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TITLE: Breast Cancer Microvesicles as a Novel Plasma Biomarker and Therapeutic Target (IDEA)

PRINCIPAL INVESTIGATOR: Kevin W. Harris, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham  
Birmingham, AL 35294-3300

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<b>14. ABSTRACT</b>  We propose to study the ability of quantitation of breast cancer tumor microvesicles (MV) to predict clinical outcomes in breast cancer patients. We also propose that treatment of HER2+ breast cancer patients with trasutuzumab results in clearing of tumor MV from the blood. In order to achieve these aims, a precise and robust clinical assay is required. Flow cytometry, as originally proposed, and as used by numerous investigators, is inadequate for this task. Thus we have developed a novel bead capture procedure to isolate and analyze tumor MV from breast cancer patients. Anti-MUC1 coated beads are used to bind and isolate tumor MV from patient plasma. We have characterized these MUC-1 tumor MV and verified the specificity of the capture. The isolated tumor MV will be assayed by commercial ELISA for MUC1, tissue factor, MMPs, and uPA. We have an active IRB approved protocol and have begun to collect patient samples for this purpose.					
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## Introduction

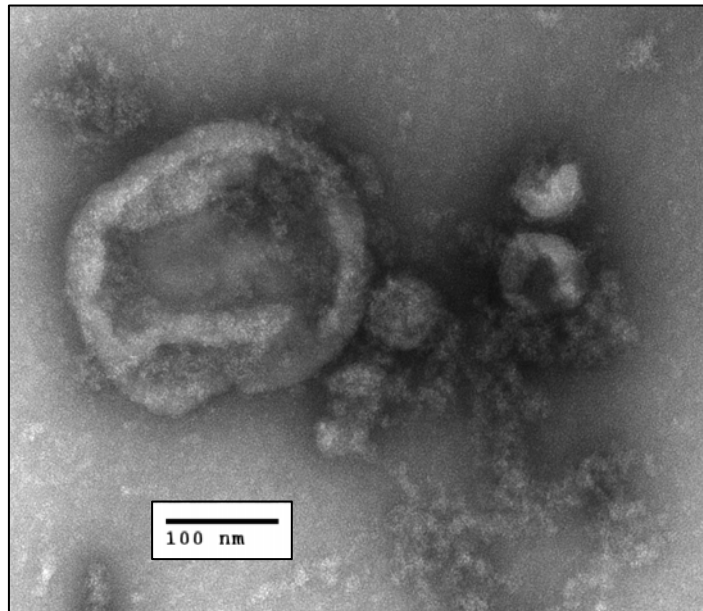
This project proposes to develop a clinical assay for tumor microvesicles (MV) from the blood of patients with breast cancer. This assay will be applied to test the hypothesis that the level of plasma tumor MV predict response to treatment and subsequent relapse in patients with locally advanced breast cancer. This pilot clinical project will enroll 35-40 patients. We and others have shown that tumor MV can express HER-2 protein. Thus, we also will use this assay to determine if trastuzumab is able to clear tumor MV from the blood of breast cancer patients.

## Body

Task 1. Develop reliable flow cytometry and immunochemical techniques to measure and analyze breast cancer microparticles from the blood of animals and humans.

In our application we proposed to use a flow cytometry platform to develop a clinical tumor MV assay. This was based on our preliminary experience (as described in the application) and previously published work from other groups (1, 2). However, after about a year of work with both cell lines and clinical samples, it became apparent that tumor MV cannot be reproducibly and robustly assayed using flow cytometry. We struggled with wildly varying results using MV obtained from the same cell line or patient (even from the same sample). The problem involves the drawing of the gates on the forward and side scattergrams by the operator for subsequent immunochemical analysis (see Figure 2B of our original grant application- the gate is marked R3). Even with the use of sizing beads, the drawing of the gate is very subjective. As one can see from the scattergram, there is not a discrete population of particles to gate on. We wondered if we were actually isolating tumor MV. However, using electron microscopy (Figure 1), we are now confident that our widely used differential centrifugation scheme was effectively isolating tumor MV. We discussed this flow cytometry problem informally with numerous investigators when we presented our research results at the American Society of Hematology meeting in December 2005. Many investigators were having the same problem. The problem is that MV are too small for analysis with flow cytometry. These particles are roughly the same size as viruses. MV are close to the size of the wavelength of the laser light used by the flow cytometry and thus discrete particles can not even be accurately detected. Flow cytometry is essentially useless for a reliable clinical assay of these particles, in our opinion. This problem is now widely accepted by experts in the field (3, see section entitled "Current problems with

measurement of microparticles” in this reference). We of course were not aware of this problem when we submitted our proposal in May 2005.



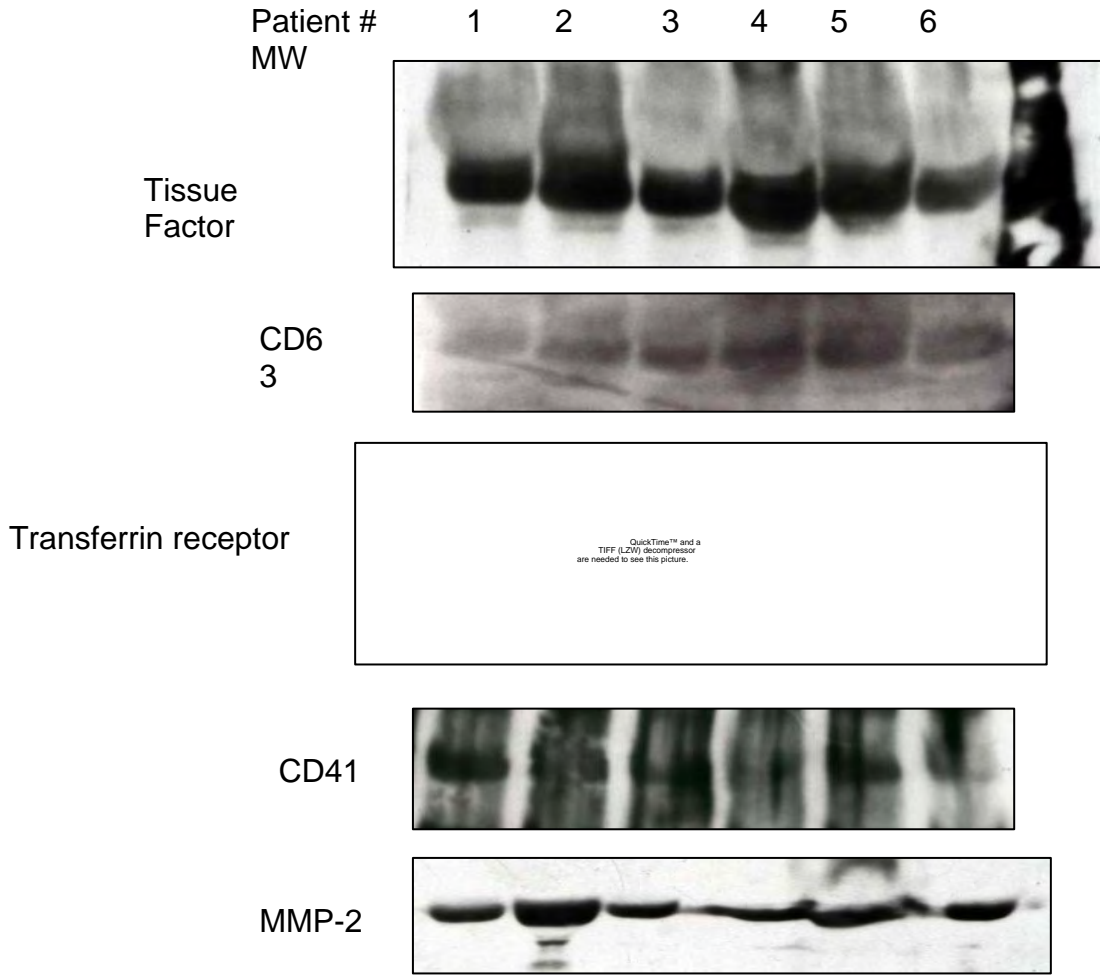
**Figure 1. Negatively stained scanning electron micrograph of breast cancer tumor MV obtained by differential centrifugation.**

MV were are described as between 50-200 nm in size. Note their characteristic “punched out” morphology.

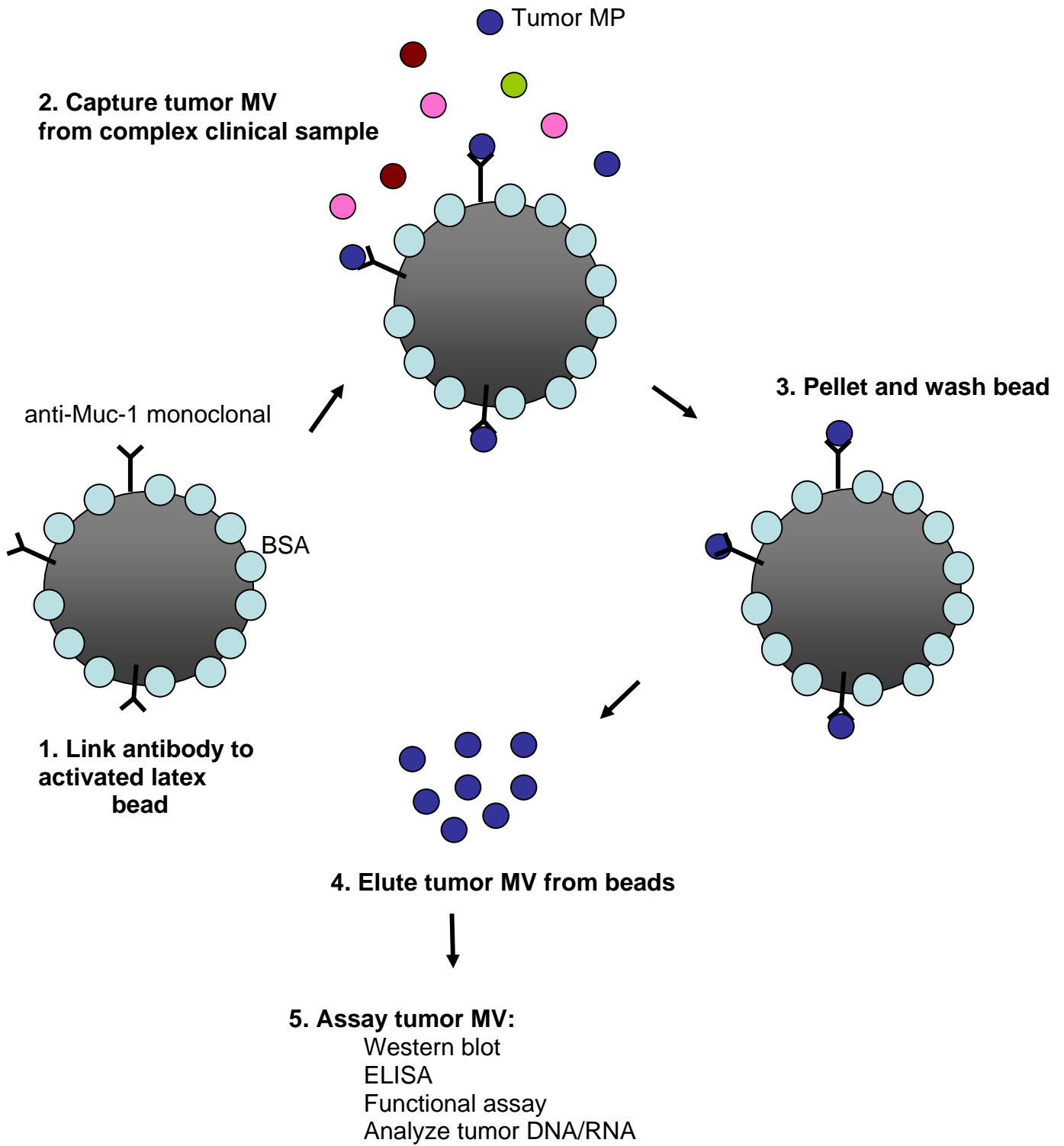
So we needed to develop a new assay for quantitation of tumor MV. This development work has occupied most of the first year of our grant. The main problem is to detect and/or isolate the tumor MV from the complex mixture of MV from the many tissue types found in blood. For instance, unfractionated MV obtained from the plasma of breast cancer patients (Figure 2) reveals markers from breast cancer and monocytes (tissue factor) and from platelets (CD41). MV of other origin (including red blood cell and endothelial cells) are also found in blood (1).

We elected to try to use antibody coated beads to capture and isolate tumor MV. See Figure 3 for a cartoon of our assay. MUC1 was chosen as a well-accepted breast cancer tumor antigen for which numerous well-characterized immunochemical reagents are available. Commercially available derivatized latex beads (Interfacial Dynamics Corporation) were irreversibly coated with a mixture of anti-MUC1 and bovine serum albumin (BSA). The BSA was used to completely coat and block all remaining binding sites. Several permutations of linkage chemistry, buffers, and amounts of MUC1 and BSA were tested before we obtained the optimum capture bead. Briefly, this involves a

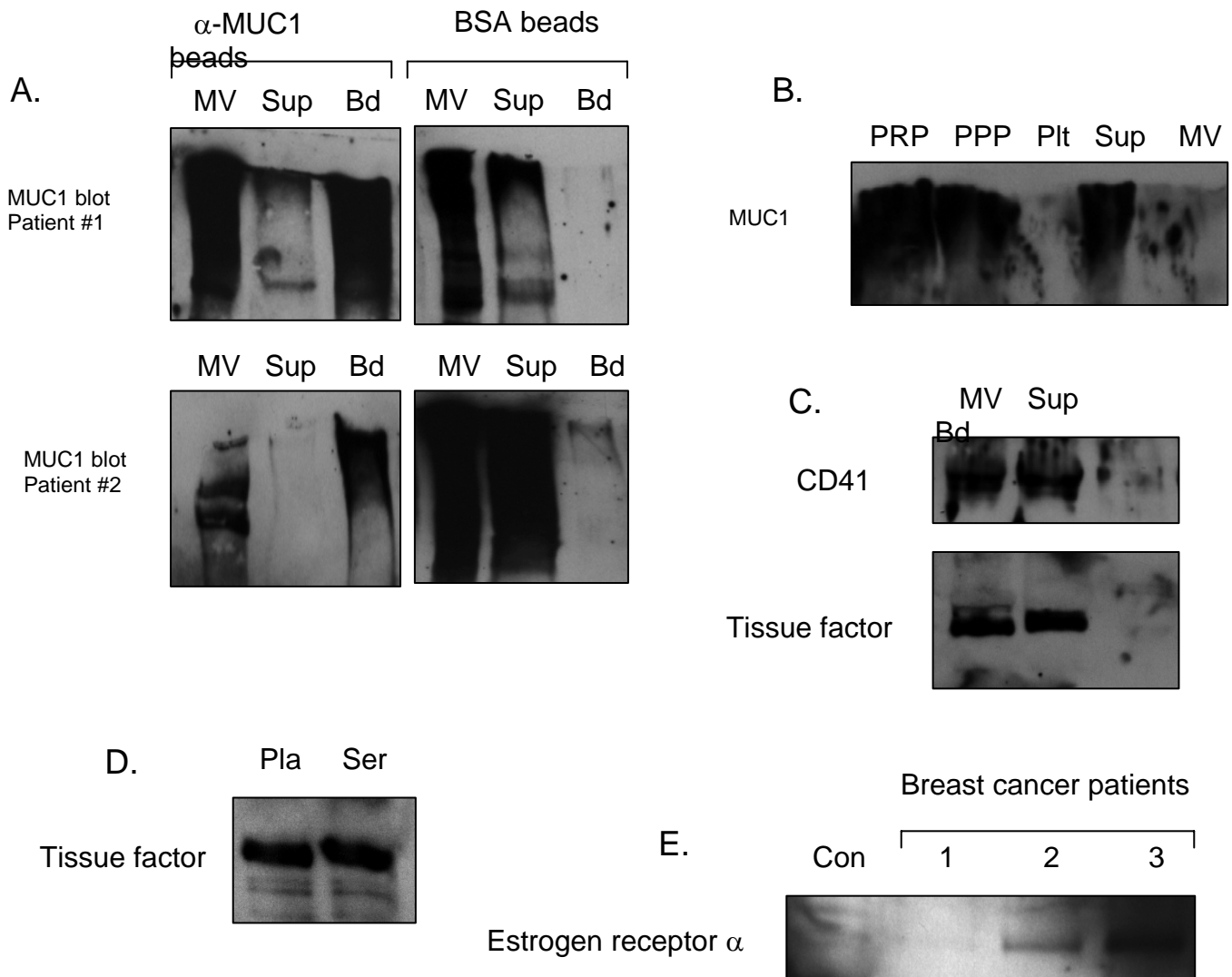
4% sulfate bead solution in 10 mM PBS with 1 ug/ml anti-MUC1 and 1 mg/ml of BSA. The beads are incubated with MV (isolated by differential centrifugation from cell culture supernatants, or clinical samples) overnight at 4° C. The beads are collected and washed by low speed centrifugation. The MV can then be eluted from the beads using routine detergent lysis buffer and analysed by western blot (see below). For our clinical



**Figure 2. Western blot of unfractionated breast cancer plasma MV obtained by differential centrifugation.** This complex mixture of microvesicles includes monocyte and tumor markers (tissue factor and MMP-2), exosomes (CD63), apoptotic bodies (transferrin receptor), and platelet MV (CD41). In our assay we will use commercially available ELISA to quantify MUC1, tissue factor, MMPs, and uPA, as described in our original application. In addition, this novel capture assay has the advantage for future studies of isolating tumor MV for tumor DNA or RNA analysis (4), or for specialized functional *in vitro* assays such as invasion or angiogenesis. (These functional assays would probably require a non-detergent-based MV elution procedure.)



**Figure 3. Cartoon of tumor MV bead capture procedure.**



**Figure 4. Characterization of tumor MV obtained using MUC1 bead**

**capture.** A. Unfractionated MV (MV), MUC1 bead-bound MV (Bd), and the cleared MV supernatant after bead capture (Sup) were blotted for MUC1 protein. MUC1 is a very large smear of protein up to  $1 \times 10^6$  kD and above. MUC1 beads were compared to BSA-only control beads in two patients. MUC1 MV quantitatively and specifically bound to the MUC1 beads. B. Example of MUC1 distribution in one breast cancer patient. MUC1 was present in platelet rich plasma (PRP), platelet poor plasma (PPP), and supernatant after MV isolation (Sup). MUC1 was not present in the platelet pellet, or in the MV in this patient. C. Breast cancer MUC1 MV do not express CD41 or tissue factor. D. Unfractionated MV are physically unchanged during conversion from plasma to serum (e.g. the MV still pellet as discrete particles at 21,000 x g for 20 minutes). E. Plasma MUC1-expressing MV were obtained from one normal control (Con) and three breast cancer patients and blotted for ER $\alpha$ . Patients 1 and 2 were clinically described as ER- and patient 3 as ER+.



Figure 4 shows the western blot characterization of this novel tumor MV capture procedure. Several important points can be demonstrated from this figure-

1. MUC1-expressing tumor MV can be quantitatively depleted from unfractionated MV preparations. This is a specific capture, since control beads coated with only BSA do not capture MUC1 MV.

2. Quantitation of MUC1 tumor MV is not the same as quantitation of soluble MUC1 in plasma. In the patient specimen shown in Figure 4B, for instance, the bulk of the plasma MUC1 is not in the MV fraction. We hypothesize that soluble serum or plasma MUC1 reflects tumor burden, while tumor MV (quantified by MUC1 ELISA) reflects metastatic potential.

3. Our tumor MV bead capture procedure is specific for tumor MV, since we do not capture any platelet (CD41) MV (Figure 4C). Interestingly, in all patients studied with our capture assay so far (about 10 patients) all the tissue factor is in non-MUC1 expressing MV. This preliminary result was not expected.

4. Preparation of MV from frozen serum rather than frozen plasma does not effect the integrity of MV (Figure 4D). This is very important for future work since many large, well-characterized tissue banks of breast cancer patient serum exist. For the current project, we will continue to study only plasma, however.

5. MUC1-expressing MV reflect the tumor of origin. For instance, in the preliminary result shown in Figure 4D, patient #3 was described as ER+, and this patients MUC1 MV have the highest ER protein expression.

Task 2. Test the ability of plasma tumor MV to predict response to treatment and subsequent relapse in locally advanced breast cancer.

We have developed and set in place mechanisms with the clinic staff to identify patients for possible enrollment in our study, and to alert us to up-coming clinic visits of those subjects already enrolled. Now that we have a characterized assay protocol ready, we have begun enrolling patients and preparing and freezing isolated tumor MV from patients with locally advanced breast cancer. We have enrolled 6 patients since our IRB application was formally approved by DOD 2/24/07. Samples will be assayed by ELISA in batch when we have at least 30 specimens to run at once.

Task 3. Determine if breast cancer MV can be cleared from the blood *in vivo* by the use of immunotherapy.

We will begin to enroll patients in this task in the upcoming year.

## Key Research Accomplishments

1. Development of a novel breast cancer MV capture protocol.
2. Characterization of MUC1-expressing tumor MV, including demonstration of lack of tissue factor on these tumor MV, and demonstration of non-equivalence of soluble serum MUC1 and MV MUC1.

## Reportable Outcomes

1. Development of a novel breast cancer MV capture protocol.
2. No manuscripts have been published from DOD-funded research in the first year of our grant. We anticipate submitting a manuscript on the MUC-1 MV capture protocol and characterization soon. However, our evolving expertise in tumor biology afforded by the DOD support contributed indirectly to our efforts on the following work published this year:

Wakchoure S, Merrell MA, Aldrich W, Millender-Swain T, **Harris KW**, Triozzi P, and Selander KS. Bisphosphonates inhibit the growth of mesothelioma cells in vitro and in vivo. *Clinical Cancer Research*, 12:2862-2868, 2006.

Merrell MA, Ilvesaro JM, Lehtonen N, Sorsa T, Gehrs B, Rosenthal E, Chen D, Shackley B, **Harris KW**, and Selander KS. Toll like receptor-9 agonists promote invasion of human breast cancer cells by increasing matrix metalloproteinase-13 activity. *Molecular Cancer Research*, 4:437-47, 2006.

Merrell MA, Wakchoure S, Ilvesaro JM, Zinn K, Gehrs B, Lehenkari PP, **Harris KW**, and Selander KS. Differential effects of Ca<sup>2+</sup> on bisphosphonate-induced growth inhibition in breast cancer and mesothelioma cells. *European Journal of Pharmacology*, 559:21-31, 2007.

Wang D, Liu Z, Li Q, Cao H, Dronadula N, Rizvi F, Kundumani-Sridharan V, Bajpai A, Zhang C, Muller-Newen G, **Harris KW**, and Rao GN. An essential role for gp130 in neointima formation following arterial injury. *Circulation Research*, 100: p. 807-816, 2007.

Ilvesaro JM, Merrell MA, Swain TM, Davidson J, Zayzafoon M, **Harris KW**, Selander KS. Toll like receptor -9 agonists stimulate prostate cancer invasion *in vitro*. In press, *The Prostate*, 2007.

## Conclusion

We have developed a novel bead capture procedure to isolate and analyze tumor MV from the blood of breast cancer patients. The implications of an easy, precise, and robust clinical assay for circulating tumor MV could include improved detection of early relapse and prediction of metastatic potential of newly diagnosed disease. The assay will also allow quantification of tumor MV in animal studies of breast cancer and improve our understanding of the activity of these particles in patients.

So What Section: This assay could be more clinically useful than the soluble MUC1 assay since the target of the assay (tumor MV) are much more likely to be involved in the process of metastasis than soluble MUC1 (this is because tumor MV are loaded with pro-angiogenic and pro-metastatic proteins). Also, this assay could be more clinically useful than the circulating tumor cell (CTC) assay since the dynamic range of the assay represents thousands of tumor MV per ml, rather than a small number of CTC in 10 ml of blood. A better understanding of tumor MV could result in therapeutic approaches (such as antibody therapy) to clear these malignant particles from the blood of patients.

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- 4 Meng S, Tripathy D, Shete S, et al. uPAR and HER-2 gene status in individual breast cancer cells from blood and tissues. *Proc Natl Acad Sci USA* 103:17361, 2006.