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A Reconstructed Metastasis Model to Recapitulate the Metastatic Spread In Vitro

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Abstract

Metastasis remains a leading cause of morbidity and mortality from solid tumors. Lack of comprehensive systems to study the progression of metastasis contributes to the low success of treatment. We developed a novel three-dimensional *in vitro* reconstructed metastasis (rMet) model that incorporates extracellular matrix (ECM) elements characteristic of the primary (breast, prostate or lung) and metastatic (bone marrow, BM) sites. A cytokine-rich liquid interphase separates the primary and distant sites further recapitulating circulation. Similar to main events underlying the metastatic cascade, the rMet model fractionated human tumor cell lines into sub-populations with distinct invasive and migratory abilities: 1) a primary tumor-like fraction mainly consisting of non-migratory spheroids; 2) an invasive fraction that invaded through the primary tumor ECM, but failed to acquire anchorage-independence and reach the BM; and 3) a highly migratory BM-colonizing population that invaded the primary ECM, survived in the ‘circulation-like’ media, and successfully invaded and proliferated within BM ECM. BM-colonizing fractions successfully established metastatic bone lesions *in vivo*, whereas the tumor-like spheroids failed to engraft the bones, showing the ability of rMet model to faithfully select for highly aggressive sub-populations with a propensity to colonize a metastatic site. By applying the rMet model to study real-time ECM remodeling, we show that tumor cells secrete collagenolytic enzymes for invading the primary site ECM but not for entering the BM ECM, indicating possible differences in ECM remodeling mechanisms at primary tumor versus metastatic sites.

Keywords

Metastasis; breast cancer; extracellular matrix; tumor microenvironment; solid tumors

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Introduction

Metastasis is responsible for significant morbidity associated with cancer and accounts for 90% of cancer-related deaths [1]. Despite the development of new treatment strategies, five-year survival rates for patients presenting distant site metastases from breast, prostate, and lung cancers remain below 30 percent [2].

Metastasis is a complex multi-step process, whereby the malignant cells escape from the primary tumor, invade through the basement membrane of the tissue, survive in circulation under the conditions of anchorage-independence, and colonize the foreign microenvironment at a secondary site [1]. Paget's seed and soil hypothesis proposes that cancers show the propensity to spread to specific secondary organs [3, 4]. Bone is one of the most common sites of the metastasis from breast, prostate, and lung cancers [3, 4]. Therefore, we focused our studies on bone as a metastatic site.

To successfully colonize the bone marrow (BM), metastatic cells have to find a BM niche that will support their survival and growth. The main niches in the BM are the endosteal and central [5, 6]. The endosteal niche is found at the bone/BM interface where the bone cavity is lined by a thin layer of connective tissue, the endosteum [7], the major extracellular matrix (ECM) components of which are fibronectin and collagen I [8]. The ECM of the human central BM niche is composed of collagens I and IV, fibronectin, laminin, and hyaluronic acid [8, 9]. Moreover, the central BM niche serves as a reservoir of soluble cytokines and growth factors [10, 11].

In vitro methods, such as the scratch assays, transwell migration assays, and invasion assays are commonly used as the surrogate measures of the metastatic capacity of cells [12-14]. However these assays evaluate the ability of cells to migrate on or through a solid substratum, and do not recapitulate the anchorage-independence required for metastatic dissemination through the circulation. Furthermore, the invasion assays, such as Matrigel invasion assay include a single type of ECM, and thus, fail to account for the differences in the ECM composition of the primary and secondary sites [15]. These shortcomings largely limit the use of standard *in vitro* methods to the studies of either dissemination of tumor cells from the primary site or invasion of the secondary organ, but not both.

In vivo models of experimental or spontaneous metastasis are invaluable for the isolation of the metastatic clones [16, 17], however, analysis of individual signaling pathways responsible for the metastatic spread is difficult in an *in vivo* setting. Additionally, the ECM of the mouse BM differs significantly from the ECM of the human BM [8, 18], raising the possibility that cellular populations engrafting mouse bones may differ from those responsible for the human bone lesions. Therefore, new models are needed to fully dissect the molecular events and understand the cellular phenotypes responsible for all steps of metastatic dissemination.

The ability of cells to degrade the ECM of a primary tissue and/or a secondary organ enables tumor cell dissemination and colonization of a distant site [1]. Matrix metalloproteinases (MMPs) serve as molecular mediators of ECM remodeling. Although multiple studies have investigated matrix degradation during metastasis [19-21], simultaneous detection of MMP

activity at both primary and secondary sites has not been possible using currently available systems. Here we present the development of a three-dimensional (3-D) *in vitro* reconstructed metastasis (rMet) model to study tumor metastasis. The unique aspects of the rMet model are incorporation of a liquid interface and the tissue specific matrix compartments that recapitulate the primary tumor site (mammary/prostate gland/lung) and the secondary organ (bone matrix). Addition of the liquid interphase forces the disseminating cells to survive in an anchorage-independent state prior to colonization of the bone marrow matrix, an element not present in the standard migration/invasion assays. The rMet model was used to isolate a population of metastatic cells with a high propensity to adapt to a foreign environment comprising BM-specific ECM elements and cytokines, after disseminating from the primary tumor site. Our data showed that the rMet system recapitulates all steps of the metastatic spread: 1) escape from the primary site, 2) invasion through the basement membrane, 3) survival under conditions of anchorage-independence, and 4) invasion/colonization of a secondary site. Furthermore, the rMet system is designed to take into account the ECM of both the primary and secondary sites, thus overcoming the major limitations of currently used systems. The ability of the rMet model to segregate heterogeneous tumor cells into distinct sub-populations capable of successful BM colonization sets the stage for future studies designed to understand the cellular populations and the genetic and signaling mechanisms responsible for solid tumor metastasis.

Materials and Methods

Cell culture

MDA-MB-231-BO cells were a kind gift from Dr. Laura Mauro (University of Minnesota). Immortalized human fetal bone marrow mesenchymal stem cells (hTERT-MSC) were generated by Dr. Carlotta Glackin (Beckman Research Institute, City of Hope National Medical Center) [22]. MDA-MB-231, MCF7, A549, LNCaP, and PC-3 cell lines were obtained from ATCC.

Human breast cancer cell lines (MDA-MB-231 and MDA-MB-231-BO) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma, St. Louis, MO). MCF7, A549, and LNCaP cells were maintained in RPMI-1640 (Sigma) supplemented with 10% FBS and 1% penicillin/streptomycin. PC-3 cells were grown in F12-K medium (ATCC, Manassas, VA) with 10% FBS. Mesenchymal stem cells (hTERT-MSC) were maintained in Minimum Essential Medium (MEM) alpha modification supplemented with 1% L-glutamine, 10% FBS and 1% penicillin/streptomycin (Sigma). All cell lines were maintained in a 5% CO₂ incubator at 37°C

Preparation of matrices

A 2mg/ml stock solution of rat-tail collagen type I (BD Biosciences, Franklin Lakes, NJ) was diluted in neutralization buffer (100mM HEPES (Sigma) in 2X PBS), pH 7.2-7.4. Reconstructed endosteum (rEnd) was a 63:5.3:1 v/v mixture of 1X PBS without CaCl₂ and MgCl₂ (Sigma), 1mg/ml human plasma fibronectin (Millipore) and 2mg/ml collagen I respectively. Reconstructed bone marrow (rBM) matrix was set-up as a 4:2.5:1 v/v mixture

of Matrigel (BD biosciences), 1mg/ml fibronectin, and 2mg/ml collagen I respectively. Reconstructed lung (rLung) matrix comprised a 2:1 v/v mixture of Matrigel and 1mg/ml fibronectin.

Assembly of the rMet model

Reconstructed bone marrow (rBM)—130 μ l/well of rEnd matrix was added to a 24-well tissue culture plate (BD Falcon) for 1hr at 37°C. The excess liquid was removed and 75 μ l/well of rBM matrix was added to the well and incubated for 1hr at 37°C. After the matrix formed a semi-solid layer, 1ml of warm BM conditioned medium (BMCM) was overlaid on top. BMCM was obtained by collecting BMGM (BM growth medium: RPMI 1640 supplemented with 6.2×10^{-4} M CaCl_2 , 1×10^{-6} M sodium succinate, 1×10^{-6} M hydrocortisone, 20% FBS, and 1% penicillin/streptomycin) that was conditioned by cultures of hTERT-MSC cells for 3 days.

Mammary/prostate 3-D model set-up in the transwell—mammary gland and prostate ECM microenvironment was set up in 24-well format cell culture inserts (8 μ m pores; BD Biosciences). Matrigel (23 μ l) was mixed with breast or prostate cancer cells at 2.5×10^4 cells/7 μ l PBS/transwell. The Matrigel/cell mixture was pipetted into the cell culture insert and allowed to gel at 37°C for 30min. Subsequently, each insert was placed in a well of a 24-well plate where the rBM matrix was previously set-up. Finally, the matrix/cell mixture in the transwell was overlaid with 0.5ml of warm epithelial cell growth medium [EGM: RPMI-1640 supplemented with 1% horse serum (Sigma) and 1% penicillin/streptomycin].

Lung 3-D model set up in the transwell—For studying lung cancer metastasis in the rMet model, 23 μ l rLung matrix was mixed with A549 lung adenocarcinoma cells at 2.5×10^4 cells/7 μ l PBS/transwell, as described above.

Microscopy

Cells were cultured in the rMet model for 12-14 days and brightfield images of top, invasive and rBM-colonizing fractions were taken using Zeiss AxioObserver inverted microscope equipped with Axiovision software 4.7.3 (Zeiss). Zeiss Axiovert 40C inverted microscope was used to observe cell migration through the rMet.

Cell viability assay

After 12-14 days of culture in the rMet model, tumor-like, invasive, and rBM-colonizing fractions were stained using the LIVE/DEAD viability/cytotoxicity kit (Life Technologies, Carlsbad, CA), per manufacturer's instructions. Briefly, cells were incubated with 1 μ M of calcein AM and ethidium homodimer-1 for 30min at 37°C. Calcein and ethidium-generated fluorescence was imaged within 1hr of staining on a Zeiss AxioObserver microscope using 493nm and 528nm filter sets respectively. Images were edited for brightness, contrast or size, and scale bar was added using ImageJ software (version 1.46r; NIH).

Xenograft model

MDA-MB-231-BO cells were cultured in the rMet model for 14 days. Tumor-like or rBM-colonizing cells isolated from the rMet model were injected into the left ventricle of NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (Jackson Labs), using a 27½-gauge needle. Mice were anesthetized using isoflurane (2.0-2.5% in 3.0L/min O₂; VetEquip tabletop vaporizer). All procedures were approved by the Purdue Animal Care and Use Committee. Animals were sacrificed using CO₂ asphyxiation/cervical dislocation when they presented with weakness, loss of mobility, weight loss, or appearance of palpable tumors >1cm³. During necropsy, images of organ involvement and metastatic lesions were acquired using Canon Powershot A650 IS digital camera.

In situ zymography

To detect matrix metalloproteinase (MMP) activity cells were cultured in the rMet model as described above, with DQ-FITC collagen I or IV (Life Technologies) incorporated into the Matrigel and rBM matrices at 50µg/ml per manufacturer's instructions. MMP secretion was monitored over 14 days with fluorescence microscopy using a Zeiss AxioObserver inverted microscope. At each time point images were acquired at the same exposure time at an excitation wavelength of 495nm. Images were edited for brightness, contrast, and size using ImageJ (version 1.46r; NIH) or Adobe Photoshop CS6 extended (version 13.0).

Statistical analysis

At least three independent biological replicas were performed for each experiment. Time taken by metastatic or non-metastatic cells to initiate colonization in the rBM was compared using a chi-square test. Statistical significance for initiation of symptoms of metastatic disease and presence of HLA positive cells in the BM, was determined using unpaired t-test and paired t-test respectively. Data were represented as mean±SD. All analyses were done using GraphPad Prism software (version 5.0a for Mac; San Diego, CA; www.graphpad.com) and p<0.05 values were considered significant. Hinton diagrams were created using Matlab (MathWorks, Natick, MA) release 2012a with Netlab toolbox.

Results

Reconstructed metastasis (rMet) model recapitulates the complexity of solid tumor metastasis

We have developed a comprehensive 3-dimensional (3-D) *in vitro* reconstructed metastasis (rMet) model that recapitulates all steps of tumor metastasis in the context of the tissue-specific ECM. The presence of specific ECM components enables successful expansion of human tumor cells and preserves the cell-cell interactions similar to those *in vivo* [23]. Hence, we incorporated organ-specific ECM components into the rMet model to study solid tumor metastasis in a physiologically relevant manner. To reconstruct the microenvironment of the mammary gland, human mammary epithelial cancer cells were embedded in Matrigel, placed inside a tissue culture insert, and overlaid with epithelial cell growth medium (EGM) (Fig. 1A). Matrigel is a collagen IV and laminin-rich commercial source of ECM and its composition resembles the basement membrane of the mammary gland [24]. To recapitulate

the BM microenvironment, a tissue culture plate was coated with rEnd matrix (collagen I and fibronectin) followed by an overlay with reconstructed BM (rBM) matrix (Matrigel, fibronectin, and collagen I) [8, 23]. We have previously shown that the ECM components used to set-up the rBM supported growth and proliferation of the primary bone marrow cells (from both healthy donors and patients with multiple myeloma), and when grown in matrices with different compositions (i.e. Matrigel alone, fibronectin, laminin, collagen I, collagen IV), the primary bone marrow cells did not survive [23]. Therefore, we used rBM to mimic the ECM of the bone marrow. To evaluate the importance of rEnd for rBM colonization, it was omitted from a set of cultures, and we observed a 50% decrease in rBM colonization by the breast cancer cells (data not shown). Interestingly, omission of rEnd from the cultures of primary BM cells drastically diminished the robustness of stromal outgrowth [23]. This suggests that rEnd is an important component of the rMet system. To evaluate any structural differences between the Matrigel and rBM that may affect cellular behavior, cryo-SEM was performed on solidified matrices (**Fig. 1B and 1C**). Both matrices provided a honey comb-like scaffold with the only observed difference in their structures being the presence of short, hair-like projections formed by Matrigel.

To complete the rBM set-up in the bottom chamber and to generate a ‘circulation-like’ environment, BM conditioned medium (BMCM), a cytokine and growth factor-rich medium, was added on top of the rBM (**Fig. 1A**). BMCM was collected from the cultures of hTERT-MSC, immortalized human BM mesenchymal stem cells, thus the factors secreted by BM stroma were present in the “bottom” compartment of the rMet model where the BM environment was being reconstructed (Materials and Methods). We have also tested the system with the human BM stromal cells plated in the rBM matrix to determine if the presence of stromal cells enhances engraftment capacity of the metastatic breast cancer cells. No difference was observed between cultures with conditioned medium versus co-cultures with stromal cells. However, a 3-fold reduction in colonization efficiency was observed when non-conditioned medium was overlaid over the rBM matrix instead of the BMCM (p-value=0.03).

To determine the specificity of the rMet system to study solid tumor metastasis, we chose MDA-MB-231 (human breast adenocarcinoma) cells and their bone-seeking variants, MDA-MB-231-BO, both of which demonstrated high metastatic capacity *in vivo* [17, 25]. As a control, we used human breast adenocarcinoma, MCF7, cells that are non-metastatic *in vivo* [25]. Culturing metastatic MDA-MB-231 and MDA-MB-231-BO cells in the rMet model gave rise to 3 distinct fractions: ‘tumor-like’, ‘invasive’ and ‘rBM-colonizing’ (**Fig. 2A**). The ‘tumor-like’ fraction consisted of spheroids trapped within the Matrigel in the transwell. The ‘invasive’ fraction comprised cells that invaded through the Matrigel and formed a monolayer on the transwell membrane. Finally, the ‘rBM-colonizing’ population invaded through the Matrigel, survived in the BMCM under the conditions of anchorage-independence, and colonized the rBM matrix. In contrast, nonmetastatic MCF7 cells formed tumor spheroids that remained in the upper chamber of the rMet system. By days 12-14 in the rMet, MCF7 cells gave rise to a minor invasive sub-population, but were unable to colonize the rBM matrix below the transwell.

Next, we determined the time taken by each cell line to start colonizing the rBM matrix. Based on the viability studies, it was determined that if the cells present in the rBM remained circular, and did not spread-out in the matrix, they eventually lost viability. Therefore, the criteria for positive rBM colonization was set as the presence of >10 non-circular cells within the rBM layer at the end of the 14 day culture period. MDA-MB-231 and MDA-MB-231-BO cells started to colonize as early as day 2 post-plating, whereas MCF7 cells failed to colonize by day 14 (**Fig. 2B**). To determine the viability of the 'tumor-like', 'invasive', and 'rBM-colonizing' fractions, the cultures were co-stained with calcein AM and ethidium homodimer-1. All fractions consisted of calcein AM positive, and thus, live cells, demonstrating that tumor cell sub-populations arising in the rMet model are viable for at least 12-14 days in the presence of the ECM components (**Fig. 2C**). To determine the proliferative potential of the rBM-colonizing population, we removed the transwell on day 8 and continued to culture the disseminated cells for additional 2 weeks (**Supplementary-figure 1A**). The rBM-colonizing cells proliferated over the course of 2 weeks and gave rise to two sub-populations: cells that formed small tumor-like spheroids within the rBM matrix (**Supplementary-figure 1B**) and cells that migrated through the rBM matrix and formed a monolayer at the rEnd (**Supplementary-figure 1C**). Thus, the rBM colonizing cells were viable and proliferating, representing a population capable of seeding distant site metastases *in vivo*. Taken together, the rMet model is set-up to reconstruct all the steps of metastatic dissemination: 1) invasion through tissue-specific ECM at the primary site, 2) dissemination in an anchorage-independent state in the liquid interface between the matrix recapitulating the primary tumor and the secondary organ, and 3) colonization of the BM-specific ECM. The unique features of the rMet model are the inclusion of the tissue-specific matrices of both the primary and secondary site, and incorporation of a liquid interphase to select for cells capable of surviving in an anchorage-independent state.

rMet model can be adapted to study metastatic dissemination of various tumor types

Prostate and lung cancers also show frequent incidence of bone metastasis, therefore, we used the rMet model to evaluate the rBM-colonization capacity of non-metastatic (LNCaP) and metastatic (PC-3) prostate cancer cell lines and a metastatic lung adenocarcinoma cell line (A549). Since the ECM composition of the prostate gland is similar to that of the mammary gland [26, 27], we used the same set up for the rMet model to culture prostate cancer cell lines as was described for the breast cancer cells. However, to culture lung cancer cells, we modified the composition of the matrix inside the transwell to mimic the lung microenvironment, the major components of which are collagen IV, laminin, and fibronectin [28].

Similar to the metastatic breast cancer cell lines, after 14 days in the rMet culture, both the PC-3 and A549 cells were able to give rise to three distinct fractions of tumor-like, invasive, and rBM-colonizing cells (**Fig. 2D**). Both PC-3 and A549 cell lines disseminated to the rBM matrix and started colonizing within 3 days of culture (**Fig. 2E**) and all populations maintained viability throughout the duration of culture (**Fig. 2F**). Similar to the non-metastatic MCF7 cells, LNCaP cells formed tumor spheroids in the cell culture insert (**Fig. 2D**). A few LNCaP cells invaded through the Matrigel and attached to the membrane of the transwell, and occasionally, LNCaP cells were able to form small aggregates of 2-4 viable

cells within the rBM matrix (**Fig. 2D**). Even though such minor population of LNCaP cells was able to disseminate to the rBM matrix, very few viable cells were seen after 14 days of culture and none were able to proliferate in the rBM, further suggesting the inability of these cells to colonize the rBM matrix compared to the metastatic PC-3 cells (**Fig. 2F**).

rBM-colonizing cells from the rMet model preferentially metastasize to the bone *in vivo*

To validate the bone metastatic capacity of the rBM-colonizing cells and to test the difference in the metastatic efficiency of the tumor-like and the rBM-colonizing sub-populations generated in the rMet model, we used a mouse model of experimental metastasis [29]. Based on the cell density and viability, we determined that 14 days in the rMet model was an ideal time point to isolate cells for the *in vivo* studies. Tumor-like and rBM-colonizing MDA-MB-231-BO cells were isolated after 14 days in the rMet culture and injected into NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice via an intra-cardiac route. Although mice injected with both populations exhibited signs of disease such as weight loss and weakness, the time to appearance of first symptoms was significantly earlier for mice injected with rBM-colonizing fraction (mean±SEM = 36 ± 3.2) compared to mice injected with tumor-like fraction (mean±SEM = 55 ± 4.9) (**Supplementary-figure 2A**). Both groups also showed very distinct trends of organ-specific metastases.

Cells injected directly from culture, without fractionation through the rMet, gave rise to lesions in the lungs, liver ovary, kidneys, bone, and occasionally thymuses (**Table 1**). However, injection of rBM-colonizing cells consistently generated tumors originating within the bones (rib cage, humerus). Occasionally, rBM-colonizing cells also gave rise to metastatic lesions in the lungs and ovary (**Table 1; Supplementary-figure 2B**). Mice injected with tumor-like cells presented enlarged thymuses. Occasionally, tumor-like fractions also gave rise to lesions within the lungs, liver, ovaries, and kidneys (**Table 1; Supplementary-figure 2C**). To confirm that the observed lesions were derived from the transplanted human cells, tumor fragments, organs with lesions, and cells flushed from the bones were stained for the human leukocyte antigen (HLA). An HLA⁺ population was detected in the tibias and femurs of animals injected with the rBM-colonizing cells (**Supplementary-figure 3A**), while tumor-like fractions failed to engraft in the bones (**Supplementary-figure 3B**). Furthermore, bone-derived tumors of the humerus and the rib cage from the rBM-colonizing cells consisted of 8-80% HLA⁺ cells (**Supplementary-figure 3C**). Thus, we show that passing tumor cells through the rMet system generates a metastatic population with bone tropism.

Tumor-like, invasive, and rBM-colonizing cells derived from the rMet possess differential collagenolytic activities

Matrix metalloproteinases play an important role in enabling tumor cells to break through the physical barrier of a tissue, mediated by degradation of ECM components present in the basement membrane. While matrix remodeling at the site of the primary tumor has been widely investigated, the studies to understand the colonization step of metastasis have been hindered by the lack of models where the engraftment step can be visualized in real time. In order to determine the ECM remodeling capacity of different cell populations generated within the rMet model, DQ collagens I or IV were used to observe their ability to degrade

collagen. DQ collagen resembles the natural MMP substrate, and when conjugated to FITC, can be used to detect the activity of collagenases in cell culture [30]. To assess the MMP activity in the rMet model, DQFITC collagen I or IV was added to the Matrigel and the rBM matrices and cells were cultured for 14 days. The presence of hydrolyzed collagen substrate was detected by day 3 in MDA-MB-231 and MDA-MB-231-BO, and at day 7 in the PC-3 cultures. Consistent with their lack of migratory ability, MCF7 and LNCaP cells did not exhibit MMP activity throughout the duration of culture (**Table 2**).

As cells disseminated through the rMet system matrix remodeling correlated with cell migration. Fluorescence was first observed in the cells migrating out of the transwell followed by the rBM-colonizing cells with its intensity increasing over the course of the culture (**Table 2**). Consistent with the heterogeneity of the metastatic cell lines where only a subset of cells have migratory capacity, only a fraction of cells in the tumor-like and invasive fractions exhibited MMP activity (**Figure 3**). This data demonstrate that the rMet model is a versatile system that can be used to study the metastatic process in its entirety and can be adapted to multiple tumor types and various metastatic sites.

Discussion

Metastatic spread is the most common cause of death from the solid tumors [1, 2], emphasizing the necessity to gain a thorough understanding of the molecular events that can be exploited therapeutically. Physiologically relevant systems must be developed to investigate the molecular mechanisms during each step of metastasis: escape from the primary site, survival in an anchorage-independent state, and colonization of a secondary site. The major shortcomings of the currently used *in vitro* models of experimental metastasis, such as migration and invasion assays, are a lack of a proper physiological context (i.e. use of a single type of ECM, or no ECM at all) and failure to incorporate all stages of metastatic spread (i.e. not taking into consideration the requirement for adhesion-independence for metastatic cell dissemination from the primary site to the secondary organ). Additionally, *in vivo* mouse models cannot provide real time view of molecular processes leading to metastatic colonization. Considering these shortcomings, we developed a reconstructed metastasis (rMet) model, where the distinct microenvironments of the primary and the secondary sites are incorporated into the same assay to recapitulate the major phases of metastasis, including the anchorage-independent step.

The matrix composition in the upper and lower chambers of the rMet was set up to mimic ECM protein expression of the primary organ (mammary and prostate glands, lung) and the distant site (BM) [24, 26, 27]. Disseminating cells are those that traverse the ECM in the upper chamber and successfully colonize the rBM, while invasive cells are those that have the capacity to migrate through the ECM, but are incapable of surviving detached from the solid substratum. The unique BMCM component of the rMet model allows us to differentiate between these two cell types. Of the 6 human breast, prostate, and lung cancer cell lines tested here, 4 with known metastatic capacity were able to colonize the rBM, while MCF7 and LNCaP cells that fail to metastasize *in vivo*, remained within the transwell or attached to its membrane. Consistent with the ability of the metastatic lesions to grow at a distant site, the rBM colonizing population was seen expanding over time. These results

demonstrate that the rMet model enables segregation of distinct populations of tumor cells into fractions with differential patterns of migration. Furthermore, the presence of the liquid phase, where cells have to survive in an anchorage-independent state, indicates its ability to capture the complexity of the metastatic process. By varying the ECM composition to recapitulate the primary tumor site of interest (i.e. breast/prostate, lung), we have demonstrated the versatility of the rMet model to study metastasis of tumors of different origins. Given that shear stress encountered by the disseminating cells in circulation likely affects cell viability, future generations of the rMet model will incorporate a dynamic liquid interphase to mimic true circulatory flow.

The presence of cellular subsets in the rMet that mimic disseminating populations required confirmation of their metastatic ability. Therefore, we introduced tumor-like or rBM-colonizing fractions into the arterial circulation of immunocompromised mice to ensure that the cells bypass the lungs, where the intravenously-injected cells get trapped [16]. As expected, the rBM-colonizing cells formed metastatic lesions, consistently giving rise to skeletal metastases (humerus/clavicle/rib cage), while the cells isolated from the transwell became trapped in the nearby tissue, i.e. the thymus. Importantly, cells from the top fraction failed to form bone lesions, suggesting that the colonization of the rBM layer of the rMet enriches for a bone-engrafting population. Although the cells remaining inside the transwell are mostly non-migratory, a subpopulation of disseminating cells remains present in the top fraction shown by the ability of tumor-like fraction to continuously “shed” rBM-colonizing cells (data not shown). Consistent with this finding, the cells from inside the transwell formed low-frequency lesions at distant sites such as lungs, liver, or ovaries. These findings are in agreement with the clinical observations that metastasis can be seeded at any stage of tumorigenesis [31, 32]. Initiation of metastatic disease, measured by occurrence of first signs of disease (weakness, immobility), was significantly faster in mice injected with rBM-colonizing fraction compared to that of mice injected with tumor-like fraction. Although the bone-seeking variants of the MDA-MB-231 cells (MDA-MB-231-BO) used in this study were originally selected for their propensity to metastasize to the bone, our studies (**Table 1**) show that these cells, in addition to skeletal metastases, exhibit a wide range of metastatic spread with lesions observed in the lungs, liver, ovaries, adrenal glands, and occasionally in the lymph nodes (**Table 1**). Therefore, the ability of the rMet system to fractionate a heterogeneous population to enrich for cells with a propensity to form skeletal lesions, demonstrates the capacity of the rMet model to mimic the metastatic process.

Engraftment of cells in the thymus was unexpected given the infrequent nature of thymic metastases with only a single case study reporting metastasis to the thymus from each breast, prostate, and lung cancers [33-35]. Thus, we concluded that the thymic lesions in mice injected with the cells from the transwell were not bona fide metastatic lesions, but an artifact of the intra-cardiac injection where the cells engrafted the most proximal organ with favorable ECM. The ECM of the thymus is similar to that of the BM, where the major components are fibronectin, laminin, and collagen IV [36] and has the direct connection to the left ventricle, the site of intra-cardiac injection, through the aorta and via the subclavian and thoracic arteries. This explanation for occurrence of thymic masses is further confirmed by additional *in vivo* studies where tumor-like MDA-MB-231-BO cells never resulted in

thymic masses when they were introduced subcutaneously near the flank of NSG mice, to test metastatic spread from an orthotopic site (data not shown).

ECM degradation is a major mechanism of tumor dissemination, and thus, is an important molecular event contributing to the metastatic spread of various tumors [1, 19-21]. We used the rMet model to study the patterns of ECM degradation by metastatic and non-metastatic cancer cells. By incorporating fluorescently conjugated DQ collagen I or IV into the Matrigel or rBM matrices, we showed that collagenases were secreted by breast and prostate cancer cells as a means to invade the basement membrane and disseminate from the 'primary tumor' site. Not surprisingly, tumor cell lines without metastatic capacity failed to degrade the ECM and spread through the rMet. However, the main contribution of the rMet model to the study of metastasis is the ability to observe not only the invasion step, but also the colonization of a secondary site, something not feasible using the conventional model systems. Interestingly, a fluorescent signal was not observed in the rBM fraction until day 10, while first cells appeared to come through the rMet system by 2-3 days post plating. It is possible that the fluorescent signal from the degradation of matrix by a few rBM-colonizing cells is below the limit of detection of this assay. However, it is also possible that the rBM-engrafting cells do not infiltrate the matrix via direct ECM degradation, and MMP secretion is only required as the cell population expands over time and spreads through the matrix. This supposition is further confirmed by studies that suggest that the metastatic site may be "set-up" prior to the arrival of disseminating cells [37, 38].

In conclusion, the comprehensive, yet modular set-up makes the rMet model a versatile tool for any solid tumor under native conditions in a real-time manner. Due to integration of organ-specific ECM components and accurate recapitulation of metastatic events, the rMet model will also be a physiologically relevant tool for future studies focusing on dissecting molecular events, genetic mechanisms, and pre-clinical drug-testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

ECM	extracellular matrix
rMet	reconstructed metastasis
BM	bone marrow
MMP	matrix metalloproteinases (MMPs)

rBM	reconstructed BM
EGM	epithelial cell growth medium
BMCM	BM conditioned medium
rEnd	reconstructed endosteum

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C

Figure 1. Reconstructed metastasis model recapitulates the complexity of solid tumor metastasis
A, Schematic diagram demonstrating the basic components and the assembly of the rMet model. Matrigel was combined with human cancer cells and the mixture was added to a cell culture transwell with 8µm pores, which was then inserted into a well of a tissue culture plate containing the rBM matrix. To complete a ‘primary tumor site’ set up in the cell culture insert, the cell/Matrigel mixture was overlaid with EGM. To assemble the microenvironment of a ‘distant metastatic site’ BMCM was added to the rBM-containing tissue culture well. **B**, Cryo-scanning electron microscopy images of solidified Matrigel and **C**, polymerized rBM matrix (scale bar: 10µm).

Figure 2. Human cancer cells give rise to distinct fractions in the rMet model

Human breast (A-C), prostate or lung cancer cells (D-F) were cultured for 14 days in the rMet model and imaged at different time points throughout the duration of the culture. All cell lines gave rise to a tumor-like fraction and an invasive fraction consisting of cells growing on the membrane of cell culture insert (dark spots: pores in the membrane) (A-B, D-E). After culturing each cell line for 14 days in the rMet model, each fraction was stained with the LIVE/DEAD kit to test cell viability (C, F). At least three independent biological replicas were performed.

A, Metastatic MDA-MB-231 and MDA-MB-231-BO cells colonized the rBM matrix (scale bar: 100µm). B, A Hinton diagram showing the time taken by each cell line to start colonizing the rBM matrix. Presence of healthy, attached, non-circular cells in the rBM matrix was considered as initiation of colonization in the rBM matrix. Size of each box is proportional to the number of replicates where cells were seen to have colonized the rBM

matrix at a given time point. Total number of samples from all independent biological replicas is denoted as 'n'. MCF7 cells never colonized the rBM matrix over the entire course of the culture. Chi-squared test was used to analyze the timing of rBM colonization by MDA-MB-231 or MDA-231BO versus MCF7 cells (*p-value<0.0001). **C**, Tumor-like, invasive, and rBM-colonizing fractions consisted of viable cells. (green: calcein AM positive, live; red: ethidium homodimer-1 positive, dead) (scale bar: 100µm). **D**, Metastatic, PC-3 and A549, cells gave rise to rBM-colonizing fractions, while, non-metastatic, LNCaP, cells failed to colonize the rBM matrix. **E**, A Hinton diagram showing the time taken by each cell line to start colonizing the rBM matrix. Presence of healthy, attached, non-circular cells in the rBM matrix was considered as initiation of colonization in the rBM matrix. Total number of samples from all independent biological replicas is denoted as 'n'. Chi-squared test was used to analyze the timing of rBM colonization by PC-3 compared to LNCaP prostate cells (*p-value=0.0002). **F**, Majority of the cells in all three fractions were viable (green: calcein AM positive, live; red: ethidium homodimer-1 positive, dead) (scale bar: 100µm).

Figure 3. MDA-MB-231-BO cells cultured in the rMet model display collagenolytic activity
MDA-MB-231-BO cells were cultured in the rMet model in the presence of FITC-labeled DQ Collagen I for 14 days. MMP secretion, visualized by the green fluorescence was observed as a portion of the green fluorescent cells as early as day 3 in the rMet in both the tumor-like and invasive fractions. At day 7, MMP secretion was still evident in the tumor-like and invasive fractions, but in addition, a small population of cells that migrated to the rBM by day 7 was also positive for MMP secretion. By day 14, there were populations of MMP secreting cells in all three fractions of the rMet model (scale bar: 100 μ m).

Table 1

Organ involvement after intracardiac injection of tumor-like and rBM-colonizing cell populations.

<i>Cell Fraction</i>	<i>Cells injected</i>	<i>Number of mice with metastatic lesions</i>					
		Bone	Thymus	Lungs	Liver	Ovary	Kidney
<i>Non-fractionated</i>	1×10^5 (n=2)	2/2 *	1/2	1/2	1/2		
	5×10^3 (n=2)	2/2 *#	1/2		2/2	2/2	1/2
<i>Tumor-like</i>	1×10^5 (n=1)		1/1				
	6×10^4 (n=2)		2/2	1/2	1/2		
	2×10^4 (n=1)		1/1				
	1×10^4 (n=4)		2/4	1/4	2/4	2/4	1/4
<i>rBM-colonizing</i>	1×10^5 (n=1)	1/1 *					
	6×10^4 (n=2)	2/2 +					
	2×10^4 (n=2)	2/2 *		1/2			
	1×10^4 (n=2)	2/2 *		1/2		1/2	

* rib cage

femur

+ humerus/clavicle

Table 2

Collagenolytic activity of cell lines cultured in the rMet model over a 14-day period.

Collagen I	Day 3			Day 5			Day 7			Day 10			Day 14		
	Tumor-like	Invasive	rBM	Tumor-like	Invasive	rBM	Tumor-like	Invasive	rBM	Tumor-like	Invasive	rBM	Tumor-like	Invasive	rBM
MCF7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
231 ^a	+	+	-	++	+	-	+	+	-	+++	++	++	+++	+++	++
231-BO ^b	+	+	-	++	+	-	+++	+	-	+++	+++	++	+++	+++	++
LNCaP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PC-3	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+

Collagen IV	Day 3			Day 5			Day 7			Day 10			Day 14		
	Tumor-like	Invasive	rBM	Tumor-like	Invasive	rBM	Tumor-like	Invasive	rBM	Tumor-like	Invasive	rBM	Tumor-like	Invasive	rBM
MCF7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
231 ^a	+	+	-	++	+	-	+	+	-	++	++	+	+++	+++	++
231-BO ^b	+	+	-	++	+	-	+++	+	-	+++	+++	-	+++	+++	++
LNCaP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PC-3	-	-	-	-	-	-	+	-	+	+	-	+	+	-	+

The scoring system of MMP activity was based on fluorescence intensity of cleaved DQ-FITC collagen (20 images, n=3 independent replicas per condition). Since the fluorescence intensity correlates with MMP activity we used the following scale: no detectable activity (-); weak activity (+); moderate activity (++); strong activity (+++). Gelatinolytic activity was also tested in all cell lines and results closely resembled collagenolytic activity (data not shown).

^aMDA-MB-231 cell line
^bMDA-MB-231-BO cell line