity and, hence, present attractive targets as prognostic or therapeutic markers. This short review focuses on those twelve metastasis suppressor genes for which functional data exist. We also outline newly identified genes that bear promising traits of having metastasis suppressor activity, but for which functional data have not been completed. We will also summarize the biochemical mechanism(s) of action (where known), and present a working model assembling potential metastasis suppression pathways.

Keywords: Metastasis; Suppression; Genes; Cancer; Tumor; Tumorigenesis; KISS1; MKK4; BRMS1; CRSP3; TXNIP; E-cadherin; CRMP1; Maspin; CD44; SSeCKS; Nm23; KAI1; TIMPs; DRG-1; Metastin; Multigene; Invasion; Prognosis; Therapeutic; Marker; Target; Anoikis; Proliferation; Apoptosis; Angiogenesis; Neovascularisation

1. Introduction

Despite better local treatments for cancer using surgery and radiotherapy, the clinical challenge remains combating systemic metastatic disease. Metastasis via the lymphatics, hematogenous system, or through the body cavities results in significant morbidity. Not only must cells leave the primary tumor, but they must also proliferate at the secondary site [1,2]. Metastasis culminates the evolution of tumor cells whereby a tumor's composition collectively becomes progressively more malignant [3,4]. Tumor progression results from genetic instability coupled with selection of subpopulations of cells [3]. Eventually some cells accumulate sufficient capacity to dissociate and spread. Depending on whether the mutations occur early or late in tumor progression determines proportions of metastatic cells within tumors of a given size. This conclusion can be appreciated when interpreted in light of classical studies of Luria and Delbrück [5]. Selection of metastatic cells varies with the nature of a tumor as well as between patients. Although it is generally true

* Corresponding author. Address: Department of Pathology, 1670 University Boulevard, Volker Hall -G-038, The University of Alabama at Birmingham, Birmingham, AL 35294-0019, USA. Tel.: +1-205-934-4612; fax: +1-205-934-1775.
E-mail address: dwelch@path.uab.edu (D.R. Welch).

0304-3835/03/$ - see front matter © 2003 Published by Elsevier Science Ireland Ltd.
doi:10.1016/S0304-3835(03)00304-5

that larger tumors are more likely to spread, size does not necessarily correlate with metastatic capacity [6,7]. In addition to accumulating mutations, there are exogenous signals that can influence metastatic efficiency.

2. Host–tumor interactions in neoplastic advancement

Tumorigenicity and metastasis are distinct, but interrelated phenotypes. Tumorigenicity is necessary, but not sufficient, for metastasis. In part, metastasis is also determined, to a great extent, by tumor–host interactions. That is, the microenvironment participates in the induction and selective proliferation of malignant cells [8].

How does the host environment at the metastatic site affect the metastatic behavior of cells? The relationship is reciprocal, and reflects both host endocrine and immunologic status. Host physiology can foster or reject neoplastic cells. In response to tumor-secreted cytokines and chemokines, diverse leukocyte populations are recruited including neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes. All inflammatory cells can produce a plethora of cytokines, proteases (e.g. MMPs), membrane-perforating agents and soluble cytotoxic mediators (e.g. TNF-α, interleukins and interferons) ([9]). For example, tumor-associated macrophages, play a dual role in tumor development. They can kill neoplastic cells following activation by IL-2, IL-12 and interferons; but they can also induce angiogenesis by growth factor, cytokine and proteinase secretion [9]. Indeed proteinases in the tumor milieu are largely stroma-derived [10]. Thus, metastatic tumor cells can modify the host environment so that tumor cells are nurtured.

Tumor–host interactions formed the basis of Sir Steven Paget's 'seed and soil' theory [11] to explain the predilection of breast cancer spread to bone. He proposed that the tumor cells (seed) are scattered in many directions by the circulatory system, but grow only in response to the microenvironments of specific organs (soil). While this review focuses on metastasis genes (i.e. in the seed), we emphasize that the regulation of those genes by the host cannot be ignored. That is, the context in which the genes function must be considered, even though the details are not yet known.

3. Stochastic and selective aspects of cancer metastasis

In order to metastasize, cells must complete a series of sequential steps, each of which is rate-limiting. Following primary tumor growth (including establishment of neovasculature or primitive vascular channels [12,13]), tumor cells detach and enter a circulatory compartment. The tumor vasculature is immature and incontinent [14], providing easier access to the vasculature. Once there, tumor cells can remain as single cells or form homo- or heterotypic emboli but they must survive shear forces as well. At the secondary site, tumor cells can arrest due to size restriction or become tethered to vascular endothelium using a variety of surface adhesion molecules. In some cases, tumor cells recognize endothelial addressins—surface molecules that designate the cells as from a particular organ, tissue or vessel structure [15–18]. Additionally, tumor cells can respond to chemoattractants produced by different tissues [9,19]. For the most part, the identity of the attractants are not yet known [20], but recent data implicate chemokines [9,21–23]. Depending upon tumor type and the tissue in which the tumor cells have arrested, cells can begin to proliferate within the vasculature or extravasate before proliferating [24–28]. Merely getting to the secondary site does not constitute a metastasis. Metastases are defined as secondary *masses*.

Overall, the process of metastasis is quite inefficient [29,30]. Cells in the vasculature are cleared biphasically [29,31]. The initial phase (6–24 h), represents an exponential decline of cell number, presumably due to mechanical trauma, oxygen toxicity, anoikis and immune clearance. A second, more gradual decline, presumably represents cell death at secondary sites [29]. Tumor cells that arrive at a second site do not necessarily proliferate immediately. Some cells may remain 'dormant' for extended periods or until conditions become favorable for proliferation [32–35].

Dormancy of pre-angiogenic metastases is more accurately described as a balance between

proliferation and apoptosis [36]. Wong et al. [37] found that the majority of cells underwent apoptosis within 24 h of intravasation. If apoptosis was inhibited, metastatic potential increased. In contrast, Luzzi et al. [33], and Cameron et al. [38] found that most cells survived, but failed to proliferate. It is not yet possible to reconcile these two apparently conflicting conclusions. However, since the tumor cells and host tissue were not identical and since the data are not mutually exclusive, it is likely that both are correct. It is probable that the rate-limiting steps of metastasis will vary by cell lines and in different tissues, reflecting yet another level of heterogeneity within tumors.

Technical advances have made it possible to detect single cancer cells or microscopic foci in experimental models [39–42]. If model data are extrapolated to the clinical setting, diagnosis and treatment decisions become significantly more complex. The issue is whether microscopic foci justify aggressive treatment because of their potential to grow into overt lesions. Or, if the percentage of cells that eventually proliferate is vanishingly small, should patients be spared toxic chemotherapy since the mere detection of cell clusters at a secondary site does not necessarily translate into establishment of macroscopic metastases?

Considerations such as these underscore the need for markers that can be used to accurately and definitively predict metastatic potential (in this case, defined as the possibility of forming macroscopic metastases) [43]. New technologies such as microdissection, microarray, real-time RT-PCR, proteomics and comparative genomic hybridization (CGH) are being evaluated to define and characterize metastatic potential of cancer specimens [44–53]. Identifying molecules that are specifically involved in metastasis (as opposed to indirect changes in gene expression due to tumor progression) presents a daunting challenge as well as significant opportunity. The difficulty relates to discriminating between mere association from causality [2,43,54–57]. Metastasis suppressor genes are attractive candidates for marker development because, by definition, their loss should be associated with the acquisition of metastatic potential [58]. Moreover, they represent potential therapeutic targets.

We emphasize that, while it takes a finely orchestrated set functions to metastasize, blockage of even one step halts the process. Since the discovery of the first metastasis suppressor gene, *nm23*, more than a decade ago, the number of metastasis suppressors identified has grown significantly (reviewed in Ref. [2]).

Various studies involving CGH, loss of heterozygosity (LOH) and karyotype analysis identified distinctively altered regions and/or genomic imbalances involving various human chromosomes [55]. Some changes correlated temporally with acquisition of metastatic propensity. By inference, then, those chromosomal regions were thought to predict the location(s) for metastasis-associated genes. In the case of genetic loss, replacement of the chromosomes by microcell-mediated transfer (MMCT) was predicted to suppress metastasis. MMCT has been instrumental in identifying several metastasis suppressor genes.

MMCT of chromosomes 2, 7, 8, 10, 11, 12, 13, 16, 17 and 20 suppressed metastasis of prostate carcinoma cells without blocking tumorigenicity (reviewed in Ref. [59]). By positional cloning regions on chromosome 17 were narrowed to an ~70 cM [60]. Yoshida et al. [34] eventually cloned the MKK4 metastasis suppressor gene. Details regarding individual genes will be provided below. The identities of the invasion-suppressing genes with regard to metastasis suppression have not been as easily forthcoming. Importantly, inhibition of invasion (unless *completely* inhibited) does not necessarily suppress metastasis. While invasion is required for metastasis, tumor cells must merely be able to accomplish the step [43,56,61,62]. They do not have to be extraordinarily efficient at component processes.

Structural alterations involving chromosome 6 are frequent in metastatic melanoma [63]. MMCT of full-length human chromosome 6 suppressed metastasis of the human metastatic melanoma cell line C8161 [64,65]. Chromosome 6 hybrids were less motile, but just as invasive [66]. Chromosome 6 hybrids engineered to express green fluorescent protein were used to demonstrate that they completed every step of the metastatic cascade except proliferation at the secondary site [67]. Using subtractive hybridization the *KISS-1* metastasis suppressor was identified [68]. Also using the C8161 melanoma, MMCT of chromosome 1 suppressed metastasis [69].

Alterations of chromosome 11 in metastatic breast carcinoma are well documented [51]. Following

MMCT of chromosome 11 into the metastatic human breast carcinoma cell line, MDA-MB-435, hybrids were significantly suppressed for lung and lymph node metastasis [70].

MMCT has been the most lucrative technique for identifying metastasis suppressors. However, other approaches (subtractive hybridization, differential display and microarrays) have been used successfully and their frequency of identification is rapidly growing.

4. NM23

By screening cDNA libraries of matched metastatic/non-metastatic K1735 murine melanoma cell lines by differential hybridization, 'non-metastatic clone 23' gene, was identified as the first metastasis suppressor gene [71]. Enforced expression in cell lines of diverse cellular origin, suppressed metastasis without altering tumor growth (reviewed in Ref. [72]). The product of the human ortholog, *NM23-H1*, was identified to be a nucleoside diphosphate kinase (NDPK). NDPKs catalyze the transphosphorylation of the γ-phosphate of a deoxynucleoside triphosphate to a deoxynucleoside diphosphate with the formation of a histidine-phosphorylated intermediate. The *Drosophila* nm23 ortholog, *awd*, is required for proper differentiation of tissues of epithelial origin (reviewed in Ref. [73]). To date, eight NM23 family members have been identified, designated *NME1* through *NME8*. Of these, *NM23-H1* and *NM23-H2* have reported metastasis suppressor activity, but NDPK activity has been dissociated from metastasis suppression [74]. Postel and colleagues identified Nm23-H2 as a PuF, a transcription-promoting factor of the *c-myc* gene [75].

Protein–protein and other Nm23 interaction studies have been complicated by the 'sticky' nature of the molecule, making it difficult to establish specificity [72]. Yet, building upon previous experiments in which histidine kinase activity of NM23 was correlated with reduced metastasis [76], Hartsough et al., showed that Nm23 immunoprecipitated kinase suppressor of Ras (KSR) [77]. KSR is a scaffold protein for the mitogen activated protein kinase (MAPK) cascade. Nm23 is phosphorylated KSR at serine 392, a 14-3-3-binding site. This, coupled with observations that Nm23 transfected MDA-MB-435 cells had lower levels of phosphorylated MAPK led to the conclusion that Nm23 signals through the ERK-MAPK pathway [78,79]. Numerous papers have documented signaling through the Ras-ERK-MAPK as important in metastasis. Therefore the KSR result is especially intriguing.

Another interesting interaction involving Nm23-H1 was recently described by Fan et al. [80]. They provide evidence that Nm23-H1 interacts with granzyme A in the process of DNA damage induction in cytotoxic T-cell apoptosis. The mechanism has not been demonstrated in tumor cells; however, the association relates to the NDPK activity of Nm23's and may offer an alternative mechanism for metastasis suppression.

Clinical studies assessing Nm23 as a marker for metastasis were recently reviewed [72]. Briefly, decreased expression (as would be expected for a metastasis suppressor) correlated in many, but not in all cancers. Higher expression in neuroblastoma correlated with aggressiveness. A few studies found no correlation with metastasis. Interpretation is sometimes complicated because each study used different antibodies and involved different criteria. Thus, Nm23 has shown promise for some cancer types, but is not yet considered an independent prognostic factor.

5. KAI-1 (CD82)

KAI-1 was identified in prostate cancer cell lines (Dunning rat AT3.1 and AT6.1) that were suppressed for metastasis following introduction of human chromosome 11 [81]. Positional cloning mapped KAI1 to 11p11.2 [82].

KAI-1 is an evolutionarily conserved member of the tetraspanin transmembrane protein family of leukocyte surface glycoproteins. It is the only tetraspanin with an internalization sequence at the C-terminus [83]. Although no allelic losses were seen, expression in the epithelial compartment was consistently down-regulated during prostate cancer progression [84]. Expression also inversely correlated with breast cancer metastasis [85]. Enforced constitutive expression suppressed metastasis of breast cancer [86] and melanoma [87]. Additionally *KAI1*

inhibited key steps in metastasis (i.e. invasion and motility) of colon cancer cells [88].

There are contradicting reports [89,90] regarding interactions between p53 the *KAI1* promoter following identification of a p53-consensus binding sequence. There is evidence of *KAI1* epigenetic regulation by methylation of CpG islands in the promoter [91]. The mechanism of action is enigmatic, in part, because KAI1 functions as an adhesion molecule on leucocytes, but does dramatically influence adhesion in tumor cells. So, other mechanisms have been proposed. KAI1 directly associates with the EGF receptor and suppresses induced lamellipodia and migration signaling [92]. Attenuation of EGF-induced signaling is accomplished by ligand-induced receptor endocytosis. Thus, KAI1 might suppress metastasis by altering the balance between KAI1 and EGFR, which might affect proliferative and migratory signals delivered. KAI1 also associates with the cytoskeleton promoting phosphorylation and association of both the guanine exchange factor Vav and the adaptor protein SLP76 leading to *de novo* actin polymerization [93]. Involvement of Rho GTPases in KAI1 signaling brings to the forefront additional pathways in KAI1 signaling.

Immunohistochemical detection of *KAI1* correlated inversely with metastasis in many different cancers [59]. Down-regulation of KAI1 was also seen in cancer lines of urogenital, gynecological, and pulmonary origin [94].

6. KISS-1, TXNIP and CRSP3

KISS-1 was identified as a melanoma metastasis suppressor using subtractive hybridization to compare chromosome 6 metastasis-suppressed melanoma hybrids with metastatic parental cells [68,95]. Surprisingly, the *KISS-1* gene mapped to the long arm of chromosome 1 [68]. Enforced expression of *KISS-1* suppressed metastasis of melanoma and breast carcinoma [96]. A deletion variant (neo6qdel; neo6-del(q16.3-q23)) of neomycin-tagged human chromosome 6 did not suppress metastasis and did not express *KISS1* [97]. Therefore, it was hypothesized that regulators of *KISS-1* were encoded on chromosome 6.

Ultimately, the mechanism of action of *KISS-1* remains unknown. Research has been stymied by an apparently short protein half-life. However, three groups studying an orphan G-protein coupled receptor (GPR54, hOT7T175, AXOR12) identified a fragment of *KISS-1* as the ligand [98–100]. KISS-1 fragments were named metastin [100] and Kisspeptins [98]. The functional peptides were amidated [100]. Ligand binding initiates hydrolysis of (PIP2) and Ca^{+2} mobilization and arachidonate release. ERK1/2 and $p38^{MAPK}$ phosphorylation have also been observed concomitant with cytoskeletal changes [98–102]. Boyd and colleagues showed that constitutive up-regulation of *KISS-1* in HT10810 cells resulted in decreased NFκB activation which, in turn, led to diminution of MMP-9 transcription [103].

While Ohtaki and colleagues showed elegant data showing that exogenous Metastin/Kisspeptin treatment of receptor-transfected B16–BL6 melanoma reduced metastasis and anchorage-independent growth [100], activity of the endogenous receptor has not been demonstrated to date in cancer cells. Likewise, endogenous receptor expression and mutation analysis still need to be done to firmly establish a connection with melanoma metastasis.

The normal physiological function(s) of *KISS-1* (and its receptor) are only recently becoming elucidated. *KISS-1* levels are higher in early placenta and molar pregnancies and are reduced in choriocarcinoma cells, favoring a predominant role in the control of the invasive and migratory properties of trophoblast cells [104].

A clinical role for KISS-1 was inferred by the experimental studies showing metastasis suppression. The following issues have made it difficult to complete a detailed study-lack of antibodies/antisera recognizing KISS-1 or Metastin/Kisspeptin; lack of reagents recognizing receptor; and short life span of the nascent protein. Nonetheless, Shirasaki and colleagues used in situ hybridization to examine *KISS-1* expression in clinical melanoma samples [105]. As expected, an inverse correlation of *KISS-1* with malignancy were found. While carefully performed, information regarding KISS-1 processing or the receptors was not possible in those studies. Importantly, the studies compared LOH on 6q loci with *KISS-1* expression [105]. The clinical studies corroborated the experimental MMCT data linking loci between 6q16.3-q23. Murine orthologs of metastin and GPR54 were used to demonstrate activation of phospholipase C following ligand binding [102].

Recently, Goldberg et al., identified two molecules (TXNIP and CRSP3) that appear to function upstream of *KISS-1* [53]. Briefly, paired microarrays compared metastatic C8161 and non-metastatic neo6/C8161 cells. Also, metastatic neo6qdel/C8161 cells were compared to neo6/C8161. The gene with greatest differential expression in both arrays was VDUP1 (Vitamin D3 upregulated protein 1). VDUP1 was first identified in HeLa cells by differential display following treatment with 1,25-dihydroxyvitamin-D3 [106]. Subsequently it was identified as an interactor of thioredoxin (TRN) in a yeast two-hybrid screen and is also known as TBP2 (TRN binding protein 2) and TXNIP (TRN-interacting protein, preferred name). TRN is a redox- signal regulating protein [107] and regulates stress-response MAPK signaling via suppression of the apoptosis signal-regulating kinase 1 (ASK1) activation and also activation of transcription factors. TXNIP binds to the reduced form of TRN to inhibit function and expression [108,109]. TXNIP also regulates stress-response apoptosis signal transduction [110,111]. Concomitant with increased *TXNIP* expression is decreased expression of TRN and arrest of cell growth [112]. Based upon trends toward increased TRN in many tumors and cell lines, TXNIP may have tumor suppressor effects as well.

CRSP3 encodes a co-factor required for SP1-mediated activation of transcription. Sp1 (Specificity protein 1) is a general transcription factor that binds to and acts through GC-boxes, widely distributed promoter elements [113,114]. *CRSP3* has no known yeast or murine orthologs [115]. Definitive clinical studies have not yet been done, but *CRSP3* and *TXNIP* expression, generally inversely correlate with melanoma progression. Additionally, sequence tagged sites adjacent to *CRSP3* in patient samples [105] suggest that the gene may indeed show changes associated with clinical outcome.

7. TIMPs

Tissue inhibitors of metalloproteinases (TIMPs) are a family of secreted proteins that selectively, but reversibly, inhibit metalloproteinases (MMPs) with 1:1 stoichiometry [10,116,117]. Modulation of MMP and TIMP levels is critical to the control of extravasation and tumor-induced angiogenesis, processes that involve basement membrane degradation. Paradoxically, TIMP-1, 2 and 4 have an anti-apoptotic effect, while TIMP-3 induces apoptosis. TIMP-2, in concert with MT1-MMP can bind to and activate proMMP-2 (reviewed in Ref. [116]). Although there are no known TIMP-specific receptors, membrane-bound molecules such as MT-MMPs and metalloproteinase disintegrins (ADAMs) serve as TIMP-binding molecules at the cell surface [117].

TIMPs are expressed in tumor tissues and are present in the sera of cancer patients, raising the possibility that TIMP levels could predict clinical outcome and risk of metastasis [118–121]. But results are complicated because the ratio of TIMPs to MMPs is the crucial parameter. Nonetheless, the possibility that serum TIMP levels could be useful in a clinical setting remains. Gene therapy studies for local or systemic delivery of TIMPs are in an exploratory phase (reviewed in Ref. [122]).

8. Cadherins

Cadherins are transmembrane glycoproteins responsible for Ca^{+2}-dependent cell adhesion. Although the family is widely expressed, E-cadherin (gene designation CAD1) is expressed on epithelial cells. A precursor protein (135 kDa) is processed to a mature 120 kDa form. The extracellular N-terminus is critical for homophilic Ca^{+2}-dependent cell–cell adhesion. The C-terminus interacts with β-catenin to mediate actin binding. E-cadherin/β-catenin binding sequesters the latter, blocking nuclear translocation and transcription of *c-myc* and *cyclin D1*.

Defining a role for E-cadherin as a metastasis suppressor is complicated. Over-expression decreases motility and invasiveness [123]. Mutations of CAD1 and α-catenin have been associated with invasion [124]. High E-cadherin levels inhibit shedding of tumor cells from the primary tumor; thus, CAD1 is a metastasis-suppressor [124–126]. However, CAD1 can also be a tumor suppressor [124,125,127]. Loss of expression occurs in many tumors and is directly associated with loss of differentiation (reviewed in Ref. [128]). Mechanisms of reduced expression include: reduction or loss of E-cadherin expression (by LOH or epigenetic silencing [129]), redistribution to different sites within the cell, shedding of E-cadherin

and competition from other proteins (reviewed in [130]). Stimulation of the EGFR by EGF, TGF-β or PP2 brings about phosphorylation of E-cadherin and β-catenin resulting in dissociation of the complex [131,132]. Other than breast and gastric cancers, with nearly 50% of the tumors affected, mutations of *CAD1* appear to be infrequent [133]. Evidence supports a role of E-cadherin in tumor suppression rather than just being an epiphenomenon of the tumor cells' phenotypic changes [134]. Since loss of E-cadherin alone, leading to decreased cell–cell adhesion is insufficient for the tumor cells to invade, it appears more than likely that down-regulation actively transduces specific signals that support tumor invasion.

Recently, Kashima et al., showed that N-cadherin and cadherin-11 (osteoblast cadherin), which are both highly expressed in osteoblasts (bone forming cells), reduce metastasis to lungs without negatively affecting tumorigenicity [135]. Reduced motility was presumably the mechanism responsible for diminished metastasis. Curiously, N-cadherin and cadherin-11 are frequently over-expressed in many metastatic breast and prostatic carcinoma cells [136–138]. Moreover, transfection and over-expression promotes invasion and metastasis in breast and melanoma cells [136,139,140]. These results highlight the complexities of interpretation because of cell origin. They further reinforce the point raised above—gene context is important.

9. MKK4

MKK4/JNKK1/SEK1 is a mitogen-activated protein kinase, which transduces signals from MEKK1 to stress-activated protein kinase/JNK1 and $p38^{MAPK}$ [59]. MKK4 transmits stress signals to nuclear transcription factors that mediate proliferation, apoptosis and differentiation. Portions of the *MKK4* gene (on chromosome 17) were deleted or altered in cancer cell lines that displayed defects in signal transduction from MEKK1 [141]. Suppression of prostate cancer cell metastasis was brought about by over-expressed *MKK4* [142]. An inverse relationship between Gleason score and MKK4 staining was established in prostate tumors [143]. *MKK4* is also a metastasis suppressor in ovarian carcinomas [144].

10. BRMS1

Following upon MMCT studies, Seraj et al., performed differential display to identify the gene(s) responsible for chromosome 11 suppression of breast cancer metastasis. Three novel cDNAs were identified. *BRMS1* suppressed metastasis in MDA-MB-231 and MDA-MB-435 [145] breast carcinomas in addition to two human melanoma (C8161 and MelJuSo, [146]) and two murine mammary carcinoma cell lines (4T1 and 66cl4 [147]). *BRMS1* transfectants were not suppressed for growth in vitro or in vivo; adhesion to extracellular components (LN, FN, collagens I or IV, Matrigel); expression of gelatinases (MMP-2, MMP-9) or heparanase, or invasion in vitro [148].

The *BRMS1* gene mapped to human chromosome 11q13.1-q13.2, a region frequently altered in metastatic breast cancer. Expression of other metastasis suppressors (i.e. *NM23*, *KAI-1*, *KISS-1*, *CAD1*) did not correlate with *BRMS1*. Motility was moderately reduced in wound assays as was the ability to grow in soft agar. The most striking change amongst transfectants was restoration of gap junctional intercellular communications (GJIC) [148,149], accompanied by increased expression of connexin (Cx) 43 and decreased expression of Cx32 [150]. Connexins are the protein subunits of gap junctions and the expression pattern in *BRMS1* transfectants more closely mimics normal breast tissue. Using real time RT-PCR, *BRMS1* expression inversely correlated with metastasis in human melanoma cells [146]. Expression of *BRMS1* also reduced T24T, metastatic the human bladder carcinoma metastasis, T24 [151]. Although a role in normal physiology has not been determined, *BRMS1* does not appear to regulate invasive and/or migratory properties of trophoblast cells [104]. BRMS1 RNA expression was detected in villous cytotrophoblasts, but the level in invasive cytotrophoblasts, the subclass of trophoblast cells that invades into the decidua was not examined, thus warranting prudence in interpreting the data.

Hunter and colleagues [152,153] using a genetic approach to identify factors predisposing to metastatic disease, co-localized the *Brms1* gene with the *Mtes1* (*Metastasis Efficiency Suppressor 1*) locus on chromosome 19 (orthologous to human chromosome 11). Later studies utilizing comparative sequence

analysis, however, suggest that *Brms1* is not likely *Mtes1* [152,154].

11. SSeCKS

SSeCKS (pronounced essex) for Src-suppressed C kinase substrate expression is down-regulated in *src*- and *ras*-transformed rodent fibroblasts [155,156]. It is the likely rodent ortholog of human Gravin/AKAP12, a cytoplasmic scaffolding protein for protein kinases A and C [157], concentrating at the cell edge and podosomes. In response to phorbol esters, *SSeCKS* controls elaboration of a cortical cytoskeletal matrix. Over-expression suppresses v-src-induced morphological transformation and tumorigenesis. ERK2 activity was induced 5- to 10-fold in presence of v-src [158], resulting in decreased cyclin D1 expression and pRb phosphorylation, thereby playing a role cell cycle progression [158,159]. While SSeCKS/Gravin protein is detected in untransformed rat and human prostate epithelial cell lines, expression is severely reduced in metastatic prostate carcinoma cell lines. Re-expression significantly decreased lung metastases, induced filopodia-like projections and decreased anchorage-independent growth [160] in vitro.

12. RhoGDI2

Rho GTPases are guanine nucleotide binding proteins, which cycle between active GTP-bound state and inactive GDP-bound state. RhoGDI (Rho *GDP dissociation inhibitors*) stabilize the GDP-bound form and sequester them in an inactive non-membrane localized, cytoplasmic compartment [161]. In an earlier bladder carcinoma study, RNA expression of RhoGDI2 was associated with decreased metastatic potential [151]. Transfection and enforced expression suppressed metastasis of T24 human bladder carcinoma variants [162]. Gene expression profiling of 105 bladder carcinomas, corroborated the expression pattern—i.e. *RhoGDI2* expression correlated inversely with the invasive phenotype of tumors.

13. Drg-1

Drg-1 (a.k.a. RTP, cap43 and rit42) was identified as a differentiation-associated gene in colon carcinomas by differential display [163]. It is orthologous to mouse TDD45 and Ndr1 and rat Bdm1. Kurdistani and colleagues showed that introduction of *Drg-1* cDNA suppressed tumorigenicity of human bladder carcinoma cells, suggesting that *Drg-1* is a tumor suppressor gene [164]. However, in vitro invasion and liver metastases are inhibited from colorectal carcinomas when expression is restored either by inhibiting DNA methylation or by transfection [165]. Likewise, Bandopadhyay et al., recently showed that prostate carcinoma cells are suppressed for metastasis, but not tumorigenicity, when Drg-1 is over-expressed [166]. The latter studies support the contention that *Drg-1* is a metastasis suppressor.

Drg-1 expression inversely correlated with Gleason score in human prostate cancer specimens [166]. While the mechanism of action of *Drg-1* is unknown, it is up-regulated by PTEN and p53 and phosphorylated by Protein Kinase A [167]. It is postulated that Drg-1 might function downstream of MKK4, since it is induced similarly to the stress activated protein kinases (JNK/SAPK) [168] via MKK4, itself a metastasis-suppressor.

14. Metastasis suppressors without functional portfolio

The above genes have functional evidence supporting classification as metastasis suppressors. We will briefly describe below several others whose evidence is suggestive, but the data are deficient with regard to classification as metastasis suppressors for two reasons. First, the data are at this time correlative, not functional. Second, functional suppression of metastasis occurs concurrent with diminished tumorigenicity. In the absence of experimental arms to accommodate differential growth rates and detailed analysis to verify expression, designation as metastasis suppressors by the strict definition is not possible.

Responding to environmental and growth stimuli, axons extend growth cones in several directions. *Semaphorins*, a large family of secreted

and membrane-bound proteins participate in a repulsive (collapse) process [169,170]. CRMP proteins aid intracellular transduction of collapse signals [171]. *CRMP-1*, for *Collapsin Response Mediator Protein-1*, is one of five proteins in the CRMP family, whose molecular mechanisms have not yet been characterized, although recent literature implicates involvement in controlling cell movement (reviewed in Ref. [172]). Recently, CRMP-1 was shown to reduce invasion of lung cancer cells [51]. Shih et al., demonstrated that CRMP-1 expression was inverse to lung carcinoma grade. Expression correlated directly with survival and time to relapse.

Gelsolin modulates actin assembly and disassembly to regulate motility. It also inhibits apoptosis [173]. Gelsolin decreases colonization in soft agar, retards spread, reduces chemotaxis to fibronectin and suppresses both tumorigenicity and metastasis of melanoma [174], bladder carcinoma [175] and lung carcinoma [176].

Following identification by DD-RT-PCR comparing normal mammary epithelium and invasive mammary carcinoma cells, *maspin* (mammary serine protease inhibitor) was reported to suppress invasion and metastasis (but no metastasis data was shown in the original paper). Complicating interpretation, tumorigenicity and growth were also reduced. [177]. The gene, *SERPINB5*, is a member of the serine protease inhibitor (serpin) gene cluster on chromosome 18q21.3. Maspin transgenic mice show attenuated tumor progression and metastasis, supporting its role against tumor spread [178]. Mechanistically, maspin also sensitizes cells to induced apoptosis [179] and reduces angiogenesis [180]. Expression of maspin is controlled at several levels. Futscher et al. [181] showed that cell-type specific expression of maspin inversely correlated with methylation of *SERPINB5*. *SERPINB5* expression can be surmounted by treatment with 5-aza-2′-deoxycytidine [182]. Regulation of maspin by p53 has also been reported using EMSA [183].

Heterochromatin-associated protein 1 ($HP1^{HS\alpha}$) expression is down-regulated in highly invasive metastatic cells compared to non-metastatic cells where it is predominantly localized in the nucleus. Although the clinical correlations show promise as a metastasis suppressor HP1 in breast carcinoma [184], no data functional evidence for metastasis suppression are yet available.

Data for *CD44* as a metastasis suppressor are controversial. Gao et al., showed CD44 to have metastasis suppressor activity in AT3.1 prostate carcinoma cells, without altering tumorigenicity [185]. Complexity exists because CD44, which encodes a membrane protein that binds the extracellular membrane components hyaluronic acid and osteopontin exists in multiple isoforms. The standard isoform, CD44-s, significantly (>60%) reduces lung metastases, but it is still not certain which are the most relevant isoforms for cancer and metastasis. Reagents to study the role(s) of particular isoforms in tumorigenicity and/or metastasis are under development. Until then, CD44 data should be interpreted cautiously.

SHP-2 is a widely expressed cytoplasmic tyrosine phosphatase that is believed to participate in signal relay downstream of growth factor receptors. SHP-2 impairs spreading of fibroblasts on fibronectin and migration (in vitro) [186]. Cells expressing mutant SHP-2 display reduced focal adhesion kinase dephosphorylation as well as decreased association with paxillin. In vivo demonstration of metastasis suppression remains to be done.

15. Remaining questions and perspectives

The critical clinical threshold for any cancer is development of metastasis. Diagnosis occurring prior to the establishment of secondary lesions means favorable prognosis and more effective treatment. As a result, earlier, more effective diagnosis has been instrumental in improving cure rates for cancer.

Unfortunately, there are many cases in which there is no evidence of cancer spread at the time of diagnosis. Treatment plans are usually based upon somewhat subjective morphologic criteria in tissue specimens submitted to the pathologist. In the case of breast cancer, approximately 25% of node-negative patients develop metastases despite being designated 'metastasis negative' at the time of diagnosis. What can be done to identify the patients whose cancers are likely to spread and those whose cancers are unlikely to form secondary lesions? The answer depends upon

a thorough understanding of the underlying genetic and biochemical basis of metastasis.

While it is not yet known how, or whether, metastasis suppressor genes will play a role in predicting the propensity to metastasize in clinical cancer, information gained by understanding the mechanisms of action of the metastasis suppressors is providing insight into the fundamental mechanisms controlling cancer spread. The metastasis suppressors identified in Table 1 and Fig. 1 were discovered in several laboratories, using different model systems, and tested using distinct experimental systems. There is variability in terms of understanding mechanism and with regard to clinical evaluation. Nonetheless, the pieces to a complex jigsaw puzzle are beginning to take form. Pathways are beginning to emerge that connect heretofore independent metastasis suppressors. The picture is still sketchy; but some common elements are apparent.

First, many metastasis suppressors have functions that amplify 'signals' (i.e. there are several branches downstream in each signaling arbor). This situation is highly desirable for controlling complex, multigenic phenotypes like metastasis. Second, metastasis suppressors exist within all cellular compartments. The situation is reminiscent of the genes controlling cell cycle, apoptosis, and differentiation. The expectation (hope?) is that, like the cell cycle genes, some higher order will become evident as the regulatory molecules are put into pathways. Moreover, it is hoped that key rate-limiting steps will be identified. Third, many metastasis suppressors function in diverse cell types (i.e. genes discovered in one tumor type also suppress metastasis in cells of other origins). Fourth, despite use of a strict definition of metastasis suppression (i.e. demonstration of a functional suppression of metastasis without inhibition of tumor formation), the number of metastasis suppressor genes is continuing to grow. How many metastasis suppressor genes are there? We do not know. Based upon similarly highly regulated phenotypes, we would predict that the number is limited within the core regulatory pathway(s). The complexity is daunting if alterations downstream are also counted.

Table 1
Characteristics of metastasis suppressor genes

Gene	Method of discovery[a]	In vitro characterization[b]				In vivo characterization		
		Soft agar colonization	Motility	Invasion	Adhesion to ECM components	Tumor growth	Metastasis	Clinical specimens
BRMS1	MMCT/DD	↔	↓	↓	↓	↔	↓	
CAD1	Clin		↓		↓	↔	↓↑	↓
Cadherin-11	MA		↓			↔	↓↑	
CD44-s	MMCT					↔	↓	↓
CRMP-1	MA			↓		↔		↓
CRSP3	MMCT/MA					↔	↓	
Drg-1	DD	↔	↓	↓	↓	↔	↓/↔	↓
Gelsolin	Clin	↓				↓	↓	
$HP1^{HS}\alpha$	Clin			↓				↓
KAI-1	SH	↓	↓	↓	↔	↓	↓	↓
KISS-1	MMCT/SH	↓	↓	↓	↔	↔	↓	↓
MKK4	MMCT/PC					↔	↓	
N-cadherin	MA					↔	↓↑	
NM23	SH	↓	↓	↓	↓	↔	↓	↓↑
RhoGDI2	MA			↓		↔	↓	↓
SERPINB5	DD		↓	↓		↓	↓	
TXNIP	MMCT/MA				↔	↔	↓	↓

[a] The method of discovery is abbreviated: Clin, clinical correlation; DD, differential display; MA, microarray; MMCT/DD, microcell-mediated chromosome transfer + differential display; or SH, subtractive hybridization.

[b] Arrows depict direction of change in behavior or expression (in clinical samples). (↔) depicts no consistent change. Fields left blank indicate that the experiments have not yet been done or have not been reported.

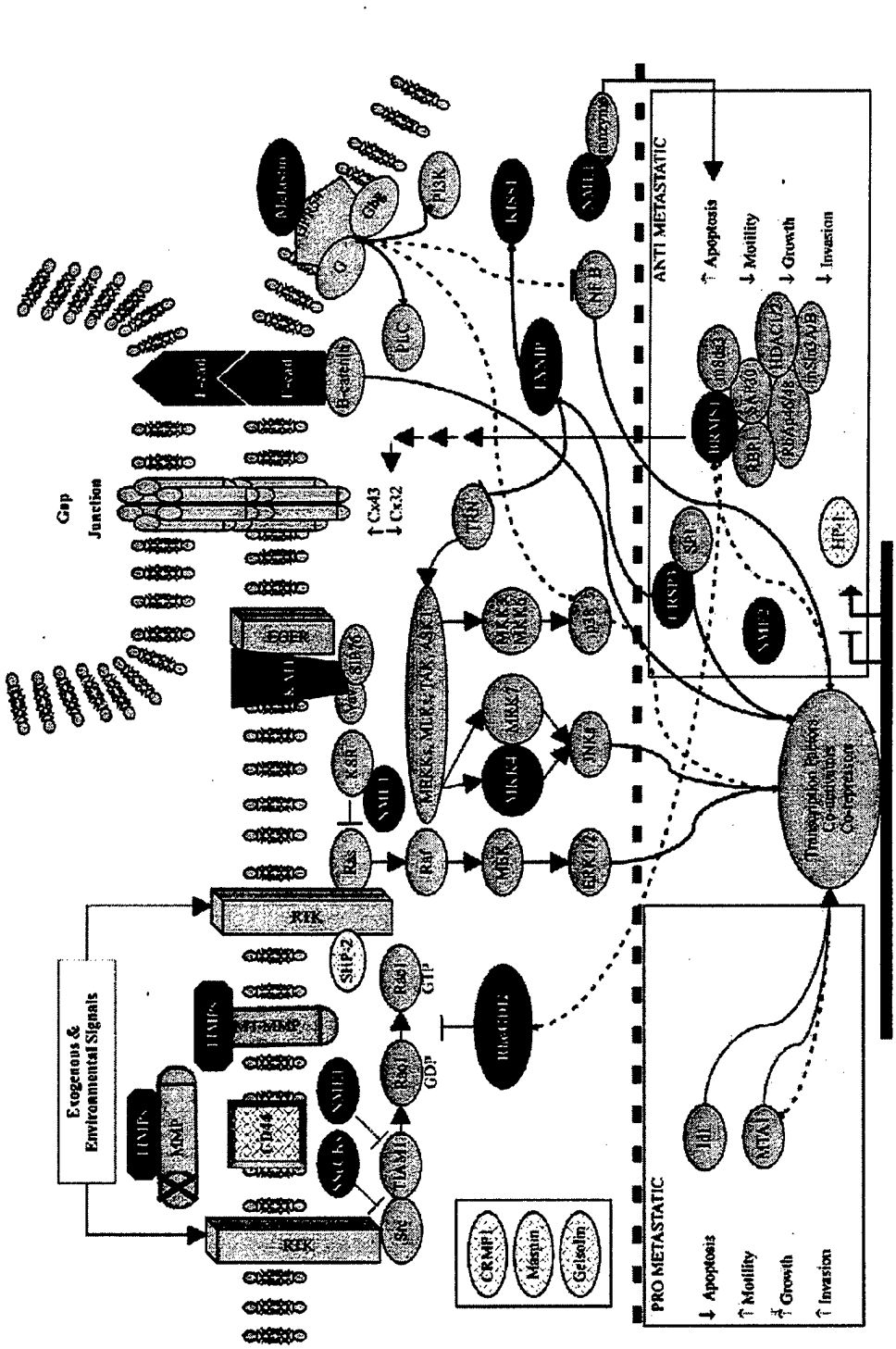


Fig. 1. Proposed model of pathways of metastasis suppression. Metastasis suppressor genes are featured in solid black shapes. Solid lines indicate pathways for which biochemical evidence has been provided. The dotted lines represent inferred/implied pathways. Putative metastasis suppressors are stippled. If the location of the proteins are known, they are placed. If the location or functional subcellular compartment are not definitively known, the proteins are placed in the box at the left. Some connections are omitted to simplify the figure. The shadowed boxes positioned within the nucleus highlight the existence of pro- and anti-metastatic genes involved in transcription. Two molecules are highlighted with regard to promoting metastasis, Id1 [199,200] and MTA1 [201,202].

The field of metastasis genetics and the existence of genes that specifically control metastasis has been called into question by some [6,7]. Yet, functional data with the metastasis suppressor genes strongly argue that there are specific genes controlling metastasis.

Our colleague, Kent Hunter has collected some very important data that support the existence of metastasis genes using breeding strategies in mice. Using a transgene-induced mouse mammary tumor model (MMTV-PyMT), mice were crossed with mice of varying genetic backgrounds. Significant differences in metastasis were found despite failure to alter tumor initiation or growth kinetics in some strains. Since all of the mouse tumors were initiated by the same oncogenic event, the differences in metastasis and gene expression are most likely due to genetic background. His data reinforce a notion that we introduced earlier—gene context is an important parameter in determining metastatic potential.

Further contributing to the argument that microenvironment is important are observations from multiple laboratories showing that many metastasis suppressors act at the terminal steps of the metastatic cascade, i.e. proliferation at the secondary site [34,67, 187]. In studies from our laboratory, we have showed, that tumor cells proliferated in some sites (i.e. orthotopic) but not others (i.e. metastatic). Furthermore, we have preliminary evidence that some metastasis suppressor genes suppress colonization in some organs, but not others (J.F. Harms and D.R. Welch unpublished). Much more work will be required to understand the interplay between metastasis-controlling genes and microenvironment; however, the importance of cellular context cannot be overstated.

An issue that has stymied the field for several years is the imprecise use of terminology. Even a cursory look at the literature finds numerous papers that claim suppression of metastasis. Many claims are unfounded because there is no biological data to support them. Metastasis is an in vivo phenotype and, quite simply, in vitro assays are not always predictive of in vivo behavior. In short, many labs suppressed steps of metastasis (i.e. invasion, motility, adhesion, resistance to apoptosis, growth) without testing the impact of changes using in vivo metastasis assays. Correlative studies are often related to promises unfulfilled. Nonetheless, we are encouraged by the emergence of new researchers in the metastasis field and the breadth of expertise that they bring. More common are claims that a gene blocks metastasis when it blocks growth—tumorigenicity. The issue was addressed above. However, the field must address the paradox that emerges when metastasis is suppressed in one cell type but tumorigenicity is suppressed in another (as for E-cadherin and DRG-1).

What do the data summarized in this review tell us about the clinical control of metastasis? Readers are cautioned to note that reliable antibodies/antisera recognizing many of the metastasis suppressors do not yet exist. As a result, many of the correlations presented are measured using RNA. While proportional expression of RNA and protein is anticipated for most, data are not yet available to definitively conclude such. Likewise, it is not known whether some metastasis suppressors are post-translationally modified. Ultimately, interpretation will depend upon identifying the functional protein responsible for metastasis suppression.

Another area of active research relates to the mechanisms responsible for loss of metastasis suppressor gene expression. Both anecdotal and published data suggest that many metastasis suppressor genes are not mutated, but are differentially expressed (reviewed in Ref. [188]). While not described in detail here, there are several levels at which expression could be regulated—protein translation [189,190], methylation [191,192], histone acetylation [192–195], mRNA protein stability [196,197]. Pat Steeg and colleagues have been pioneering the notion that metastasis suppressor genes may be re-expressed in a clinical setting. Recent data from her laboratory show that dexamethasone and medroxyprogesterone acetate can enhance expression of Nm23 [198]. They have also presented evidence that hypomethylation by 5-azacytidine can restore Nm23 expression as well [79]. While data were not collected for the other metastasis suppressors, their data support the possibility of pharmacologic regulation of metastasis via metastasis suppressor genes. Given that the drugs used for their experiments are first line, the possibility for therapeutic intervention in the near term is very real.

Acknowledgements

Work from the Welch lab has been supported by grants from the NIH (CA62168; CA88728,

and CA89109), the US Department of Defense Medical Research and Materiel Command (DAMD-17-96-1-6152 and DAMD17-02-1-0541) and the National Foundation for Cancer Research Center for Metastasis Research. L.R.S. is the recipient of a Susan G. Komen Postdoctoral Fellowship (PDF-2000-218). We also appreciate our colleagues Pat Steeg, Carrie Rinker-Schaeffer and Kent Hunter for stimulating conversations and inspiration. Finally, we ask the forbearance of authors whose work was not cited for space considerations.

References

[1] S.A. Stacker, M.G. Achen, L. Jussila, M.E. Baldwin, K. Alitalo, Metastasis: lymphangiogenesis and cancer metastasis, Nature Rev. Cancer 2 (2002) 573–583.

[2] P.S. Steeg, Metastasis suppressors alter the signal transduction of cancer cells, Nature Rev. Cancer 3 (2003) 55–63.

[3] D.R. Welch, S.P. Tomasovic, Implications of tumor progression on clinical oncology, Clin. Exptl. Metastasis 3 (1985) 151–188.

[4] P. Nowell, The clonal evolution of tumor cell populations, Science 194 (1976) 23–28.

[5] S.E. Luria, M. Delbruck, Mutations of bacteria from virus sensitivity to virus resistance, Genetics 28 (1943) 491–511.

[6] R. Bernards, R.A. Weinberg, Metastasis genes: a progression puzzle, Nature (London) 418 (2002) 823.

[7] W.C. Hahn, R.A. Weinberg, Rules for making human tumor cells, N. Engl. J. Med. 347 (2002) 1593–1603.

[8] L.A. Liotta, E.C. Kohn, The microenvironment of the tumour–host interface, Nature (London) 411 (2001) 375–379.

[9] L.M. Coussens, Z. Werb, Inflammation and cancer, Nature (London) 420 (2002) 860–867.

[10] M. Egeblad, Z. Werb, New functions for the matrix metalloproteinases in cancer progression, Nature Rev. Cancer 2 (2002) 161–174.

[11] S. Paget, The distribution of secondary growths in cancer of the breast, Lancet 1 (1889) 571–573.

[12] R. Folberg, M.J.C. Hendrix, A.J. Maniotis, Vasculogenic mimicry and tumor angiogenesis, Am. J. Pathol. 156 (2000) 361–381.

[13] A.J. Maniotis, R. Folberg, A. Hess, E.A. Seftor, L.M.G. Gardner, J. Pe'er, J.M. Trent, P.S. Meltzer, M.J.C. Hendrix, Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry, Am. J. Pathol. 155 (1999) 739–752.

[14] R.K. Jain, L.L. Munn, D. Fukumura, Dissecting tumour pathophysiology using intravital microscopy, Nature Rev. Cancer 2 (2002) 266–276.

[15] C. Kieda, M. Paprocka, A. Krawczenko, P. Zalecki, P. Dupuis, M. Monsigny, C. Radzikowski, D. Dus, New human microvascular endothelial cell lines with specific adhesion molecules phenotypes, Endothelium New York 9 (2002) 247–261.

[16] P. Laakkonen, K. Porkka, J.A. Hoffman, E. Ruoslahti, A tumor-homing peptide with a targeting specificity related to lymphatic vessels, Nature Med. 8 (2002) 751–755.

[17] R. Pasqualini, W. Arap, D.M. McDonald, Probing the structural and molecular diversity of tumor vasculature, Trends Mol. Med. 8 (2002) 563–571.

[18] E. Ruoslahti, Specialization of tumour vasculature, Nature Rev. Cancer 2 (2002) 83–90.

[19] S.J. Youngs, S.A. Ali, D.D. Taub, R.C. Rees, Chemokines induce migrational responses in human breast carcinoma cell lines, Int. J. Cancer 71 (1997) 257–266.

[20] G.L. Nicolson, Cancer metastasis. Organ colonization and the cell-surface properties of malignant cells, Biochim. Biophys. Acta 695 (1982) 113–176.

[21] A. Müller, B. Homey, H. Soto, N.F. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verástegui, A. Zlotnik, Involvement of chemokine receptors in breast cancer metastasis, Nature (London) 410 (2001) 50–56.

[22] R.S. Taichman, C. Cooper, E.T. Keller, K.J. Pienta, N.S. Taichman, L.K. McCauley, Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone, Cancer Res. 62 (2002) 1832–1837.

[23] F. Balkwill, Chemokine biology in cancer, Seminars Immunol. 15 (2003) 49–55.

[24] A.F. Chambers, I.C. MacDonald, E.E. Schmidt, S. Koop, V.L. Morris, R. Khokha, A.C. Groom, Steps in tumor metastasis: new concepts from intravital videomicroscopy, Cancer Metastasis Rev. 14 (1995) 279–301.

[25] S. Koop, I.C. MacDonald, K. Luzzi, E.E. Schmidt, V.L. Morris, M. Grattan, R. Khokha, A.F. Chambers, A.C. Groom, Fate of melanoma cells entering the microcirculation: over 80% survive and extravasate, Cancer Res. 55 (1995) 2520–2523.

[26] H.M. Qiu, F.W. Orr, D. Jensen, H.H. Wang, A. McIntosh, B.B. Hasinoff, D.M. Nance, S. Pylypas, K. Qi, C. Song, R.J. Muschel, A.B. Al Mehdi, Arrest of B16 melanoma cells in the mouse pulmonary microcirculation induces endothelial nitric oxide synthase-dependent nitric oxide release that is cytotoxic to the tumor cells, Am. J. Pathol. 162 (2003) 403–412.

[27] A.B. Al Mehdi, K. Tozawa, A.B. Fisher, A. Shientag, R.J. Lee, R.J. Muschel, Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis, Nature Med. 6 (2000) 100–102.

[28] A.F. Chambers, A.C. Groom, I.C. MacDonald, Metastasis: dissemination and growth of cancer cells in metastatic sites, Nature Rev. Cancer 2 (2002) 563–572.

[29] L. Weiss, Metastatic inefficiency, Adv. Cancer Res. 54 (1990) 159–211.

[30] L. Weiss, Concepts of metastasis, Cancer Metastasis Rev. 19 (2000) 219–234.

[31] I.J. Fidler, Selection of successive tumor lines for metastasis, Nature New Biol. 242 (1973) 148–149.

[32] L. Holmgren, Antiangiogenesis restricted tumor dormancy, Cancer Metastasis Rev. 15 (1996) 241–245.
[33] K.J. Luzzi, I.C. MacDonald, E.E. Schmidt, N. Kerkvliet, V.L. Morris, A.F. Chambers, A.C. Groom, Multistep nature of metastatic inefficiency—dormancy of solitary cells after successful extravasation and limited survival of early micrometastases, Am. J. Pathol. 153 (1998) 865–873.
[34] B.A. Yoshida, Z. Dubauskas, M.A. Chekmareva, M.M. Zaucha, T.R. Christiano, A.P. Christiano, W.M. Stadler, C.W. Rinker-Schaeffer, Identification and characterization of candidate prostate cancer metastasis-suppressor genes encoded on human chromosome 17, Cancer Res. 59 (1999) 5483–5487.
[35] G.N. Naumov, I.C. MacDonald, P.M. Weinmeister, N. Kerkvliet, K.V. Nadkarni, S.M. Wilson, V.L. Morris, A.C. Groom, A.F. Chambers, Persistence of solitary mammary carcinoma cells in a secondary site: a possible contributor to dormancy, Cancer Res. 62 (2002) 2162–2168.
[36] L. Holmgren, M.S. O'Reilly, J. Folkman, Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression, Nature Med. 1 (1995) 149–153.
[37] C.W. Wong, A. Lee, L. Shientag, J. Yu, Y. Dong, G. Kao, A.B. Al Mehdi, E.J. Bernhard, R.J. Muschel, Apoptosis: an early event in metastatic inefficiency, Cancer Res. 61 (2001) 333–338.
[38] M.D. Cameron, E.E. Schmidt, N. Kerkvliet, K.V. Nadkarni, V.L. Morris, A.C. Groom, A.F. Chambers, I.C. MacDonald, Temporal progression of metastasis in lung: cell survival, dormancy, and location dependence of metastatic inefficiency, Cancer Res. 60 (2000) 2541–2546.
[39] T. Chishima, M. Yang, Y. Miyagi, L. Li, Y. Tan, E. Baranov, H. Shimada, A.R. Moossa, S. Penman, R.M. Hoffman, Governing step of metastasis visualized in vitro, Proc. Natl Acad. Sci. 94 (1997) 11573–11576.
[40] R.M. Hoffman, Visualization of GFP-expressing tumors and metastasis in vivo, Biotechniques 30 (2001) 1016–1020.
[41] J.F. Harms, L.R. Budgeon, N.D. Christensen, D.R. Welch, Maintaining green fluorescent protein tissue fluorescence through bone decalcification and long-term storage, Biotechniques 33 (2002) 1197–1200.
[42] J.F. Harms, D.R. Welch, MDA-MB-435 human breast carcinoma metastasis to bone, Clin. Exptl Metastasis (2003) in press.
[43] D.R. Welch, C.W. Rinker-Schaeffer, What defines a useful marker of metastasis in human cancer?, J. Natl Cancer Inst. 91 (1999) 1351–1353.
[44] S. Ramaswamy, K.N. Ross, E.S. Lander, T.R. Golub, A molecular signature of metastasis in primary solid tumors, Nat. Genet. 33 (2003) 49–54.
[45] T. Nishizaki, S. Devries, K. Chew, W.H. Goodson, B.M. Ljung, A. Thor, F.M. Waldman, Genetic alterations in primary breast cancers and their metastases—direct comparison using modified comparative genomic hybridization, Genes Chromosomes Cancer 19 (1997) 267–272.
[46] K. Nakao, M. Shibusawa, A. Ishihara, H. Yoshizawa, A. Tsunoda, M. Kusano, A. Kurose, T. Makita, K. Sasaki, Genetic changes in colorectal carcinoma tumors with liver metastases analyzed by comparative genomic hybridization and DNA ploidy, Cancer 91 (2001) 721–726.
[47] R. Redon, D. Muller, K. Caulee, K. Wanherdrick, J. Abecassis, S. Du Manoir, A simple specific pattern of chromosomal aberrations at early stages of head and neck squamous cell carcinomas: PIK3CA but not p63 gene as a likely target of 3q26-qter gains, Cancer Res. 61 (2001) 4122–4129.
[48] W.G. Wu, X.M. Tang, W. Hu, R. Lotan, W.K. Hong, L. Mao, Identification and validation of metastasis-associated proteins in head and neck cancer cell lines by two-dimensional electrophoresis and mass spectrometry, Clin. Exptl Metastasis 19 (2002) 319–326.
[49] T. Nakayama, B. Taback, R. Turner, D.L. Morton, D.S.B. Hoon, Molecular clonality of in-transit melanoma metastasis, Am. J. Pathol. 158 (2001) 1371–1378.
[50] D. Massi, I. Sardi, C. Urso, A. Franchi, L. Borgognoni, A. Salvador, A. Giannini, U.M. Reali, M. Santucci, Microsatellite analysis in cutaneous malignant melanoma, Melanoma Res. 12 (2002) 577–584.
[51] J.Y. Shih, S.C. Yang, T.M. Hong, A. Yuan, J.J. Chen, C.J. Yu, Y.L. Chang, Y.C. Lee, K. Peck, C.W. Wu, P.C. Yang, Collapsin response mediator protein-1 and the invasion and metastasis of cancer cells, J. Natl Cancer Inst. 93 (2001) 1392–1400.
[52] J. Reifenberger, C.B. Knobbe, M. Wolter, B. Blaschke, K.W. Schulte, T. Pietsch, T. Ruzicka, G. Reifenberger, Molecular genetic analysis of malignant melanomas for aberrations of the Wnt signaling pathway genes CTNNB1, APC, ICAT and BTRC, Int. J. Cancer 100 (2002) 549–556.
[53] S.F. Goldberg, M.E. Miele, N. Hatta, M. Takata, C.A. Paquette-Straub, L.P. Freedman, D.R. Welch, Melanoma metastasis suppression by chromosome 6: Evidence for a pathway regulated by CRSP3 and TXNIP, Cancer Res. (2003) 63.
[54] R.V. Gopalkrishnan, D.C. Kang, P.B. Fisher, Molecular markers and determinants of prostate cancer metastasis, J. Cell Physiol. 189 (2001) 245–256.
[55] D.R. Welch, L.L. Wei, Genetic and epigenetic regulation of human breast cancer progression and metastasis, Endocrine-related Cancer 5 (1998) 155–197.
[56] B.A. Yoshida, M. Sokoloff, D.R. Welch, C.W. Rinker-Schaeffer, Metastasis-suppressor genes: a review and perspective on an emerging field, J. Natl Cancer Inst. 92 (2000) 1717–1730.
[57] C.W. Rinker-Schaeffer, D.R. Welch, M. Sokoloff, Defining the biologic role of genes that regulate prostate cancer metastasis, Curr. Opin. Urol. 10 (2001) 397–401.
[58] H.H. Luu, G.P. Zagaja, Z. Dubauskas, S.L. Chen, R.C. Smith, K. Watabe, Y. Ichikawa, T. Ichikawa, E.M. Davis, M.M. Le Beau, C.W. Rinker-Schaeffer, Identification of a novel metastasis-suppressor region on human chromosome 12, Cancer Res. 58 (1998) 3561–3565.
[59] E.C. Kauffman, V.L. Robinson, W.M. Stadler, M.H. Sokoloff, C.W. Rinker-Schaeffer, Metastasis suppression: the evolving role of metastasis suppressor genes for regulating

cancer cell growth at the secondary site, J. Urol. 169 (2003) 1122–1133.
[60] M.A. Chekmareva, C.P. Hollowell, R.C. Smith, E.M. Davis, M.M. LeBeau, C.W. Rinker-Schaeffer, Localization of prostate cancer metastasis-suppressor activity on human chromosome 17, Prostate 33 (1997) 271–280.
[61] I.J. Fidler, R. Radinsky, Genetic control of cancer metastasis, J. Natl Cancer Inst. 82 (1990) 166–168.
[62] D.R. Welch, Technical considerations for studying cancer metastasis in vivo, Clin. Exptl Metastasis 15 (1997) 272–306.
[63] D.R. Welch, S.F. Goldberg, Molecular mechanisms controlling human melanoma progression and metastasis, Pathobiology 65 (1997) 311–330.
[64] D.R. Welch, P. Chen, M.E. Miele, C.T. McGary, J.M. Bower, B.E. Weissman, E.J. Stanbridge, Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity, Oncogene 9 (1994) 255–262.
[65] M.E. Miele, A. de la Rosa, J.H. Lee, D.J. Hicks, J.U. Dennis, P.S. Steeg, D.R. Welch, Suppression of human melanoma metastasis following introduction of chromosome 6 is independent of NME1 (Nm23), Clin. Exptl Metastasis 15 (1997) 259–265.
[66] J. You, M.E. Miele, C. Dong, D.R. Welch, Suppression of human melanoma metastasis by introduction of chromosome 6 may be partially due to inhibition of motility, but not to inhibition of invasion, Biochem. Biophys. Res. Comm. 208 (1995) 476–484.
[67] S.F. Goldberg, J.F. Harms, K. Quon, D.R. Welch, Metastasis-suppressed C8161 melanoma cells arrest in lung but fail to proliferate, Clin. Exptl Metastasis 17 (1999) 601–607.
[68] J.-H. Lee, M.E. Miele, D.J. Hicks, K.K. Phillips, J.M. Trent, B.E. Weissman, D.R. Welch, KiSS-1, a novel human malignant melanoma metastasis-suppressor gene, J. Natl Cancer Inst. 88 (1996) 1731–1737.
[69] M.E. Miele, G. Lee, J.-H. Robertson, A. Coleman, C.T. McGary, P.B. Fisher, T.G. Lugo, D.R. Welch, Metastasis is suppressed in human melanoma cell line MelJuSo following introduction of chromosomes 1 or 6 but tumorigenicity and local invasiveness are unaffected, Proc. Am. Assoc. Cancer Res. 37 (1996) 527.
[70] K.K. Phillips, D.R. Welch, M.E. Miele, J.-H. Lee, L.L. Wei, B.E. Weissman, Suppression of MDA-MB-435 breast carcinoma cell metastasis following the introduction of human chromosome 11, Cancer Res. 56 (1996) 1222–1226.
[71] P.S. Steeg, G. Bevilacqua, L. Kopper, U.P. horgeirsson, J.E. Talmadge, L.A. Liotta, M.E. Sobel, Evidence for a novel gene associated with low tumor metastatic potential, J. Natl Cancer Inst. 80 (1988) 200–204.
[72] M. Salerno, T. Ouatas, D. Palmieri, P.S. Steeg, Inhibition of signal transduction by the nm23 metastasis suppressor: Possible mechanisms, Clin. Exptl Metastasis 20 (2003) 3–10.
[73] D. Lombardi, M.L. Lacombe, M.G. Paggi, nm23: Unraveling its biological function in cell differentiation, J. Cell Physiol. 182 (2000) 144–149.
[74] Y. Otsuki, M. Tanaka, S. Yoshii, N. Kawazoe, K. Nakaya, H. Sugimura, Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1, Proc. Natl Acad. Sci. 98 (2001) 4385–4390.
[75] E.H. Postel, S.J. Berberich, S.J. Flint, C.A. Ferrone, Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis, Science 261 (1993) 478–480.
[76] P.D. Wagner, P.S. Steeg, N.D. Vu, Two-component kinase-like activity of nm23 correlates with its motility-suppressing activity, Proc. Natl Acad. Sci. 94 (1997) 9000–9005.
[77] M.T. Hartsough, D.K. Morrison, M. Salerno, D. Palmieri, T. Ouatas, M. Mair, J. Patrick, P.S. Steeg, Nm23-H1 metastasis suppressor phosphorylation of kinase suppressor of ras via a histidine protein kinase pathway, J. Biol. Chem. 277 (2002) 32389–32399.
[78] P.S. Steeg, D. Palmieri, T. Ouatas, M. Salerno, Histidine kinases and histidine phosphorylated proteins in mammalian cell biology, signal transduction and cancer, Cancer Lett. 190 (2003) 1–12.
[79] M.T. Hartsough, S.E. Clare, M. Mair, A.G. Elkahloun, D. Sgroi, C.K. Osborne, G. Clark, P.S. Steeg, Elevation of breast carcinoma Nm23-H1 metastasis suppressor gene expression and reduced motility by DNA methylation inhibition, Cancer Res. 61 (2001) 2320–2327.
[80] Z. Fan, P.J. Beresford, D.Y. Oh, D. Zhang, J. Lieberman, Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor, Cell 112 (2003) 659–672.
[81] T. chikawa, Y. Ichikawa, J. Dong, A.L. Hawkins, C.A. Griffin, W.B. Isaacs, M. Oshimura, J.C. Barrett, J.T. Isaacs, Localization of metastasis suppressor gene(s) for prostatic cancer to the short arm of human chromosome 11, Cancer Res. 52 (1992) 3486–3490.
[82] J.T. Dong, P.W. Lamb, C.W. Rinker-Schaeffer, J. Vukanovic, T. Ichikawa, J.T. Isaacs, J.C. Barrett, KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2, Science 268 (1995) 884–886.
[83] C.C. Quinn, G.E. Gray, S. Hockfield, A family of proteins implicated in axon guidance and outgrowth, J. Neurobiol. 41 (1999) 158–164.
[84] J.T. Dong, H. Suzuki, S.S. Pin, G.S. Bova, J.A. Schalken, W.B. Isaacs, J.C. Barrett, J.T. Isaacs, Down-regulation of the KAI1 metastasis suppressor gene during the progression of human prostatic cancer infrequently involves gene mutation or allelic loss, Cancer Res. 56 (1996) 4387–4390.
[85] X.H. Yang, D.R. Welch, K.K. Phillips, B.E. Weissman, L.L. Wei, KAI1, a putative marker for metastatic potential in human breast cancer, Cancer Lett. 119 (1997) 149–155.
[86] K.K. Phillips, A.E. White, D.J. Hicks, D.R. Welch, J.C. Barrett, L.L. Wei, B.E. Weissman, Correlation between reduction of metastasis in the MDA-MB-435 model system and increased expression of the Kai-1 protein, Molec. Carcinog. 21 (1998) 111–120.
[87] A. Takaoka, Y. Hinoda, S. Sato, F. Itoh, M. Adachi, M. Hareyama, K. Imai, Reduced invasive and metastatic

potentials of KAI1-transfected melanoma cells, Jpn. J. Cancer Res. 89 (1998) 397–404.
[88] A. Takaoka, Y. Hinoda, S. Satoh, Y. Adachi, F. Itoh, M. Adachi, K. Imai, Suppression of invasive properties of colon cancer cells by a metastasis suppressor KAI1 gene, Oncogene 16 (1998) 1443–1453.
[89] C. Duriez, N. Falette, U. Cortes, C. Moyret-Lalle, A. Puisieux, Absence of p53-dependent induction of the metastatic suppressor KAI1 gene after DNA damage, Oncogene 19 (2000) 2461–2464.
[90] T. Mashimo, M. Watabe, S. Hirota, S. Hosobe, K. Miura, P.J. Tegtmeyer, C.W. Rinker-Schaeffer, K. Watabe, The expression of the KAI1 gene, a tumor metastasis suppressor, is directly activated by p53, Proc. Natl Acad. Sci. 95 (1998) 11307–11311.
[91] N. Sekita, H. Suzuki, T. Ichikawa, H. Kito, K. Akakura, T. Igarashi, T. Nakayama, M. Watanabe, T. Shiraishi, M. Toyota, O. Yoshie, H. Ito, Epigenetic regulation of the KAI1 metastasis suppressor gene in human prostate cancer cell lines, Jpn. J. Cancer Res. 92 (2001) 947–951.
[92] E. Odintsova, T. Sugiura, F. Berditchevski, Attenuation of EGF receptor signaling by a metastasis suppressor, the tetraspanin CD82/KAI-1, Curr. Biol. 10 (2000) 1009–1012.
[93] A. Delaguillaumie, C. Lagaudriere-Gesbert, M.R. Popoff, H. Conjeaud, Rho GTPases link cytoskeletal rearrangements and activation processes induced via the tetraspanin CD82 in T lymphocytes, J. Cell Sci. 115 (2002) 433–443.
[94] H.T. Lynch, M.J. Casey, J. Lynch, T.E.K. White, A.K. Godwin, Genetics and ovarian carcinoma, Sem. Oncol. 25 (1998) 265–280.
[95] J.-H. Lee, D.R. Welch, Identification of highly expressed genes in metastasis-suppressed chromosome 6/human malignant melanoma hybrid cells using subtractive hybridization and differential display, Int. J. Cancer 71 (1997) 1035–1044.
[96] J.-H. Lee, D.R. Welch, Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1, Cancer Res. 57 (1997) 2384–2387.
[97] S.F. Goldberg, M.E. Miele, C.A. Paquette, D.R. Welch, Identifying metastasis suppressor genes in human melanoma, Anticancer Res. (2001).
[98] M. Kotani, M. Detheux, A. Vandenbogaerde, D. Communi, J.M. Vanderwinden, E. Le Poul, S. Brezillon, R. Tyldesley, N. Suarez-Huerta, F. Vandeput, C. Blanpain, S.N. Schiffmann, G. Vassart, M. Parmentier, The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54, J. Biol. Chem. 276 (2001) 34631–34636.
[99] A.I. Muir, L. Chamberlain, N.A. Elshourbagy, D. Michalovich, D.J. Moore, A. Calamari, P.G. Szekeres, H.M. Sarau, J.K. Chambers, P. Murdock, K. Steplewski, U. Shabon, J.E. Miller, S.E. Middleton, J.G. Darker, C.G.C. Larminie, S. Wilson, D.J. Bergsma, P. Emson, R. Faull, K.L. Philpott, D.C. Harrison, AXOR12: a novel human G protein-coupled receptor, activated by the peptide KiSS-1, J. Biol. Chem. 276 (2001) 28969–28975.
[100] T. Ohtaki, Y. Shintani, S. Honda, H. Matsumoto, A. Hori, K. Kanehashi, Y. Torao, S. Kumano, Y. Takatsu, Y. Matsuda, Y. Ishibashi, T. Watanabe, M. Asada, T. Yamada, M. Suenaga, C. Kitada, S. Usuki, T. Kurokawa, H. Onda, O. Nishimura, M. Fujino, Metastasis suppressor gene KiSS1 encodes peptide ligand of a G-protein-coupled receptor, Nature (London) 411 (2001) 613–617.
[101] M.D. Ringel, E. Hardy, V.J. Bernet, H.B. Burch, F. Schuppert, K.D. Burman, M. Saji, Metastin receptor Is overexpressed in papillary thyroid cancer and activates MAP Kinase in thyroid cancer cells, J. Clin. Endocrin. Metab. 87 (2002) 2399.
[102] L.J. Stafford, C.Z. Xia, W.B. Ma, Y. Cai, M.Y. Liu, Identification and characterization of mouse metastasis-suppressor KiSS1 and its G-protein-coupled receptor, Cancer Res. 62 (2002) 5399–5404.
[103] C.H. Yan, H. Wang, D.D. Boyd, KiSS-1 represses 92 kDa type IV collagenase expression by down- regulating NFκB binding to the promoter as a consequence of IκB α-induced block of p65/p50 nuclear translocation, J. Biol. Chem. 276 (2001) 1164–1172.
[104] J.L. Janneau, J. Maldonado-Estrada, G. Tachdjian, I. Miran, N. Motte, P. Saulnier, J.C. Sabourin, J.F. Cote, B. Simon, R. Frydman, G. Chaouat, D. Bellet, Transcriptional expression of genes involved in cell invasion and migration by normal and tumoral trophoblast cells, J. Clin. Endocrin. Metab. 87 (2002) 5336–5339.
[105] F. Shirasaki, M. Takata, N. Hatta, K. Takehara, Loss of expression of the metastasis suppressor gene KiSS1 during melanoma progression and its association with LOH of chromosome 6q16.3-q23, Cancer Res. 61 (2001) 7422–7425.
[106] K.S. Chen, H.F. DeLuca, Isolation and characterization of a novel cDNA from HL-60 cells treated with 1,25-dihydroxyvitamin D-3, Biochim. Biophys. Acta 1219 (1994) 26–32.
[107] H. Nakamura, K. Nakamura, J. Yodoi, Redox regulation of cellular activation, Ann. Rev. Immunol. 15 (1997) 351–369.
[108] A. Nishiyama, M. Matsui, S. Iwata, K. Hirota, H. Masutani, H. Nakamura, Y. Takagi, H. Sono, Y. Gon, J. Yodoi, Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression, J. Biol. Chem. 274 (1999) 21645–21650.
[109] H. Yamanaka, F. Maehira, M. Oshiro, T. Asato, Y. Yanagawa, H. Takei, Y. Nakashima, A possible interaction of thioredoxin with VDUP1 in HeLa cells detected in a yeast two-hybrid system, Biochem. Biophys. Res. Comm. 271 (2000) 796–800.
[110] T. Saitoh, S. Tanaka, T. Koike, Rapid induction and Ca(2 +) influx-mediated suppression of vitamin D3 up- regulated protein 1 (VDUP1) mRNA in cerebellar granule neurons undergoing apoptosis, J. Neurochem. 78 (2001) 1267–1276.
[111] E. Junn, S.H. Han, J.Y. Im, Y. Yang, E.W. Cho, H.D. Um, D.K. Kim, K.W. Lee, P.L. Han, S.G. Rhee, I. Choi, Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function, J. Immunol. 164 (2000) 6287–6295.
[112] L.M. Butler, X. Zhou, W.S. Xu, H.I. Scher, R.A. Rifkind,

P.A. Marks, V.M. Richon, The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin, Proc. Natl Acad. Sci. 99 (2002) 11700–11705.

[113] A.M. Naar, P.A. Beaurang, S. Zhou, S. Abraham, W. Solomon, R. Tjian, Composite co-activator ARC mediates chromatin-directed transcriptional activation, Nature (London) 398 (1999) 828–832.

[114] D.J. Taatjes, A.M. Naar, F. Andel III, E. Nogales, R. Tjian, Structure, function, and activator-induced conformations of the CRSP coactivator, Science 295 (2002) 1058–1062.

[115] S. Ryu, S. Zhou, A.G. Ladurner, R. Tjian, The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1, Nature (London) 397 (1999) 446–450.

[116] Y.F. Jiang, I.D. Goldberg, Y.E. Shi, Complex roles of tissue inhibitors of metalloproteinases in cancer, Oncogene 21 (2002) 2245–2252.

[117] C. Chang, Z. Werb, The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis, Trends Cell Biol. 11 (2001) S37–S43.

[118] G. Giannelli, C. Bergamini, F. Marinosci, E. Fransvea, M. Quaranta, L. Lupo, O. Schiraldi, S. Antonaci, Clinical role of MMP-2/TIMP-2 imbalance in hepatocellular carcinoma, Int. J. Cancer 97 (2002) 425–431.

[119] S. Ylisirnio, M. Hoyhtya, R. Makitaro, P. Paaakko, J. Risteli, V.L. Kinnula, T. Turpeenniemi-Hujanen, A. Jukkola, Elevated serum levels of type I collagen degradation marker ICTP and tissue inhibitor of metalloproteinase (TIMP) 1 are associated with poor prognosis in lung cancer, Clin. Cancer Res. 7 (2001) 1633–1637.

[120] P. Pellegrini, I. Contasta, A.M. Berghella, E. Gargano, C. Mammarella, D. Adorno, Simultaneous measurement of soluble carcinoembryonic antigen and the tissue inhibitor of metalloproteinase TIMP1 serum levels for use as markers of pre-invasive to invasive colorectal cancer, Cancer Immunol. Immunother. 49 (2000) 388–394.

[121] S. Ylisirnio, M. Hoyhtya, T. Turpeenniemi-Hujanen, Serum matrix metalloproteinases -2, -9 and tissue inhibitors of metalloproteinases -1, -2 in lung cancer—TIMP-1 as a prognostic marker, Anticancer Res. 20 (2000) 1311–1316.

[122] L. Blavier, P. Henriet, S. Imren, Y.A. DeClerck, Tissue inhibitors of matrix metalloproteinases in cancer, Ann. NY Acad. Sci. 878 (1999) 108–119.

[123] M. Mareel, T. Boterberg, V. Noe, L. van Hoorde, S. Vermeulen, E. Bruyneel, M. Bracke, E-cadherin/catenin/cytoskeleton complex: a regulator of cancer invasion, J. Cell Physiol. 173 (1997) 271–274.

[124] S.J. Vermeulen, E.A. Bruyneel, M.E. Bracke, G.K. De Bruyne, K.M. Vennekens, K.L. Vleminckx, G.J. Berx, F.M. van Roy, M.M. Mareel, Transition from the noninvasive to the invasive phenotype and loss of α-catenin in human colon cancer cells, Cancer Res. 55 (1995) 4722–4728.

[125] U.H. Frixen, J. Behrens, M. Sachs, G. Eberle, B. Voss, A. Warda, D. Lochner, W. Birchmeier, E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells, J. Cell Biol. 113 (1991) 173–185.

[126] A.K. Perl, P. Wilgenbus, U. Dahl, H. Semb, G. Christofori, A causal role for E-cadherin in the transition from adenoma to carcinoma, Nature (London) 392 (1998) 190–193.

[127] G. Christofori, H. Semb, The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene, Trends Biochem. Sci. 24 (1999) 73–76.

[128] I.R.G. Beavon, The E-cadherin–catenin complex in tumour metastasis: structure, function and regulation, Eur. J. Cancer 36 (2000) 1607–1620.

[129] S. Nakayama, A. Sasaki, H. Mese, R.E. Alcalde, T. Tsuji, T. Matsumura, The E-cadherin gene is silenced by CpG methylation in human oral squamous cell carcinomas, Int. J. Cancer 93 (2001) 667–673.

[130] W.G. Jiang, E-cadherin and its associated protein catenins, cancer invasion and metastasis, Br. J. Surg. 83 (1996) 437–446.

[131] H. Shiozaki, H. Oka, M. noue, S. Tamura, M. Monden, E-cadherin mediated adhesion system in cancer cells, Cancer 77 (1996) 1605–1613.

[132] J.S. Nam, Y. Ino, M. Sakamoto, S. Hirohashi, Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis, Clin. Cancer Res. 8 (2002) 2430–2436.

[133] G. Berx, A.M. Cleton-Jansen, K. trumane, W.J.F. De Leeuw, F. Nollet, F. Van Roy, C. Cornelisse, E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain, Oncogene 13 (1996) 1919–1925.

[134] U. Cavallaro, G. Christofori, Cell adhesion in tumor invasion and metastasis: loss of the glue is not enough, Biochim. Biophys. Acta Rev. Cancer 1552 (2001) 39–45.

[135] T. Kashima, K. Nakamura, J. Kawaguchi, M. Takanashi, T. Ishida, H. Aburatani, A. Kudo, M. Fukayama, A.E. Grigoriadis, Overexpression of cadherins suppresses pulmonary metastasis of osteosarcoma in vivo, Int. J. Cancer 104 (2003) 147–154.

[136] M.T. Nieman, R.S. Prudoff, K.R. Johnson, M.J. Wheelock, N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression, J. Cell Biol. 147 (1999) 631–643.

[137] N.L. Tran, R.B. Nagle, A.E. Cress, R.L. Heimark, N-Cadherin expression in human prostate carcinoma cell lines, an epithelial-mesenchymal transformation mediating adhesion with stromal cells, Am. J. Pathol. 155 (1999) 787–798.

[138] M.J. Pishvaian, C.M. Feltes, P. Thompson, M.J. Bussemakers, J.A. Schalken, S.W. Byers, Cadherin-11 is expressed in invasive breast cancer cell lines, Cancer Res. 59 (1999) 947–952.

[139] G. Li, K. Satyamoorthy, M. Herlyn, N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells, Cancer Res. 61 (2001) 3819–3825.

[140] R.B. Hazan, G.R. Phillips, R.F. Qiao, L. Norton, S.A. Aaronson, Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis, J. Cell Biol. 148 (2000) 779–790.

[141] D.H.F. Teng, W.L. Perry III, J.K. Hogan, M. Baumgard, R.

Bell, S. Berry, T. Davis, D. Frank, C. Frye, T. Hattier, R. Hu, S. Jammulapati, T. Janecki, A. Leavitt, J.T. Mitchell, R. Pero, D. Sexton, M. Schroeder, P.H. Su, B. Swedlund, J.M. Kyriakis, J. Avruch, P. Bartel, A.C. Wong, Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor, Cancer Res. 57 (1997) 4177–4182.

[142] B.A. Yoshida, Z. Dubauskas, M.A. Chekmareva, T.R. Christiano, W.M. Stadler, C.W. Rinker-Schaeffer, Mitogen-activated protein kinase kinase 4/stress-activated protein/Erk kinase 1 (MKK4/SEK1), a prostate cancer metastasis suppressor gene encoded by human chromosome 17, Cancer Res. 59 (1999) 5483–5487.

[143] H.L. Kim, D.J. Van der Griend, X. Yang, D.A. Benson, Z. Dubauskas, B.A. Yoshida, M.A. Chekmareva, Y. Ichikawa, M.H. Sokoloff, P. Zhan, T. Karrison, A. Lin, W.M. Stadler, T. Ichikawa, M.A. Rubin, C.W. Rinker-Schaeffer, Mitogen-activated protein kinase kinase 4 metastasis suppressor gene expression is inversely related to histological pattern in advancing human prostatic cancers, Cancer Res. 61 (2001) 2833–2837.

[144] S.D. Yamada, J.A. Hickson, Y. Hrobowski, D.J. Vander-Griend, D. Benson, A. Montag, T. Karrison, D.Z. Huo, J. Rutgers, S. Adams, C.W. Rinker-Schaeffer, Mitogen-activated protein kinase kinase 4 (MKK4) acts as a metastasis suppressor gene in human ovarian carcinoma, Cancer Res. 62 (2002) 6717–6723.

[145] M.J. Seraj, R.S. Samant, M.F. Verderame, D.R. Welch, Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13, Cancer Res. 60 (2000) 2764–2769.

[146] L.A. Shevde, R.S. Samant, S.F. Goldberg, T. Sikaneta, A. Alessandrini, H.J. Donahue, D.T. Mauger, D.R. Welch, Suppression of human melanoma metastasis by the metastasis suppressor gene, BRMS1, Exp. Cell Res. 273 (2002) 229–239.

[147] R.S. Samant, M.T. Debies, L.A. Shevde, M.F. Verderame, D.R. Welch, Identification and characterization of murine ortholog (Brms1) of breast cancer metastasis suppressor 1 (BRMS1), Int. J. Cancer 97 (2002) 15–20.

[148] R.S. Samant, M.J. Seraj, M.M. Saunders, T. Sakamaki, L.A. Shevde, J.F. Harms, T.O. Leonard, S.F. Goldberg, L.R. Budgeon, W.J. Meehan, C.R. Winter, N.D. Christensen, M.F. Verderame, H.J. Donahue, D.R. Welch, Analysis of mechanisms underlying BRMS1 suppression of metastasis, Clin. Exptl Metastasis 18 (2001) 683–693.

[149] M.M. Saunders, M.J. Seraj, Z.Y. Li, Z.Y. Zhou, C.R. Winter, D.R. Welch, H.J. Donahue, Breast cancer metastatic potential correlates with a breakdown in homospecific and heterospecific gap junctional intercellular communication, Cancer Res. 61 (2001) 1765–1767.

[150] Z. Li, Z. Zhou, M.M. Saunders, G. Casey, D.R. Welch, H.J. Donahue, Connexin and osteopontin expression correlate with breast cancer metastatic potential, Proc. Am. Assoc. Cancer Res. (2001) 42.

[151] M.J. Seraj, M.A. Harding, J.J. Gildea, D.R. Welch, D. Theodorescu, The relationship of BRMS1 and RhoGDI2 gene expression to metastatic potential in lineage related human bladder cancer cell lines, Clin. Exptl Metastasis 18 (2001) 519–525.

[152] K.W. Hunter, K.W. Broman, T. LeVoyer, L. Lukes, D. Cozma, M.T. Debies, J. Rouse, D.R. Welch, Predisposition to efficient mammary tumor metastatic progression is linked to the breast cancer metastasis suppressor gene Brms1, Cancer Res. 61 (2001) 8866–8872.

[153] T. LeVoyer, T. Lifsted, M. Williams, K. Hunter, Identification and Mapping of a Mammary Tumor Metastasis Susceptibility Genes, Era of Hope—Department of Defense Breast Cancer Research Program, Vol. 2, 2000, p. 625.

[154] Y.G. Park, L. Lukes, H. Yang, M.T. Debies, R.S. Samant, D.R. Welch, M. Lee, K.W. Hunter, Comparative sequence analysis in eight inbred strains of the metastasis modifier QTL candidate gene Brms1, Mamm. Genome 13 (2002) 289–292.

[155] X. Lin, E. Tombler, P.J. Nelson, M. Ross, I.H. Gelman, A novel src- and ras-suppressed protein kinase C substrate associated with cytoskeletal architecture, J. Biol. Chem. 271 (1996) 28430–28438.

[156] I.H. Gelman, The role of SSeCKS/gravin/AKAP12 scaffolding proteins in the spaciotemporal control of signaling pathways in oncogenesis and development, Front. Biosci. 7 (2002) d1782–d1797.

[157] J.B. Nauert, T.M. Klauck, L.K. Langeberg, J.D. Scott, Gravin, an autoantigen recognized by serum from myasthenia gravis patients, is a kinase scaffold protein, Curr. Biol. 7 (1997) 52–62.

[158] P.J. Nelson, I.H. Gelman, Cell-cycle regulated expression and serine phosphorylation of the myristylated protein kinase C substrate, SSeCKS: correlation with culture confluency, cell cycle phase and serum response, Mol. Cell. Biochem. 175 (1997) 233–241.

[159] X. Lin, P. Nelson, I.H. Gelman, SSeCKS, a major protein kinase C substrate with tumor suppressor activity, regulates G(1) → S progression by controlling the expression and cellular compartmentalization of cyclin D, Molec. Cell. Biol. 20 (2000) 7259–7272.

[160] W. Xia, P. Unger, L. Miller, J. Nelson, I.H. Gelman, The Src-suppressed C kinase substrate, SSeCKS, is a potential metastasis inhibitor in prostate cancer, Cancer Res. 61 (2001) 5644–5651.

[161] B. Boettner, L. VanAelst, The role of Rho GTPases in disease development, Gene 286 (2002) 155–174.

[162] J.J. Gildea, M.J. Seraj, G. Oxford, M.A. Harding, G.M. Hampton, C.A. Moskaluk, H.F. Frierson, M.R. Conaway, D. Theodorescu, RhoGD12 is an invasion and metastasis suppressor gene in human cancer, Cancer Res. 62 (2002) 6418–6423.

[163] N. van Belzen, W.N. Dinjens, M.P. Diesveld, N.A. Groen, A.C. van der Made, Y. Nozawa, R. Vlietstra, J. Trapman, F.T. Bosman, A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms, Lab. Invest. 77 (1997) 85–92.

[164] S.K. Kurdistani, P. Arizti, C.L. Reimer, M.M. Sugrue, S.A. Aaronson, S.W. Lee, Inhibition of tumor cell growth by RTP/

rit42 and its responsiveness to p53 and DNA damage, Cancer Res. 58 (1998) 4439–4444.
[165] R.J. Guan, H.L. Ford, Y. Fu, Y. Li, L.M. Shaw, A.B. Pardee, Drg-1 as a differentiation-related, putative metastatic suppressor gene in human colon cancer, Cancer Res. 60 (2000) 749–755.
[166] S. Bandyopadhyay, S.K. Pai, S.C. Gross, S. Hirota, S. Hosobe, K. Miura, K. Saito, T. Coomes, S. Hayashi, M. Watabe, K. Watabe, The Drg-1 gene suppresses tumor metastasis in prostate cancer, Cancer Res. (2003) 63.
[167] K.L. Agarwala, K. Kokame, H. Kato, T. Miyata, Phosphorylation of RTP, an ER stress-responsive cytoplasmic protein, Biochem. Biophys. Res. Comm. 272 (2000) 641–647.
[168] M. Motwani, F.M. Sirotnak, Y.H. She, T. Commes, G.K. Schwartz, Drg1, a novel target for modulating sensitivity to CPT-11 in colon cancer cells, Cancer Res. 62 (2002) 3950–3955.
[169] V. Castellani, G. Rougon, Control of semaphorin signaling, Curr. Opin. Neurobiol. 12 (2002) 532–541.
[170] B.J. Dickson, Molecular mechanisms of axon guidance, Science 298 (2002) 1959–1964.
[171] Y. Goshima, T. Ito, Y. Sasaki, F. Nakamura, Semaphorins as signals for cell repulsion and invasion, J. Clin. Invest. 109 (2002) 993–998.
[172] P.M. Comoglio, L. Trusolino, Invasive growth: from development to metastasis, J. Clin. Invest. 109 (2002) 857–862.
[173] T.A. Endo, M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, T. Miyazaki, N. Leonor, T. Taniguchi, T. Fujita, Y. Kanakura, S. Komiya, A. Yoshimura, A new protein containing an SH2 domain that inhibits JAK kinases, Nature (London) 387 (1997) 921–924.
[174] H. Fujita, F. Okada, J. Hamada, M. Hosokawa, T. Moriuchi, R.C. Koya, N. Kuzumaki, Gelsolin functions as a metastasis suppressor in B16-BL6 mouse melanoma cells and requirement of the carboxyl-terminus for its effect, Int. J. Cancer 93 (2001) 773–780.
[175] M. Tanaka, L. Müllauer, Y. Ogiso, H. Fujita, S. Moriya, K. Furuuchi, T. Harabayashi, N. Shinohara, T. Koyanagi, N. Kuzumaki, Gelsolin: a candidate for suppressor of human bladder cancer, Cancer Res. 55 (1995) 3228–3232.
[176] N. Sagawa, H. Fujita, Y. Banno, Y. Nozawa, H. Katoh, N. Kuzumaki, Gelsolin suppresses tumorigenicity through inhibiting PKC activation in a human lung cancer cell line, PC10, Br. J. Cancer 88 (2003) 606–612.
[177] Z. Zou, A. Anisowicz, M.J.C. Hendrix, A. Thor, M. Neveu, S. Sheng, K. Rafidi, E. Seftor, R. Sager, Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells, Science 263 (1994) 526–529.
[178] K.B. Reddy, R. McGowen, L. Schuger, D. Visscher, S.J. Sheng, Maspin expression inversely correlates with breast tumor progression in MMTV/TGF-alpha transgenic mouse model, Oncogene 20 (2001) 6538–6543.
[179] N. Jiang, Y.H. Meng, S.L. Zhang, E. Mensah-Osman, S.J. Sheng, Maspin sensitizes breast carcinoma cells to induced apoptosis, Oncogene 21 (2002) 4089–4098.
[180] M. Zhang, O. Volpert, Y.H. Shi, N. Bouck, Maspin is an angiogenesis inhibitor, Nature Med. 6 (2000) 196–199.
[181] B.W. Futscher, M.M. Oshiro, R.J. Wozniak, N. Holtan, C.L. Hanigan, H. Duan, F.E. Domann, Role for DNA methylation in the control of cell type-specific maspin expression, Nat. Genet. 31 (2002) 175–179.
[182] J.F. Costello, P.M. Vertino, Methylation matters: a new spin on maspin, Nat. Genet. 31 (2002) 123–124.
[183] Z. Zou, C. Gao, A.K. Nagaich, T. Connell, S. Saito, J.W. Moul, P. Seth, E. Appella, S. Srivastava, p53 regulates the expression of the tumor suppressor gene maspin, J. Biol. Chem. 275 (2000) 6051–6054.
[184] D.A. Kirschmann, R.A. Lininger, L.M.G. Gardner, E.A. Seftor, V.A. Odero, A.M. Ainsztein, W.C. Earnshaw, L.L. Wallrath, M.J.C. Hendrix, Down-regulation of $HP1^{Hs1}$ expression is associated with the metastatic phenotype in breast cancer, Cancer Res. 60 (2000) 3359–3363.
[185] A.C. Gao, W. Lou, J.T. Dong, J.T. Isaacs, CD44 is a metastasis suppressor gene for prostatic cancer located on human chromosome 11p13, Cancer Res. 57 (1997) 846–849.
[186] D.H. Yu, C.K. Qu, O. Henegariu, X. Lu, G.S. Feng, Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion, J. Biol. Chem. 273 (1998) 21125–21131.
[187] M.A. Chekmareva, M.M. Kadkhodaian, C.M.P. Hollowell, H. Kim, B.A. Yoshida, H.H. Luu, W.M. Stadler, C.W. Rinker-Schaeffer, Chromosome 17-mediated dormancy of AT6.1 prostate cancer micrometastases, Cancer Res. 58 (1998) 4963–4969.
[188] P.S. Steeg, T. Ouatas, D. Halverson, D. Palmieri, M. Salerno, Metastasis suppressor genes: Basic biology and potential clinical use, Clin. Breast Cancer (2003) in press.
[189] S.G. Zimmer, J.R. Graff, The emerging role for the mRNA cap-binding protein, EIF-4E, in metastatic progression, in: D.R. Welch (Ed.), Cancer Metastasis: Biology and Treatment, Kluwer Academic Publishers, Dordrecht, 2002, pp. 257–278.
[190] J.R. Graff, S.G. Zimmer, Translational control and metastatic progression: enhanced activity of the mRNA cap-binding protein eIF-4E selectively enhances translation of metastasis-related mRNAs, Clin. Exptl Metastasis 20 (2003) 265–273.
[191] C. Plass, P.D. Soloway, DNA methylation imprinting and cancer, Eur. J. Hum. Genet. 10 (2002) 6–16.
[192] C. Plass, Cancer epigenomics, Hum. Molec. Genet. 11 (2002) 2479–2488.
[193] A. Imhof, P.B. Becker, Modifications of the histone N-terminal domains, evidence for an epigenetic code?, Mol. Biotechnol. 17 (2001) 1–13.
[194] A.R. Karpf, D.A. Jones, Reactivating the expression of methylation silenced genes in human cancer, Oncogene 21 (2002) 5496–5503.
[195] W.K. Kelly, O.A. O'Connor, P.A. Marks, Histone deacety-

lase inhibitors: from target to clinical trials, Expert Opin. Investig. Drugs 11 (2002) 1695–1713.
[196] R.M. Elledge, W.H. Lee, Life and death by p53, BioEssays 17 (1995) 923–930.
[197] N.J. Mabjeesh, D.E. Post, M.T. Willard, B. Kaur, E.G. VanMeir, J.W. Simons, H. Zhong, Geldanamycin induces degradation of hypoxia-inducible factor 1α protein via the proteosome pathway in prostate cancer cells, Cancer Res. 62 (2002) 2478–2482.
[198] T. Ouatas, D. Halverson, P.S. Steeg, Dexamethasone and medroxyprogesterone acetate elevate Nm23-H1 metastasis suppressor expression in metastatic human breast carcinoma cells via glucocorticoid receptor-dependent, transcriptional and post-transcriptional mechanisms: new uses for old compounds, Clin, Cancer Res. (2003) in press.
[199] P.Y. Desprez, C.Q. Lin, N. Thomasset, C.J. Sympson, M.J. Bissell, J. Campisi, Novel pathway for mammary epithelial cell invasion induced by the helix-loop-helix protein Id-1, Mol. Cell. Biol. 18 (1998) 4577–4588.
[200] J. Singh, K. Murata, Y. Itahana, P.Y. Desprez, Constitutive expression of the Id-1 promoter in human metastatic breast cancer cells is linked with the loss of NF-1/Rb/HDAC-1 transcription repressor complex, Oncogene 21 (2002) 1812–1822.
[201] Y. Toh, S.D. Pencil, G.L. Nicolson, A novel candidate metastasis-associated gene, mta1, differentially expressed in highly metastatic mammary adenocarcinoma cell lines, cDNA cloning, expression, and protein analyses, J. Biol. Chem. 269 (1994) 22958–22963.
[202] G.L. Nicolson, A. Nawa, Y. Toh, S. Taniguchi, K. Nishimori, A. Moustafa, Tumor metastasis-associated human MTA1 gene and its MTA1 protein product: role in epithelial cancer cell invasion, proliferation and nuclear regulation, Clin. Exptl Metastasis 20 (2003) 19–24.

Breast Cancer Cells Downregulate Alkaline Phosphatase Production in Osteoblasts

R. R. Mercer, C. V. Gay, D. R. Welch, A. M. Mastro, J. Gittlen

Department of Biochemistry and Molecular Biology, Penn State University, University Park, Pennsylvania, Cancer Research Institute, Penn State University School of Medicine, Hershey, Pennsylvania

Biotechnology Grant, Life Sciences Consortium, Penn State University

R.R. Mercer

Osteolytic lesions resulting from metastatic breast cancer can be limited through treatment with bisphosphonates. However, osteoblasts do not synthesize new bone to restore regions resorbed by osteoclasts. We hypothesize that breast cancer cells affect the differentiation process of the osteoblasts and prevent them from being fully functional. We have begun to test this possibility by determining if breast cancer cells produce factors that affect osteoblast differentiation.

We chose bone alkaline phosphatase, a characteristic protein produced by differentiating osteoblasts. Primary osteoblasts were isolated from rat tibia. At confluence, either breast cancer conditioned media from the human breast cancer MDA-MB-231 cell line or control media was added to the osteoblasts, which were subsequently cultured for an additional 5 and 12 days. The cells were then removed from culture and stained for alkaline phosphatase production. Analysis was performed microscopically, and cells were reported as being stained weakly, moderately, or intensely, with the intensity of the stain directly correlating to the amount of alkaline phosphatase present.

Only 30% of the osteoblasts cultured 12 days postconfluence in the presence of breast cancer conditioned media had moderate or intense staining, while 70% of osteoblasts cultured in control media stained intensely.Results were similar for osteoblasts cultured 5 days postconfluence and with conditioned media from the MDA-MB-435 human breast cancer cell line at both 5 and 12 days postconfluence.

CONCLUSION: These data indicate that alkaline phosphatase production is decreased by a secreted product from breast cancer cells and suggest that breast cancer cells have the ability to slow osteoblast differentiation.

tively, relative to 435 cells. ELISA revealed a 50% reduction in matrix metalloproteinase-1 (MMP-1) release, relative to 435 cells. Interestingly, MMP-1, osteopontin, and Cx32 expression have been found to correlate with breast cancer cell metastatic potential.

CONCLUSION: Therefore, these results strongly suggest that GJIC and Cx43 expression contribute to the metastatic potential of breast cancer cells to bone.

Differences Between Osteoblast-Secreted and Breast Cancer–Secreted Osteonectin: N-Linked Glycosylation May Be Key in Chemoattraction

D. A. Campo, D. M. Sosnoski, A. M. Mastro, D. R. Welch, C. V. Gay

Intercollege Program in Physiology and Department of Biochemistry and Molecular Biology, Penn State University, University Park, Pennsylvania; Jake Gittlen Cancer Research Institute, Penn State University School of Medicine, Hershey, Pennsylvania

Osteonectin, first identified in bone, has wide tissue distribution, varying degrees of glycosylation, and has been shown to be a chemoattractant for breast cancer cells. Here we report differences between bone-derived osteonectin and breast cancer–derived osteonectin and show differential chemoattraction.

In one experiment, individual cultures of the human fetal osteoblast cell line (hFOB1.19) and a metastatic breast cancer cell line (MDA-MB-435) were grown with or without tunicamycin, a potent inhibitor of N-linked glycosylation. Conditioned, serum-free media (CM) were collected from the cultures. Aliquots of CM were subjected to SDS-page gel electrophoresis and blotted on a nitrocellulose membrane. Immunostaining with mouse antihuman osteonectin was used to detect osteonectin bands. In untreated hFOB1.19 cells, a doublet of osteonectin (~39 kDa and ~38 kDa) was detected; the MDA-MB-435 cells also secreted osteonectin of two sizes (~41 kDa and ~38 kDa).

Upon treatment with tunicamycin, the hFOB1.19 doublet decreased in size (~36 kDa and ~35 kDa), whereas the MDA-MB-435 osteonectin was unchanged. The data show that osteoblast-derived osteonectin is heavily glycosylated through the N-linkage, whereas osteonectin from breast cancer cells has no detectable N-linked glycosylation. One consequence of altered glycosylation is a change in protein folding, which could account chemotactic potentials of osteonectin.

In another experiment which was assess the chemotactic potential of the osteonectin, breast cancer cells (5 × 1(were placed in the upper chamber of chamber insert (12-μm pore size) coate gel. CM from untreated hFOB1.19 or M cells was placed in the lower chamber. A the number of MDA-MB-435 cells migr the transwell membrane toward the hF was fourfold greater than toward MDA-I

CONCLUSION: Collectively, the re that bone-derived osteonectin is distii breast cancer osteonectin in molecula glycosylation. Furthermore, bone-deriv tin has an enhanced chemotactic potent cancer cells.

SDF-1/CXCR4 and Prostate Ca Metastases

R. S. Taichman, C. Cooper, E. T. Keller, K. N. S. Taichman, L. K. McCauley

Departments of Periodontics, Internal Med. thology, University of Michigan, Ann Arbor, M. partment of Pathology, University of Pennsylv Dental Medicine, Philadelphia, Pennsylvania

Neoplasms have a striking tendency t or "home" to bone. Hematopoietic cells to bone during embryonic developmen dence points to the chemokine stromal tor-1 (SDF-1 or CXCL12) (expressed b and endothelial cells), and its receptor key elements in these processes.

We hypothesized that metastatic pros mas also utilize the SDF-1/CXCR4 path ize to the bone. To test the hypothesis th of CXCR4 in several human prostate car by reverse transcriptase polymerase ch and by Western Blot was determined. Po were obtained with the PC-3 and DU1 derived from malignancies that had sp and brain, respectively. Hormone-refrac carcinoma cell lines cloned from a lymp CaP) and marrow (LNCaP C4-2B) als CXCR4.

Activation of phosphorylated ERK-1 teins was observed within 5 minutes of

The Expression of Metalloproteinases Capable of Type I Collagen Degradation in Bone Metastases by Cancer Cells Is Independent of Primary Tumor

L. Costa, A. Fernandes, E. Frauenhoffer, A. G. Oliveira, K. Leitzel, L. Demers, S. Ali, J. Schaller, E. B. Costa, M. C. de Moura, A. Lipton

Hospital de Santa Maria, Lisbon, Portugal; Hershey Medical Center, Penn State University, Hershey, Pennsylvania; Medical University of South Carolina, Charleston, South Carolina

The capacity of cancer cells to degrade bone directly is not yet well established. Using immunohistochemical localization in bone metastases we studied metalloproteinases (MMPs) known to be capable of type I collagen degradation.

Bone pathologic fractures or bone metastases biopsies from 35 patients, median age 67 (range: 40–85), 18 female, were analyzed. Fifteen had breast cancer, 4 colorectal, 3 unknown primary tumor, 2 prostate, 2 lung, 2 thyroid, 2 renal, and 5 had several other types of cancer. The lesions were lytic in 24, blastic in 6, and mixed in 5. Formalin-fixed, decalcified, paraffin-embedded sections of metastatic lesions were stained with routine hematoxylin and eosin and by immunoperoxidase methods with antibodies (Oncogene Research Products) to MMP1, 2, 8, and 9. The expression of MMPs in cancer cells was graded according to the percentage of cells staining (0, 1: < 1/3, 2: 1/3–2/3, or 3: > 2/3) and the intensity of staining (1, 2, or 3). A final score (0 to 9) was obtained for each MMP in each patient.

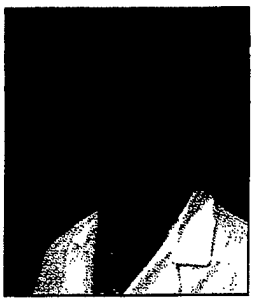
MMP1 had the highest expression in cancer cells (median score: 6.0) followed by MMP2 (5.5), MMP8 (2.96), and MMP9 (0.11); this difference is statistically significant ($P < .0001$) by two-way ANOVA test. The difference among MMP median scores remained significant irrespective of primary tumor (breast vs nonbreast) or the x-ray pattern of bone metastases. MMP9 was rarely expressed in cancer cells but commonly observed in osteoclasts.

CONCLUSION: Cancer cells in bone metastases express MMPs capable of bone collagen degradation. This expression is independent of primary tumor and of x-ray pattern. Among the MMPs analyzed in this study, MMP1 had the highest immunohistochemical score.

Connexin 43 and Breast Ca Metastasis to Bone

H. J. Donahue, P. Kapoor, Z. Li, D. R.

Musculoskeletal Research Laboratory, Cancer Research Institute, Pennsylvania S College of Medicine, Hershey, Pennsylvar



H.J. Donahue

Metastat cells intera grate throu teoblastic t prior to est ondary tum cellular mat hypothesis ic gap junc lar commu between br and osteoblastic cells initiates subse facilitate breast cancer cell transost tion.

Therefore, we examined GJIC ar gap junction protein) expression in a cancer cell line, MDA-MB-435 (a expressing the metastasis suppress (435-BRMS1), vector controls an osteoblastic cell line (hFOB). 435 gap junction protein Cx32 but no 435-BRMS1, nonmetastatic, and breast epithelial cells and normal tissue express Cx43 but not Cx32.

All of the following relative cha icant at $P < .05$. As assessed by dual dye transfer followed by flow cyto displayed very little homotypic G selves, a characteristic of many tu However, expressing BRMS1 in 43 homotypic GJIC nearly 6-fold. Wh not communicate with themselves, 2-fold greater, relative to 435-BRM GJIC with hFOB cells.

When Cx43 cDNA, which is und type 435 cells, was transfected int Cx43+), these cells displayed a 40 homotypic GJIC with themselves crease in heterotypic GJIC with hFO cells. Additionally, as revealed by transcriptase polymerase chain rea cells displayed a 75% and 80% red state levels of Cx32 and osteopontir

tively, relative to 435 cells. ELISA revealed a 50% reduction in matrix metalloproteinase-1 (MMP-1) release, relative to 435 cells. Interestingly, MMP-1, osteopontin, and Cx32 expression have been found to correlate with breast cancer cell metastatic potential.

CONCLUSION: Therefore, these results strongly suggest that GJIC and Cx43 expression contribute to the metastatic potential of breast cancer cells to bone.

Differences Between Osteoblast-Secreted and Breast Cancer–Secreted Osteonectin: N-Linked Glycosylation May Be Key in Chemoattraction

D. A. Campo, D. M. Sosnoski, A. M. Mastro, D. R. Welch, C. V. Gay

Intercollege Program in Physiology and Department of Biochemistry and Molecular Biology, Penn State University, University Park, Pennsylvania; Jake Gittlen Cancer Research Institute, Penn State University School of Medicine, Hershey, Pennsylvania

Osteonectin, first identified in bone, has wide tissue distribution, varying degrees of glycosylation, and has been shown to be a chemoattractant for breast cancer cells. Here we report differences between bone-derived osteonectin and breast cancer–derived osteonectin and show differential chemoattraction.

In one experiment, individual cultures of the human fetal osteoblast cell line (hFOB1.19) and a metastatic breast cancer cell line (MDA-MB-435) were grown with or without tunicamycin, a potent inhibitor of N-linked glycosylation. Conditioned, serum-free media (CM) were collected from the cultures. Aliquots of CM were subjected to SDS-page gel electrophoresis and blotted on a nitrocellulose membrane. Immunostaining with mouse antihuman osteonectin was used to detect osteonectin bands. In untreated hFOB1.19 cells, a doublet of osteonectin (~39 kDa and ~38 kDa) was detected; the MDA-MB-435 cells also secreted osteonectin of two sizes (~41 kDa and ~38 kDa).

Upon treatment with tunicamycin, the hFOB1.19 doublet decreased in size (~36 kDa and ~35 kDa), whereas the MDA-MB-435 osteonectin was unchanged. The data show that osteoblast-derived osteonectin is heavily glycosylated through the N-linkage, whereas osteonectin from breast cancer cells has no detectable N-linked glycosylation. One consequence of altered glycosylation is a change in protein folding, which could account chemotactic potentials of osteonectin.

In another experiment which was assess the chemotactic potential of the osteonectin, breast cancer cells (5 × 1(were placed in the upper chamber of chamber insert (12-μm pore size) coate gel. CM from untreated hFOB1.19 or M cells was placed in the lower chamber. A the number of MDA-MB-435 cells migr the transwell membrane toward the hF was fourfold greater than toward MDA-1

CONCLUSION: Collectively, the re that bone-derived osteonectin is disti breast cancer osteonectin in molecula glycosylation. Furthermore, bone-deriv tin has an enhanced chemotactic potent cancer cells.

SDF-1/CXCR4 and Prostate Ca Metastases

R. S. Taichman, C. Cooper, E. T. Keller, K. N. S. Taichman, L. K. McCauley

Departments of Periodontics, Internal Med thology, University of Michigan, Ann Arbor, M partment of Pathology, University of Pennsylv Dental Medicine, Philadelphia, Pennsylvania

Neoplasms have a striking tendency t or "home" to bone. Hematopoietic cells to bone during embryonic developmen dence points to the chemokine stromal tor-1 (SDF-1 or CXCL12) (expressed b and endothelial cells), and its receptor key elements in these processes.

We hypothesized that metastatic pros mas also utilize the SDF-1/CXCR4 path ize to the bone. To test the hypothesis th of CXCR4 in several human prostate car by reverse transcriptase polymerase ch and by Western Blot was determined. Po were obtained with the PC-3 and DU1 derived from malignancies that had sp and brain, respectively. Hormone-refrac carcinoma cell lines cloned from a lymp CaP) and marrow (LNCaP C4-2B) als CXCR4.

Activation of phosphorylated ERK-1 teins was observed within 5 minutes of