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13. ABSTRACT (Maximum 200) We had previously shown that the MDA-MB-231 breast cancer cells selected for rapid adherence to primary bone marrow stroma metastasize to bone more rapidly than cancer cells which do not adhere to the stromal matrix. Comparison of surface integrins between stroma adherent and non-adherent cells indicated that the adherent cells expressed lower levels of the integrin $\alpha_v\beta_3$. Based on this observation we isolated cancer cells which expressed either high or undetectable levels of the integrin. When these cells were tested for bone metastasis in vivo, it was determined that the high $\alpha_v\beta_3$ expressing cancer cells metastasized to bone up to ten times less frequently than the cancer cells which do not express the integrin. Thus expression of $\alpha_v\beta_3$ inversely correlates with frequency of bone metastasis. In addition to $\alpha_v\beta_3$, we have determined no significant change in the expression levels of $\alpha 1-6$, $\beta 1$ and $\beta 5$ integrins between the parental MDA-MB-231 cells and bone tumors derived from them. In addition, by serial passage of metastatic tumors in vivo, we have isolated populations of MDA-MB-231 cells which are highly metastatic to bone, liver or kidney. These organ selective metastatic cells provide a new tool for identifying molecules responsible for bone metastasis.			
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INTRODUCTION

Skeletal metastasis is virtually ubiquitous among breast cancer patients dying of their disease. In fact, 80% of all metastatic lesions to bone are derived from either a breast or prostate primary. The fatal consequences of such metastasis include fracture and hypercalcemia, the latter occurring in one third of breast cancer patients with bone involvement. Thus, prevention of skeletal metastasis would greatly impact on the morbidity of breast cancer.

While the precise mechanisms by which breast cancer metastasizes to the skeleton and subsequently destroys bone are incompletely understood, osteoclasts probably play a critical role. As will be discussed, it appears that breast cancer metastases in mineralized bone are consequent upon establishment of tumor within marrow. Once having achieved sufficient mass, the cancer promotes differentiation of marrow-residing osteoclast precursors into mature, skeleton-resorbing, polykaryons. These cells, in turn, degrade bone, permitting extension of breast cancer into mineralized matrix.

In light of the above, we hypothesize that:

- 1. MOLECULES EXIST ON THE SURFACE OF BREAST CANCER CELLS WHICH MEDIATE THEIR ATTACHMENT TO COMPONENTS OF THE MARROW MICROENVIRONMENT.**
- 2. BREAST CANCER CELLS PRODUCE OSTEOCLASTOGENIC FACTORS WHICH CAN BE IDENTIFIED, IN VITRO.**
- 3. INHIBITION OF OSTEOCLAST DIFFERENTIATION AND/OR FUNCTION DIMINISHES BREAST CANCER-INDUCED BONE DESTRUCTION.**

Therefore, our specific aims are to:

- 1. CHARACTERIZE THE MOLECULES ON METASTATIC BREAST CANCER CELLS WHICH MEDIATE THEIR ATTACHMENT TO THE CELLULAR OR MATRICAL MARROW MICROENVIRONMENT.**
- 2. DEFINE THE MECHANISMS BY WHICH BREAST CANCER CELLS PROMOTE OSTEOCLASTOGENESIS, IN VITRO.**
- 3. DETERMINE IF INHIBITION OF OSTEOCLAST DIFFERENTIATION AND/OR FUNCTION, IN VIVO, DIMINISHES BREAST CANCER-INDUCED BONE DESTRUCTION.**

STUDIES AND RESULTS

Last year we showed that human breast cancer cell lines MCF-7 and T47D can support osteoclastogenesis in vitro. This finding provides an explanation as to why breast cancer cells in the marrow promote osteolysis, i.e. through recruitment of osteoclasts, without an increase in osteoblastic activity. We found that breast cancer cell induced osteoclast differentiation is mediated primarily through cell-cell contact between the cancer cells and the osteoclast precursors. This finding is contrary to our initial, preliminary, results that breast cancer cells induce osteoclast differentiation through a secretory factor. We have chosen not to further pursue the osteoclastogenic factor(s) expressed on the surface of breast cancer cells, because identification of such cell-cell mediated osteoclastogenic factor(s) is both risky and difficult. For example, despite many attempts, we and others have not been able to identify the factor(s) on surface of the ST2 stromal cells responsible for promoting osteoclast differentiation.

We have focused considerable effort on identifying the mechanisms of preferential homing of breast cancer cells to bone. We are using the MDA-MB-231 human breast cancer cells for this purpose. Last year we reported that the MDA-MB-231 cells selected for adherence to primary human bone marrow stroma metastasized to bone more frequently than cancer cells selected for non-adherence to the same stroma. We showed that integrins were involved in the attachment of the cancer cells to stroma and thus screened the stroma-adherent and non-adherent cells for changes in integrin expression. Our results demonstrated that the stroma-adherent cells, which preferentially metastasized to bone, expressed lower levels of the integrin $\alpha_v\beta_3$.

Based on the above observation, we isolated by FACS sorting, from the parental MDA-MB-231 cells, cancer cells which expressed high levels of $\alpha_v\beta_3$ or did not express the integrin. The high $\alpha_v\beta_3$ expressing cells were isolated by FACS sorting the highest 0.5% of the parental population expressing $\alpha_v\beta_3$. Those cells were expanded in vitro, and used to repeat the FACS sorting for a total four times resulting in our high $\alpha_v\beta_3$ population (Fig. 1). The same approach to isolate an $\alpha_v\beta_3$ non-expressing population failed. Because of difficulty in isolating populations of $\alpha_v\beta_3$ non-expressors, we resorted to cell cloning and obtained four clonal sublines of the MDA-MB-231 which do not express detectable levels of $\alpha_v\beta_3$ (Fig.2). We tested the metastatic potential of the high $\alpha_v\beta_3$ expressing cells and the low $\alpha_v\beta_3$ expressing clone MDA-MB-231C4 by injection of 10^5 cells into the left ventricle of nude mice. The results indicate that the low $\alpha_v\beta_3$ expressing MDA-MB-231C4 clone metastasizes to bone approximately three times

more frequently than the high $\alpha_v\beta_3$ expressing cell population (Table 1). In addition, the low $\alpha_v\beta_3$ expressing cells produce, on average, 2-3 times more osteolytic lesions in each animal and the lesion are detectable in a shorter period of time than those produced by the high $\alpha_v\beta_3$ expressing MDA-MB-231 cells (Table 1).

In order to substantiate that the increased bone metastasis by the low $\alpha_v\beta_3$ expressing cells is not an artifact of the MDA-MB-231C4 clonal cell population, we injected nude mice, intracardiac, with a second low $\alpha_v\beta_3$ expressing clonal line. The results in case were more dramatic. Thirty out of forty mice developed bone metastases with an average of 12 osteolytic lesions per mouse (Table 2). Similar to results obtained with the MDA-MB-231C4 cells, the lesions produced by the MDA-MB-231C5 clone was detectable in 4-6 weeks after injection of the cells. Thus our data provide strong support for the conclusion that low $\alpha_v\beta_3$ expression leads to increased bone metastasis in vivo.

In order to prove that increased $\alpha_v\beta_3$ expression directly leads to decreased incidence of bone metastasis we are re-expressing the integrin in our $\alpha_v\beta_3$ non-expressing clones. We expect that these transfected cells will metastasize to bone less frequently.

We have isolated cancer cells from many of the bone tumors. Analysis of $\alpha_v\beta_3$ expression indicates that these tumor cells maintain the same level of $\alpha_v\beta_3$ expression as their parental cells (Fig. 3). We have also analyzed a number of the bone tumor derived cells for changes in expression of the integrins α_1 through α_6 , β_1 , and β_5 . Our results do not indicate any significant change in expression of those integrins. During the coming year we will expand this analysis to other cell surface molecules such as the cadherins, and CD44 and its splice variants.

During the course of experiments designed to measure frequency of bone metastasis by high and low $\alpha_v\beta_3$ expressing breast cancer cells, we made the following observation. Mice injected with the high $\alpha_v\beta_3$ expressing cells develop fewer bone metastases, but are generally more cachectic, suggesting they suffer from more visceral tumors. In contrast, mice injected with the low $\alpha_v\beta_3$ expressing cells develop more osteolytic lesions, but generally do not suffer from cachexia. Based on this observation, we suggest that high $\alpha_v\beta_3$ expression leads to metastasis to the viscera while low $\alpha_v\beta_3$ expression leads to metastasis to the skeleton (Fig.4). One test of this model is to re-express $\alpha_v\beta_3$, by transfection, on the surface of the low $\alpha_v\beta_3$ expressing cells, and if the model is correct we would expect the transfected cells to metastasize more frequently to the viscera and less frequently to bone.

Our experiments, to date, suggest that the level of $\alpha_v\beta_3$ expression may serve as a

diagnostic marker for identifying tumors with a propensity to metastasize to the viscera or the skeleton. Thus an important set of experiments planned for the next two years is to obtain human breast tumor biopsy material, grow the tumor cells and initially characterize the expression level of $\alpha_v\beta_3$ on the surface of the tumor cells. Selected low and high $\alpha_v\beta_3$ expressing cells will be injected, intracardiac, into nude mice and their metastatic potential determined. Because many tumors consist of a heterogeneous cell population, we may resort to cell sorting to isolate subpopulations of high or low $\alpha_v\beta_3$ expressing tumor cells for these experiments. Alternatively, we could inject the parental tumor cells into mice, allow bone metastases to develop, isolate the metastatic cells and characterized the $\alpha_v\beta_3$ expression level of those cells to determine if organ selective metastasis leads to selection of low $\alpha_v\beta_3$ expressing cells in vivo. Positive results in these experiments could be used, in the future, to design more appropriate chemotherapy strategies based on predicted metastatic potential of the primary tumor. For example, a patient presented with a high $\alpha_v\beta_3$ expressing primary tumor may be treated more aggressively to prevent lung metastasis.

Expression level of $\alpha_v\beta_3$, while potentially useful as a diagnostic marker, correlates negatively with bone metastasis. The ultimate goal of the following experiments is to identify new cell surface markers whose expression is responsible for organ selective, ie bone, metastasis. One approach we are using is to continue characterizing the expression level of known cell adhesion molecules on the surface of metastatic tumor cells derived from bone. To date we have measured expression levels of $\alpha 1$ through $\alpha 6$, $\beta 1$ and $\beta 5$ integrins in addition to α_v and $\alpha_v\beta_3$. We will expand this analysis to the cadherin family, and to CD44 and its variants.

It is possible that a novel molecule(s) is responsible for selective metastasis of breast cancer cells to bone. We are using a second approach to address this possibility. We have shown that tumor cells isolated from bone, once re-injected, intracardiac, into mice will re-metastasize to bone more quickly and generate more osteolytic lesions than their parental cells (Table 3, and Fig.5). Thus, serial in vivo passage of tumor cells from a specific target organ, eg bone, leads to preferential metastasis to that organ. One interpretation of these results is that serial passage of tumor cells, in vivo, leads to selection of a subpopulation expressing the optimal combination of adhesion molecules responsible for organ specific targeting. Currently, we are isolating, by serial passage in vivo, MDA-MB-231 cells which preferentially metastasize to bone, kidney or liver. We have in hand fifth passage bone metastatic cells, and third passage kidney and liver metastasizing cells. We will next determine changes in expression levels cell adhesion molecules, such as integrins, in these tumor derived cells. Once we have identified a molecule whose expression correlated with metastasis to bone (or liver), we will isolated, by FACS sorting, cells which express high and low levels of that molecule and

test their bone (or liver) metastatic potential. If we fail to identify candidate molecules by this approach, we will assume the molecule(s) responsible for selective bone metastasis is novel. In this case we will differential display PCR (or subtractive hybridization) to identify genes which are expressed by the bone metastasizing and not by the liver (or kidney) metastasizing cells.

In the third specific aim of our proposal, we ask if inhibition of osteoclast differentiation or function diminishes breast cancer-induced osteolysis. We now have highly bone metastatic cells for this purpose. We will focus on whether bone residing tumor cells, directly or indirectly, promote osteoclast differentiation by producing tumor necrosis factor α (TNF α), a potent osteoclastogenic cytokine. Initial experiments will determine TNF α expression at the site of bone tumors by immunohistochemical staining. In the future, TNF α levels in the plasma and the bone marrow of tumor bearing animals will be determined. If these experiments support the notion that TNF α is important to development of osteolytic lesions, we will test the metastatic potential of our bone seeking tumor cells in TNF receptor 1 (TNFR1), TNFR2, and TNFR1+2 knockout mice. If TNFR deleted animals are spared from bone metastasis, then we will determine if TNF α inhibitors, such as thalidomide, prevent tumor induced osteolysis.

CONCLUSIONS

We have demonstrated that: i) human breast cancer cells which do not express the integrin $\alpha_v\beta_3$ metastasize to bone 3-7 times more frequently than those expressing high levels of the integrin. ii) Metastatic bone tumor cells maintain the low $\alpha_v\beta_3$ expression level of their parental cells. The generality of these results will be determined by testing the metastasis potential of high and low $\alpha_v\beta_3$ expressing cells isolated from patient tumor biopsies. iii) Significant changes in expression levels of the integrin α_1 - α_6 , β_1 , or β_5 were not observed between MDA-MB-231 breast cancer cells and their metastatic bone tumor derivatives. iv) Tumor cells isolated from bone (or liver or kidney) metastasize to the same organ more quickly and frequently than their parental cells.

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American Society for Bone and Mineral Research Meeting - September 7-11, 1996 -Seattle, Washington

Tondravi, M., Quiroz, M., Wang, M., and Teitelbaum, S.L.: Increased rate of skeletal metastases by human breast cancer cells expressing low levels of $\alpha_v\beta_3$ integrin. *Journal of Bone and Mineral Research* 11:S98, 1996.

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Tondravi, M.M., Quiroz, M., and Teitelbaum, S.L.: $\alpha_v\beta_3$ -integrin expression by human breast cancer cells is associated with diminished skeletal metastasis. *Breast and Prostate Cancer: Basic Mechanisms*.

Figure Legends

- Fig. 1** Isolation of high $\alpha_v\beta_3$ expressing MDA-MB-231 breast cancer cells. Parental MDA-MB-231 cells were stained with the $\alpha_v\beta_3$ complex specific antibody LM609 and fluorescein-labeled secondary antibody. The highest $\alpha_v\beta_3$ expressing 0.5% of the population was sorted by FACS, expanded and resorted for a total of four times. The selected population is shown as the thick-lined histogram, while the parental population is shown as the thin-lined histogram. Unstained control cells are indicated by the dash-lined histogram.
- Fig. 2** Isolation of $\alpha_v\beta_3$ non-expressing MDA-MB-231 breast cancer cells. Because repeated FACS sorting did not yield a low $\alpha_v\beta_3$ expressing population, the parental MDA-MB-231 cells were cloned and the level of $\alpha_v\beta_3$ expression determined by FACS analysis. The top panel shows fluorescence intensity histogram of parental MDA-MB-231 cells (dashed line). The four histograms below show the fluorescence intensity of four low $\alpha_v\beta_3$ expressing clonal lines (dashed lines) compared to unstained controls (solid lines). Based on this FACS analysis, the four clones do not express any detectable $\alpha_v\beta_3$.
- Fig. 3** Bone metastatic tumor cells maintain the same level of $\alpha_v\beta_3$ expression as their parental cells. Tumor cells were excised from osteolytic lesion, expanded in vitro, stained with LM609 and their fluorescence intensity measured by FACS analysis. Top panel shows fluorescence intensity of the low $\alpha_v\beta_3$ expressing MDA-MB-231 C4 parental cells which overlaps the fluorescence intensity of unstained controls. Lower panel shows overlapping fluorescence intensity of bone tumor cells derived from MDA-MB-231 C4 parental cells compared to the same unstained control. Note that both cells do not express detectable levels of $\alpha_v\beta_3$.
- Fig. 4** High $\alpha_v\beta_3$ expressing MDA-MB-231 cells appear to metastasize more frequently to the viscera while low $\alpha_v\beta_3$ expressing cells metastasize more frequently to the skeleton.
- Fig. 5** Bone derived tumor cells remetastasize to bone frequently than their parents. Top panel shows osteolytic lesions (arrows) resulting from parental MDA-MB-231 cells. Some of these tumor cells were expanded in vitro and injected into nude mice. Lower panel shows that these first passage tumor cells produce significantly more osteolytic lesions than the parental cells (arrows).

Fig. 1

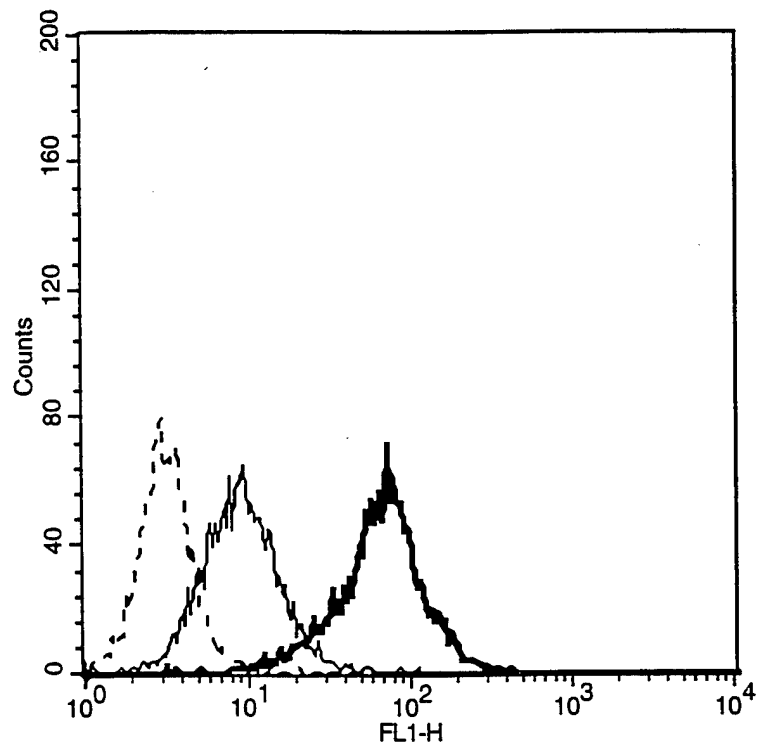


Fig. 2

isolation of MDA-231 clones that do not express the integrin $\alpha_v\beta_3$

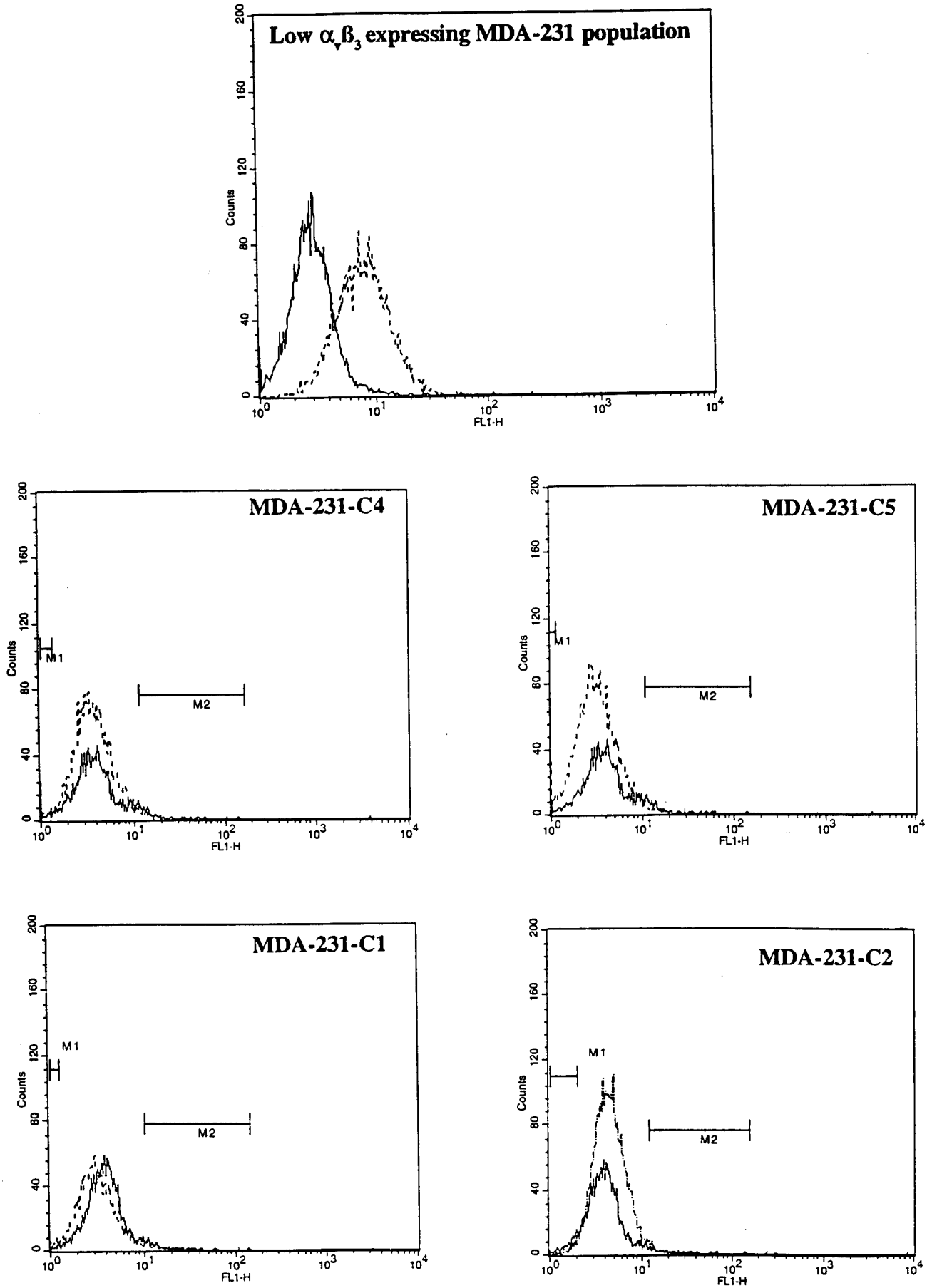


Fig. 3

Bone metastatic tumor cells maintain the same level of $\alpha_v\beta_3$ expression as their parental cells

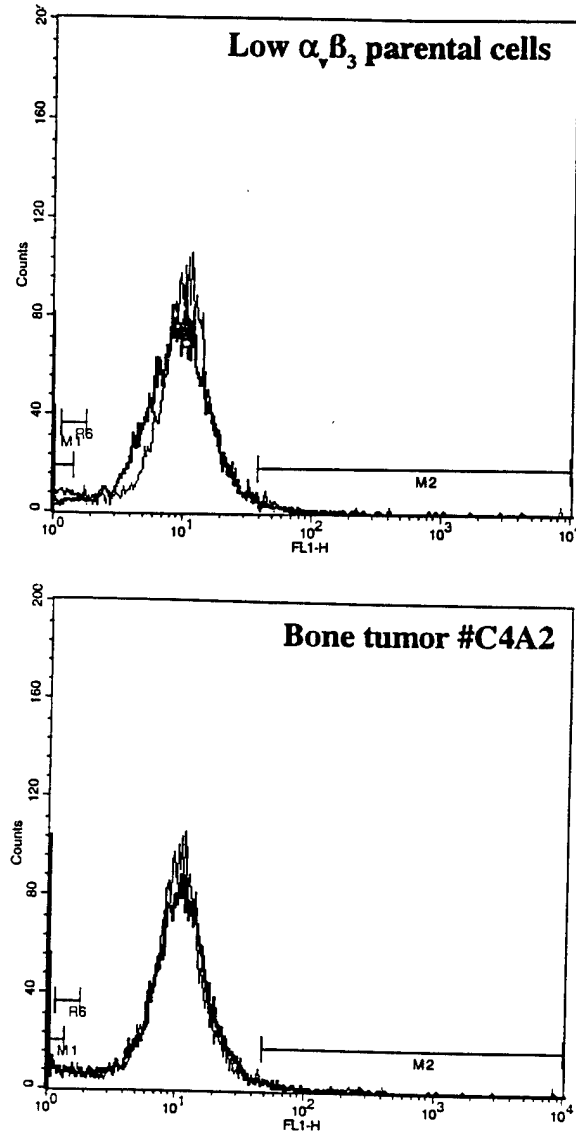


Fig. 4

MODEL

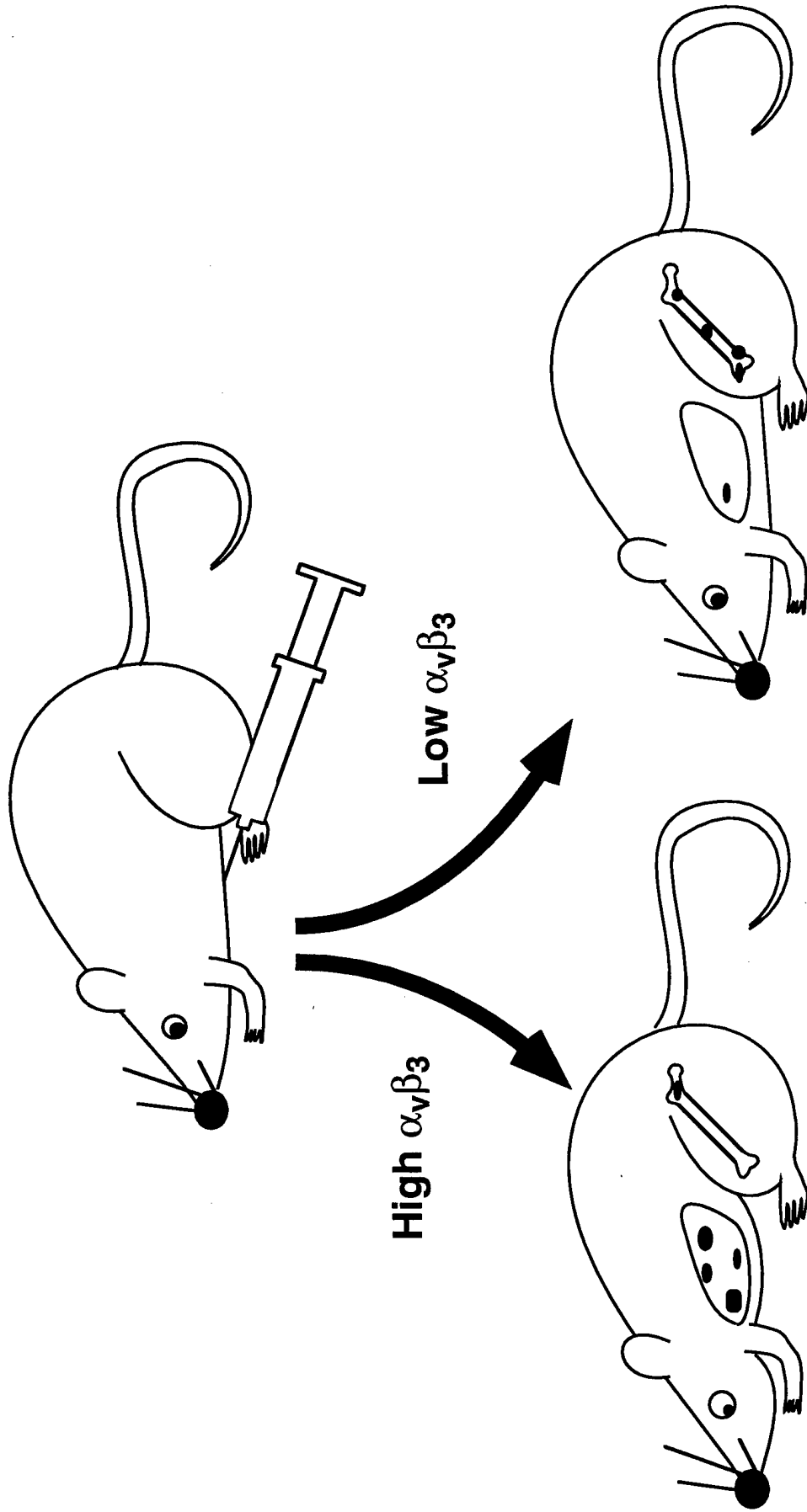
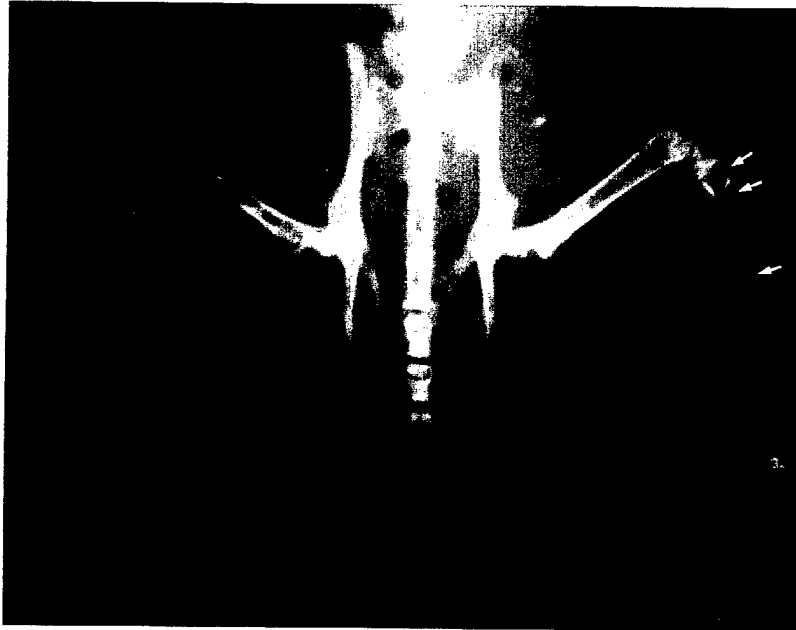


Fig. 5

Bone derived tumor cells remetastatize to bone more frequently

Primary tumor



First passage tumor



Table 1**Low $\alpha_v\beta_3$ expressing breast cancer cells demonstrate increased bone metastasis**

MDA-231 Subpopulation	Number of mice injected	Number of mice with osteolytic lesions	Average number of osteolytic lesions per mouse	Number of weeks to detection of metastases
High $\alpha_v\beta_3$ MDA-231	26	3	2.0	6-8
Low $\alpha_v\beta_3$ MDA-231 Clone C4	27	10	5.4	4-6

Table 2

Low $\alpha_v\beta_3$ expression by human breast cancer cells leads to increased frequency of bone metastasis

MDA-231 subpopulation	Number of mice injected	Number of mice with osteolytic lesions	Average number of osteolytic lesions per mouse	Number of weeks to detection of metastases
High $\alpha_v\beta_3$ MDA-231	26	3	2.0	6-8
Low $\alpha_v\beta_3$ MDA-231 Clone C4	27	10	5.4	4-6
Low $\alpha_v\beta_3$ MDA-231 Clone C5	40	30	12.2	4-6

Table 3

Low $\alpha_v\beta_3$ expressing breast cancer cells demonstrate increased bone metastasis

MDA-231 Subpopulation	Number of mice injected	Number of mice with osteolytic lesions	Number of weeks to detection of metastases
High $\alpha_v\beta_3$ MDA-231	26	3	6-8
Low $\alpha_v\beta_3$ MDA-231 Clone C4	27	10	4-6
MDA-231-C4 First Passage Tumor	5	4	3-4