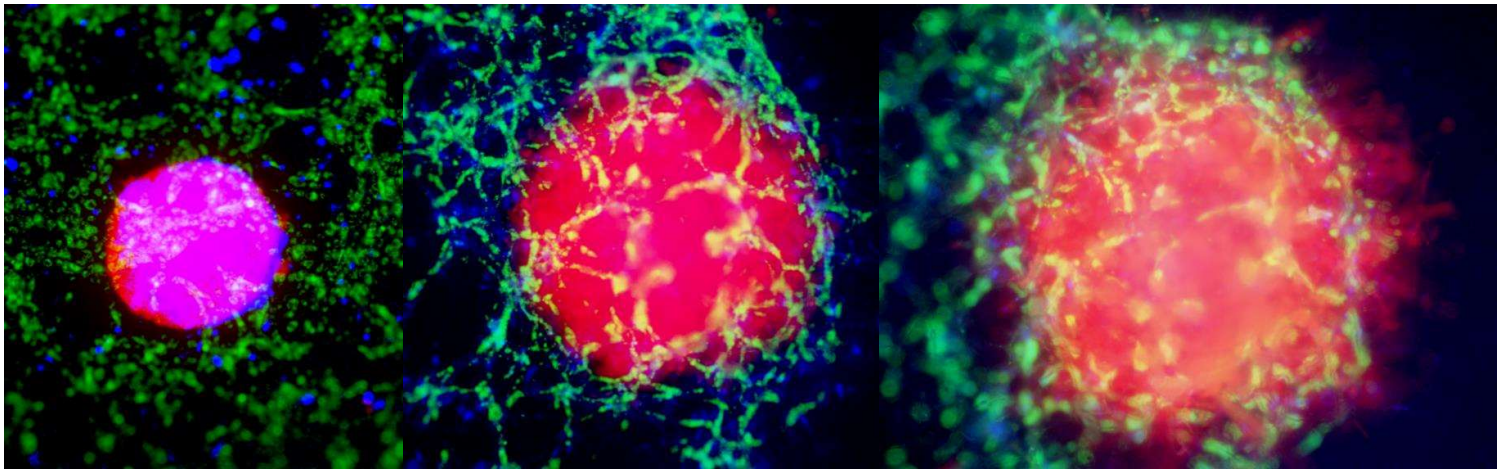
3D Triculture Model for Evaluating Breast Cancer Progression

- **Breast cancer cell lines (MCF7 / MDA-MB-231)**
- **Human Umbilical Vein Endothelial Cells (HUVECs)**
- **Human adipose-derived Mesenchymal Stem Cells (hMSCs)**

Application Note

Overview

Current models for evaluating breast cancer progression lack a comprehensive, physiological approach to modeling the complex tumor microenvironment. There has been much evidence supporting the use of tumor spheroids to mimic tumor physiology. In the outer layers, they exhibit cell-cell bond formation, comparable morphology, elevated cell survival and proliferation; whereas in the inner layers, they have reduced proliferation rates and a hypoxic core. While Multi-Cellular Tumor Spheroids (MCTS) provide a physiological tumor model, other cell types within the surrounding tumor microenvironment are essential for tumor behavior and subsequent cancer progression. The tissue vasculature provides a critical component for tumor progression given the metabolic requirements of a growing tumor and known tumor-vascular interactions, and by incorporating endothelial tubules with the MCTS, we are able to model interactions between vascular networks and growing tumors. At the same time, there is also an important stromal component involved in cancer progression where stromal cells have been shown to promote cell proliferation, dissemination, and drug resistance during cancer development. By using extracellular matrix proteins, we are able to promote the proper physiology for each of these cell types and assess their activities. To evaluate interactions, each cell type is fluorescently labeled with fluorophores with different excitation and emission spectra; MCF7 and MDA-MB-231, human breast cancer cell lines, express a red fluorescence protein, while human umbilical vein endothelial cells (HUVECs) and human adipose-derived mesenchymal stem cells (hMSCs) are labeled with stable lipophilic membrane dyes. MCTS are formed using low adhesion microwells and deposited into fully formed HUVEC tubular networks, and the hMSCs are then added within a hydrogel overlay matrix. Cellular interactions and dissemination are monitored via fluorescence microscopy, and breast cancer cell proliferation is monitored using a fluorescence plate reader. The breast cancer MCTS exhibit cell-cell interactions with endothelial tubules and stromal cells, and there is an increase in both cell proliferation and invasion over standard spheroid monoculture model.



Background

Many anticancer drugs fail in human trials despite showing efficacy in in vitro studies and in animal models. It has become clear that the in vitro assays involving 2D monoculture do not reflect the complex cellular and matrix microenvironment of the tumor tissue, and this may explain the failure of 2D models to predict clinical efficacy (1). The 3D environment of the tumor has a different architecture; cells are adherent on all surfaces; there is stiffness to the matrix; and the matrix is tumor-specific and unique (2,3). Furthermore, it is well known that by changing the in vitro culture conditions to accommodate some of these aspects of the tumor microenvironment, the biological response of the cells is altered (4-6). Development of an in vitro screen that exhibits the physiology of the tumor will provide a more accurate assessment of candidate drugs for cancer therapy, saving time and money for drug development and delivering more effective therapies for patients.

The ideal model would incorporate extracellular matrix, cellular, and biochemical components that are present in tumor tissues. Extracellular matrix proteins provide specific structural and cell binding motifs that direct gene expression determining protein production, attachment, morphology, motility, and survival (7). The basement membrane underlies epithelial and endothelial tissues while collagen is the primary component of the mesenchyme. Basement membrane extract (BME) promotes a tumor phenotype while collagen I promotes a motile phenotype (8). Basement membrane proteins can be used to promote assembly of multicellular tumor spheroids (MCTS), Figures 1A-1D, that exhibit the appropriate size and structure to create the physiological gradients present in tumors providing a more predictive response , and mixtures of BME and collagen I may be used to model a metastasizing tumor (9,10). Similarly, these proteins direct the assembly of endothelial cells into vascular, tubule networks, Figure 1C, giving rise to in vivo structure and function (11-13). The addition of endothelial cells and other cell types provide factors that influence tumor cell behavior. For example, studies with cocultures on BME have already shown that breast cancer cells are more invasive when either resident adipose-derived stem cells, Figure 1D, or macrophages are present (14,15) and that stromal cells increase proliferation (16). Tumor microenvironments also exhibit acidity (low pH), hypoxia (low O2), and low glucose concentrations (17,18). By incorporating all of these elements into one model, we can more accurately recapitulate the tumor microtissue to provide a more predictive model for evaluating drug response.

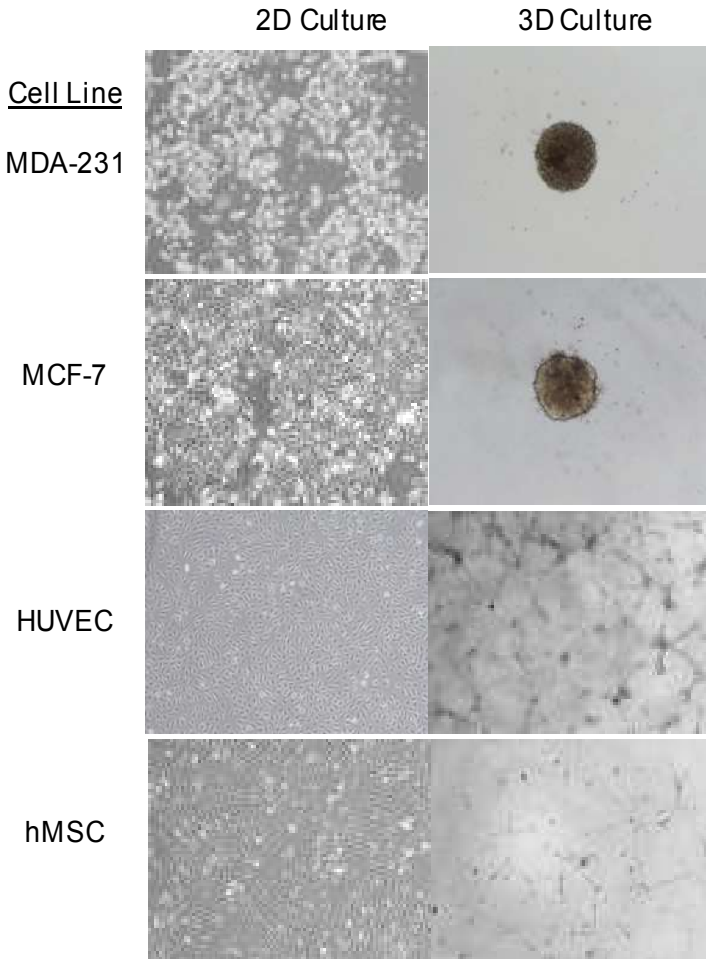


Fig 1. The morphology for different cell types are more physiological in 3D than 2D culture.

In 2D culture, breast cancer cells, MDA-MB-231 (A) and MCF-7 (C), grow in clusters and exhibit a cobblestone morphology, similar to HUVECs (E) and hMSC (G). In 3D culture, breast cancer MCTS, MDA-MB-231 (B) and MCF-7 (D), most accurately depict tumors based on size and the establishment of physiological gradients. HUVECs form tubules representative of vascular networks (F), and hMSCs form branching structures (H).

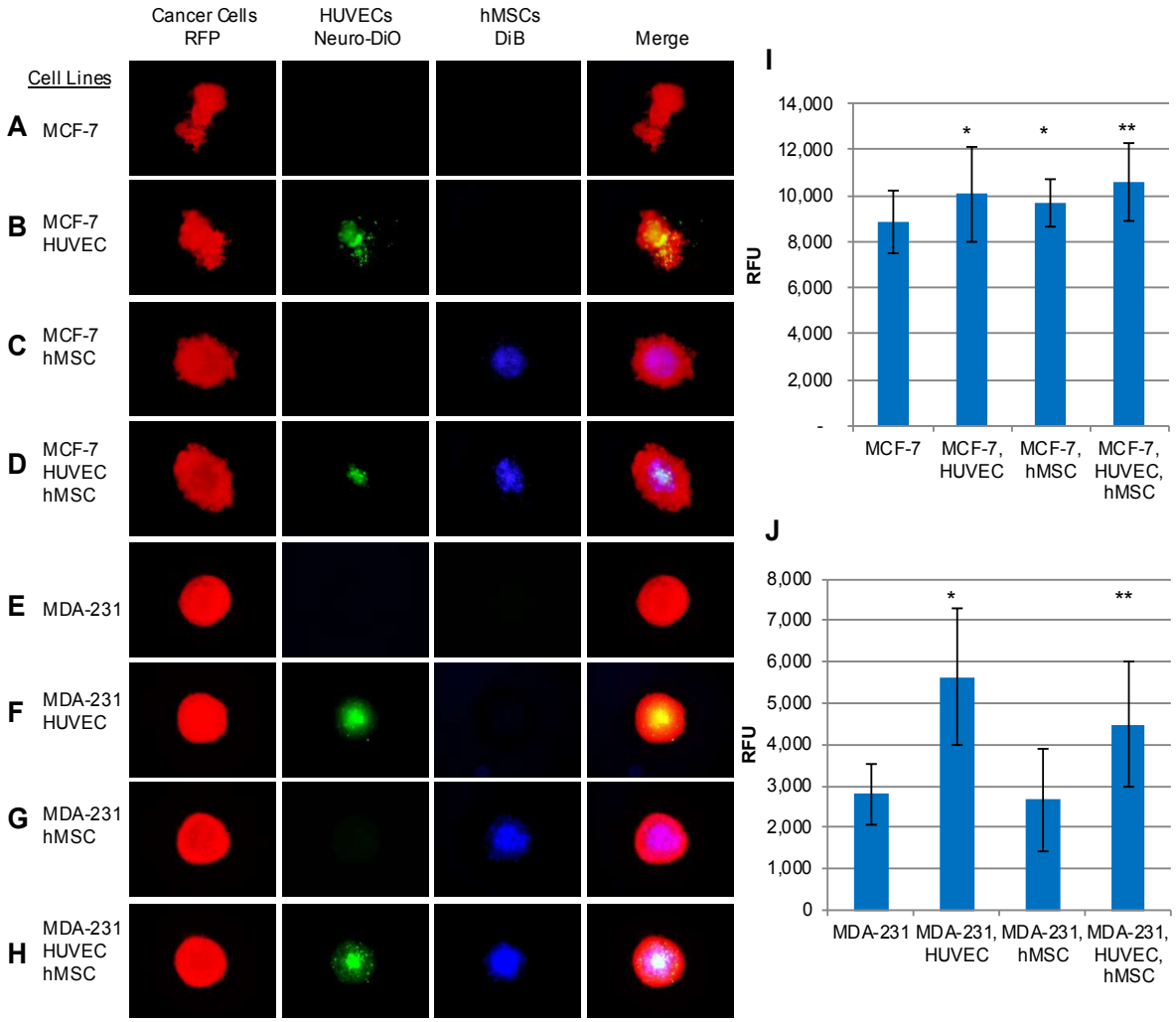


Fig 2. Coculture of breast cancer cells, HUVECs, and hMSCs during MCTS formation increases breast cancer cell proliferation.

*MCTS were formed in 96 well low adhesion plates using breast cancer cells (2,000 cells/well); HUVECs (500 cells/well); and/or hMSCs (500 cells/well) over a 72 hour period. MCF-7 (A-D) and MDA-MB-231 (E-H) expressed red fluorescent protein; HUVECs (B,D,F,H) were labeled with Neuro-DiO (green); and hMSCs (C,D,G,H) were labeled with DiB (blue). Plates were read in a 96 well plate reader at excitation 540 nm/ 587 nm emission to compare proliferation of MCF-7 (I) and MDA-MB-231 (J). *P < 0.05, **P < 0.01.*

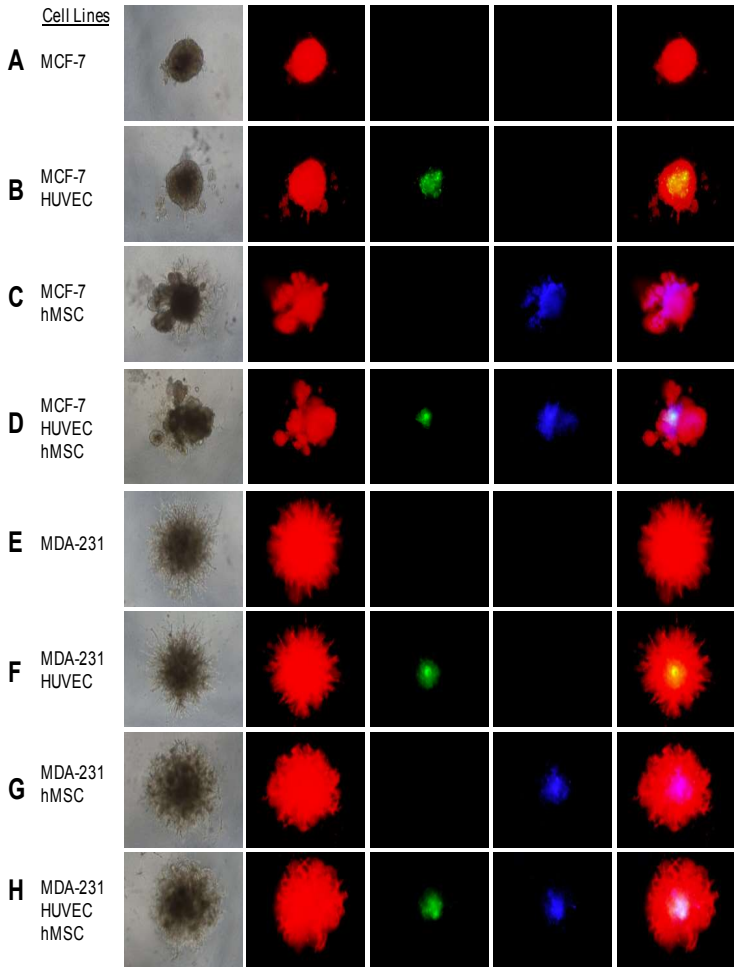
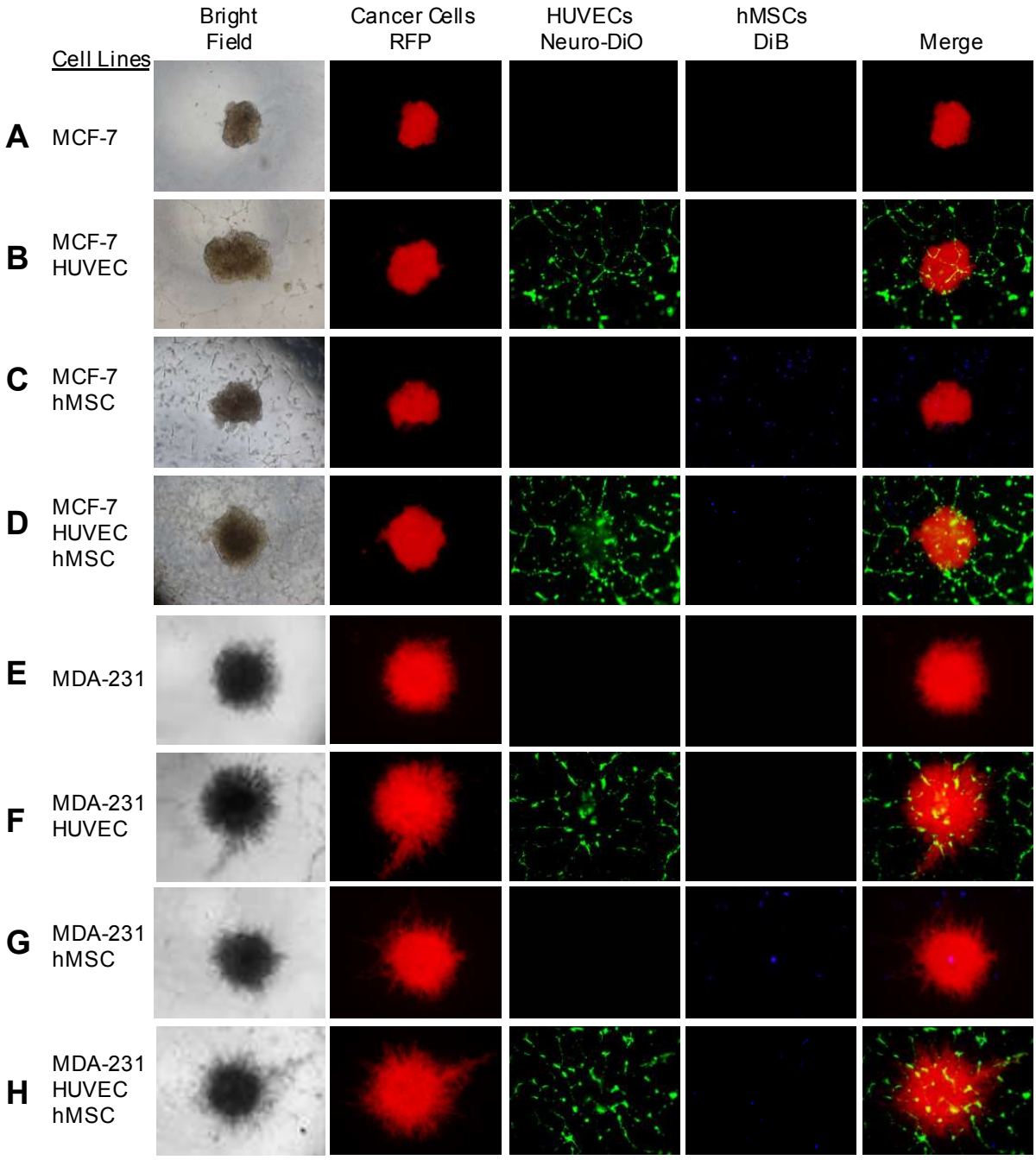


Fig 3. Coculture of Breast Cancer Cells, HUVEC, and hMSC for MCTS formation and subsequent invasion into ECM hydrogels demonstrate that MCTS containing hMSC promote a more physiological phenotype.

MCTS were formed in 96 well low adhesion plates using breast cancer cells (2,000 cells/well); HUVECs (500 cells/well); and/or hMSCs (500 cells/well) over a 72 hour period. MCTS were then embedded within an ECM hydrogel to facilitate cell invasion over 96 hours. MCF-7 (A-D) and MDA-MB-231 (E-H) expressed red fluorescent protein; HUVECs (B,D,F,H) were labeled with Neuro-DiO (green); and hMSC (C,D,G,H) were labeled with DiB (blue).


Bright Field
Cancer Cells RFP
HUVECs Neuro-DiO
hMSCs DiB
Merge
Cell Lines
A MCF-7
B MCF-7 HUVEC
C MCF-7 hMSC
D MCF-7 HUVEC hMSC
E MDA-231
F MDA-231 HUVEC
G MDA-231 hMSC
H MDA-231 HUVEC hMSC

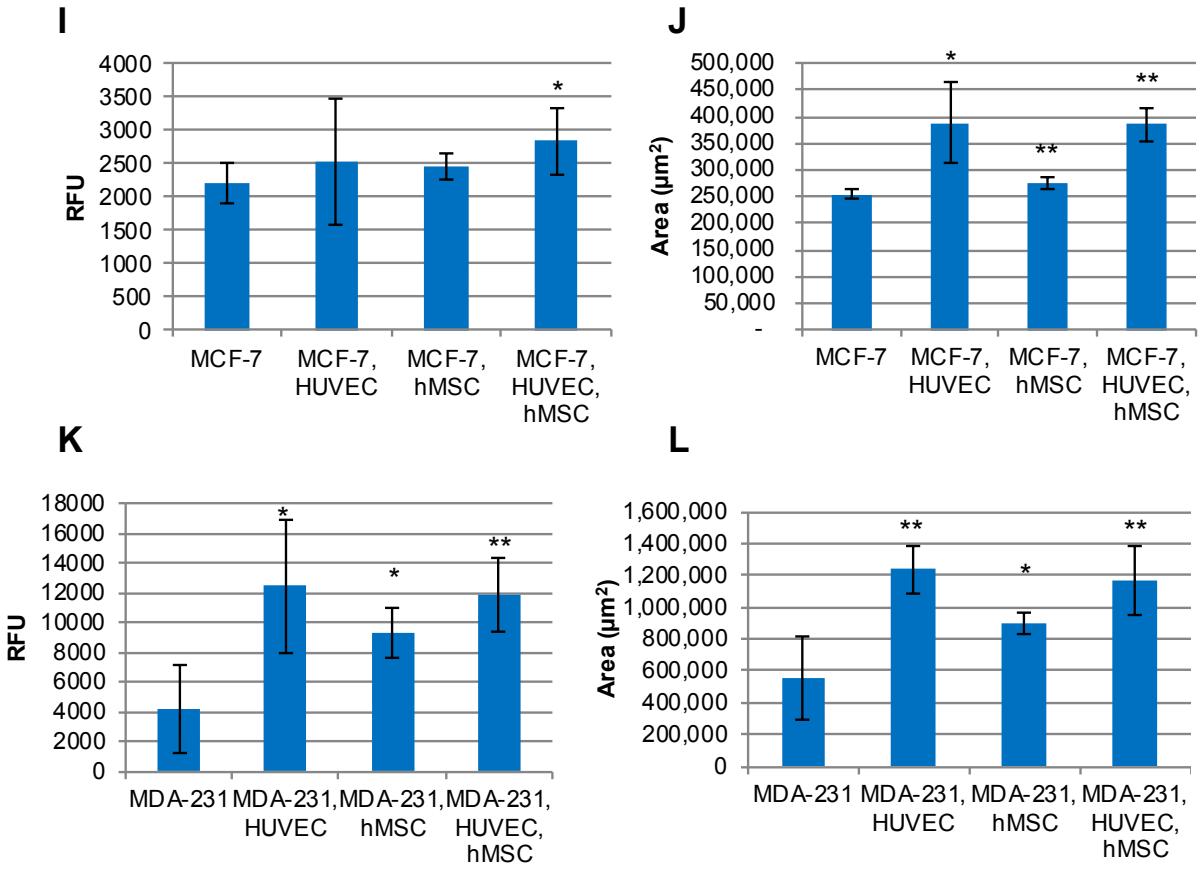


Fig 4. Tricultures of MCTS, hMSC, and endothelial tubules increase proliferation and invasion for breast cancer spheroids.

*MCTS were generated by culturing breast cancer cells (2,000 cells/well) at 37 °C under hypoxia in 96 well, ultra-low adhesion plates for 72 hours. Then 96 well, flat bottom plates were coated with 50 µl of tubule formation matrix and incubated for one hour at 37 °C, 5% CO2 to polymerize hydrogel. For HUVECs, 12,500 cells were added to each well (B,D,F,H), and for remaining samples, EGM-2 was added. HUVECs were allowed to assemble into tubules for two hours. One MCTS, MCF-7 (A-D) or MDA-MB-231 (E-F), was transferred to each of the wells in the plate containing tubules. MCTS were allowed to settle for one hour. At this time, 100 µl of medium was aspirated from each well. hMSCs were suspended in invasion matrix (10,000 cells/ml), and 50 µl was added to each well (C,D,G,H). For the remaining samples, 50 µl of tumor aligned Invasion Matrix was added to each well. The plates were then incubated at 37 °C, 5% CO2 for one hour to polymerize hydrogel, and 100 µl of tumor aligned RPMI, 10% FBS as added to each well. Cultures were conducted under hypoxia for 96 hours and photographed using bright field, TRITC, FITC, and DAPI filters (A-H), and photographs were analyzed using ImageJ to determine MCTS area (J and L). Plates were then read in a 96 well plate reader at excitation 540 nm/ 587 nm emission to compare proliferation of breast cancer cells (I and K). *P < 0.05, **P < 0.01.*

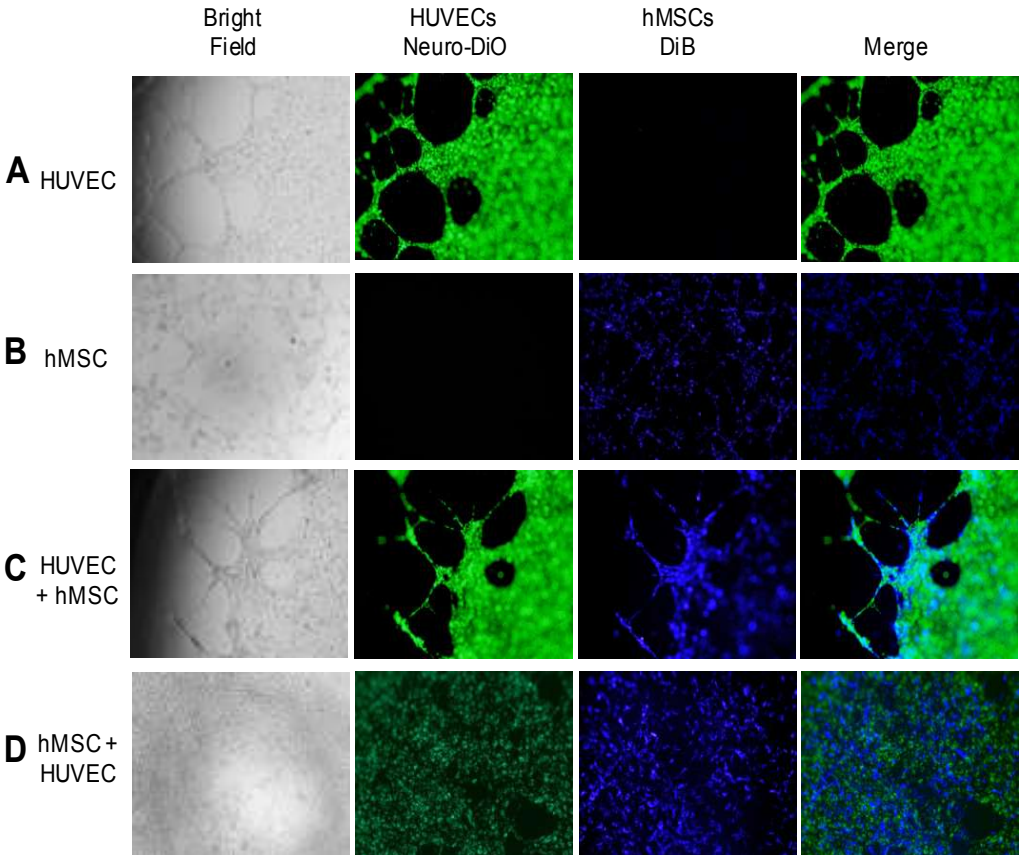


Fig 5. HUVECs and hMSCs colocalize in 3D culture suggesting a role for hMSCs to support and stabilize HUVEC tubules.

96 well plates were coated with 50 µl of BME hydrogel and polymerized for one hour at 37 °C. HUVECs were seeded at 15,000 cells/well (A, C, D), and hMSCs were seeded at 3,000 cells/well (B, C, D). When HUVEC were added first (A, C) tubules would form, and hMSC colocalized with HUVECs (C). When hMSCs were added first, there was no tubule formation (B, D); however, HUVECs colocalized with hMSC (D).

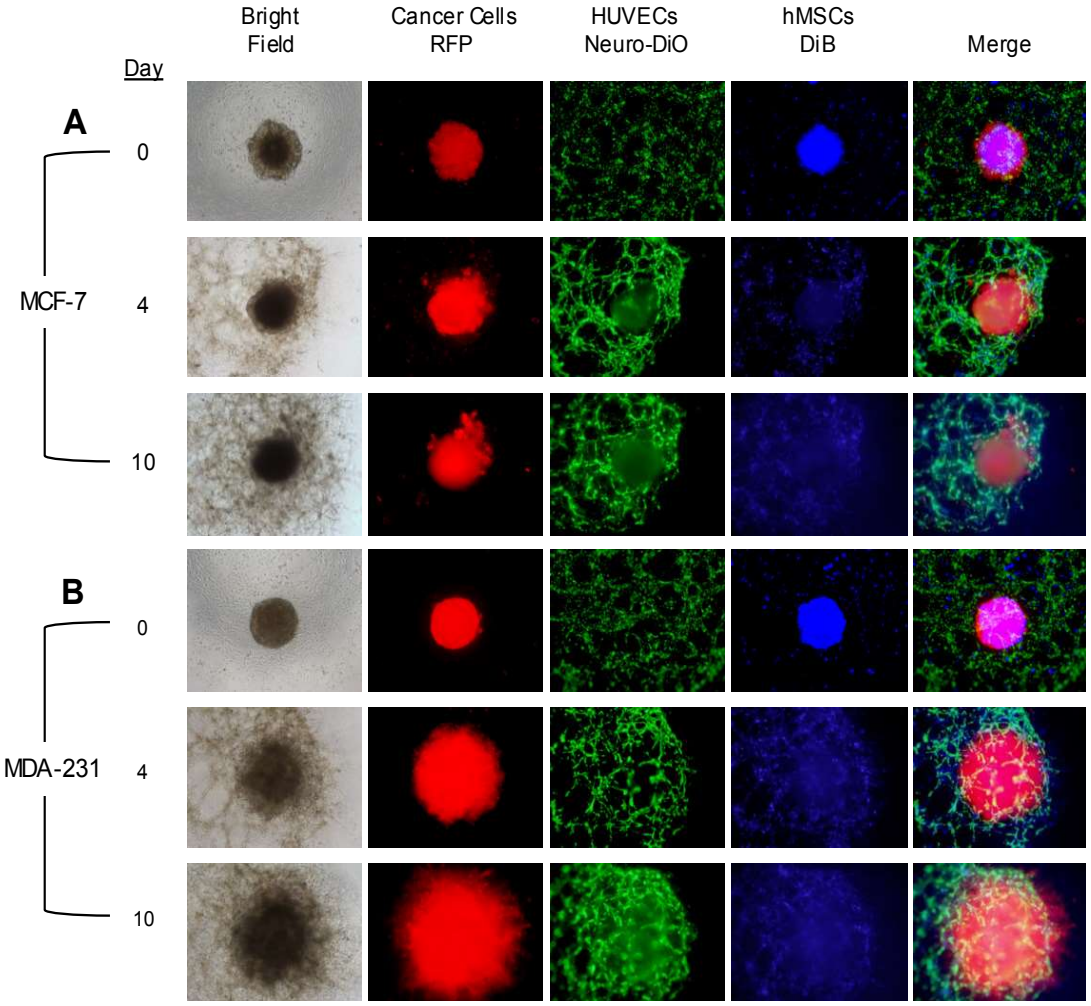


Fig 6. Tricultures of MCTS, hMSC, and endothelial tubules produce physiological breast cancer niche with tumor growth, tumor invasion, and endothelial recruitment.

MCTS were generated by culturing breast cancer cells (2,000 cells/well) and hMSCs (500 cells/well) at 37 °C under hypoxia in 96 well, ultra-low adhesion plates for 72 hours. Then 96 well, flat bottom plates were coated with 50 µl of tubule formation matrix and incubated for one hour at 37 °C, 5% CO2 to polymerize hydrogel. HUVECs (at 12,500 cells/ml) were added to each well and were allowed to assemble into tubules for two hours. hMSCs (1,000 cells/well) were added to HUVEC networks, and incubate for one hour. Then one MCTS was transferred to each well in the 96 well, flat bottom plate. MCTS were allowed to settle for one hour. At this time, 100 µl of medium was aspirated from each well, and 50 µl of Tumor Aligned Invasion Matrix was added to each well. The plates were then incubated at 37 °C, 5% CO2 for one hour to polymerize hydrogel, and 100 µl of Tumor Aligned RPMI, 10% FBS as added to each well. Tricultures were conducted under hypoxia and photographed using bright field, TRITC, FITC, and DAPI filters (A and B) at days 0, 4, and 10.

Summary

By properly mimicking the tumor extracellular environment and cellular architecture, breast cancer Multi-Cellular Tumor Spheroids (MCTS), endothelial tubules, and stromal cells coordinate a physiological response and provide a more predictable model for evaluating clinical efficacy for known effective cancer agents. In developing a functional model, we made the following determinations:

HUVECs and hMSCs provide factors that promote breast cancer proliferation and invasion:

▪ HUVECs and/or hMSCs promote proliferation for MCF-7 (Figure 2A), and HUVECs with or without hMSC promote proliferation of MDA-MB-231 (Figure 2B).

▪ When MCTS were embedded in an invasion matrix, MCTS containing hMSCs promote lobule formation for MCF-7 and an invasive morphology for MDA-MB-231 while there was little discernable difference for MCTS containing HUVECs (Figure 3).

▪ For both MCF-7 and MDA-MB-231 cells, the incorporation of both HUVEC tubules and dispersed hMSC increased both proliferation and invasion (Figure 4).

Direct cell-cell interactions are necessary to elicit the physiological phenotypes:

▪ Dispersed hMSCs (Figure 4) did not induce the same phenotypic properties as experiments where the hMSCs were incorporated into the MCTS (Figure 3).

▪ HUVEC tubules without hMSC appeared quiescent (Figure 4). HUVECs and hMSCs demonstrated an affinity for colocalization, and adding hMSCs directly to preformed HUVEC networks yielded the desired tubule phenotype (Figure 5).

Incorporation of hMSCs into the MCTS and endothelial tubules, separately, provided a more dynamic model, allowing for invasion and proliferation of MCTS with a more physiological tumor phenotype, as well as recruitment of endothelial tubules (Figure 6).

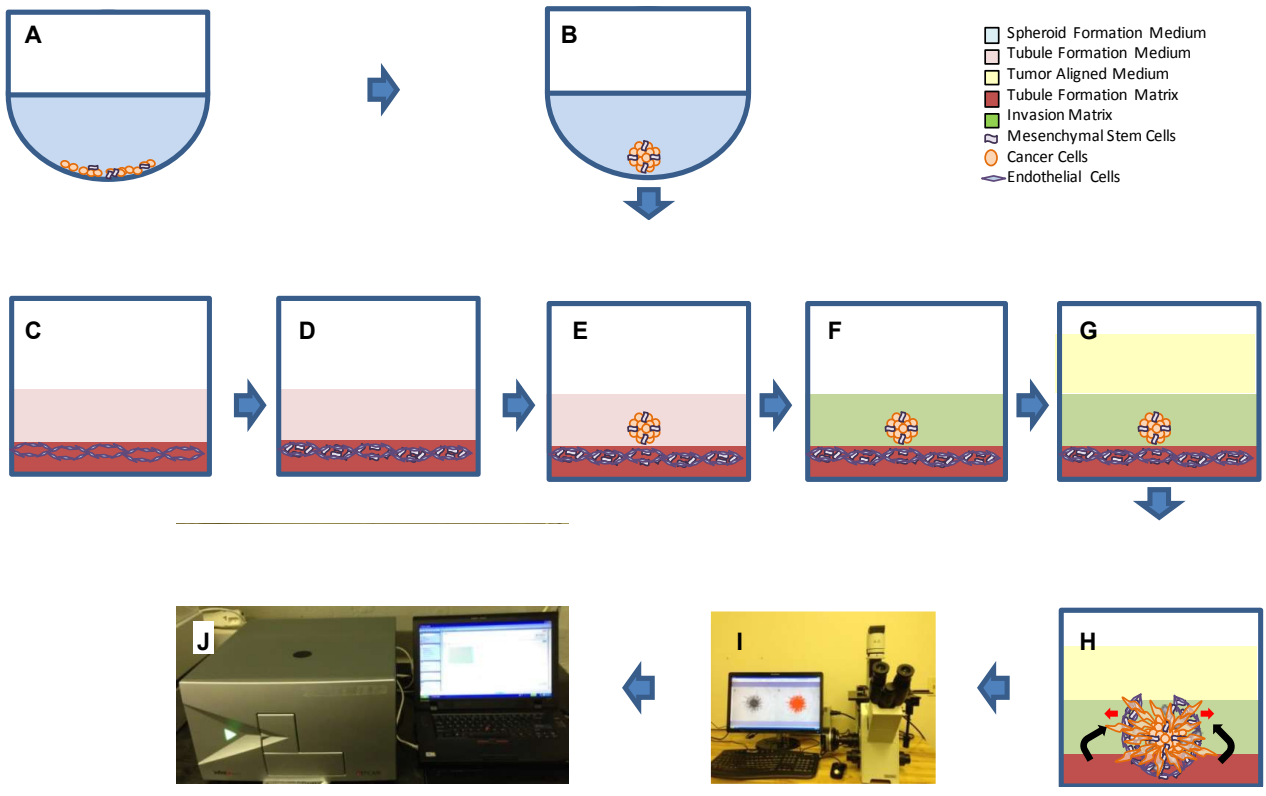


Figure 7. The stepwise process for the 3D triculture assay for evaluating breast cancer progression:

(1) Breast cancer cells and hMSCs in Spheroid Formation Medium are seeded in a low adhesion plate.(2) Over the course of 72 hours MCTS spontaneously assemble.(3) HUVECs form networks on tubule formation matrix over a two hour period. hMSCs are added to HUVEC networks to form tubules.(4) MCTS are transferred on top of the tubules. (5) The MCTS and tubules are embedded in a tumor aligned invasion matrix. (6) Tumor aligned medium with or without treatment is added to culture. (7) Triculture is conducted under hypoxia. Breast cancer cells proliferate and invade; tubules are recruited. (8) Tricultures may be evaluated for spheroid invasion and/or high content screening using microscopy. (9) Tricultures may be evaluated for breast cancer MCTS proliferation by evaluating fluorescence intensity in a plate reader.

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