

Trevigen’s Extracellular Matrix Proteins for Triculture of Breast Cancer Spheroids, Endothelial Tubules, and Human Mesenchymal Stem Cells

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Here we describe the 3D triculture model which recapitulates the breast cancer tumor microenvironment. Such a model will be useful for drug screening, basic research, and personalized medicine providing one of many needed tools to facilitate more physiologically relevant assessments of cancer cell behavior.

Currently, the high cost and limited capacity for bringing new drugs to market is attributed to ineffective methods utilized early in the drug development process. The most commonly used practice involves culturing a single cell type in an artificial environment and evaluating the impact of drug treatment on cell viability. This approach vastly oversimplifies the complexity of the tumor tissue.

Within these monocultures, the cells adhere to hard plastic through non-specific charged interactions, and as a result, the cells grow in monolayers and exhibit a cobblestone-like morphology. Both the conditions and the resulting cultures are unlike a tumor, so why would they function or respond like one?

To craft a predictive model, it must possess all of the key attributes of the system that it represents. At the time of diagnosis, tumors have sufficient size where multiple cell layers restrict diffusion within the tumor leading to the formation of physiological gradients for nutrients, waste, and oxygen. Within the tumor, the environment is glucose-deprived, acidic, and hypoxic [1-3]. The cells within these regions have different metabolic properties, and they exhibit different proliferation rates and therapeutic responses [4]. Also, the tumor extracellular matrix (ECM) undergoes remodeling with increased collagen deposition, resulting in a more rigid environment that favors cancer progression and metastasis [5-7]. Furthermore, interactions between tumor cells and other cells within the existing tissues are also known to influence

cancer progression through both cell-cell and soluble interactions [8-10]. For a cell culture model to accurately predict tumor tissue behavior, it must recreate the tumor microenvironment by taking into account key biochemical, ECM, and cellular properties (Figure 1).

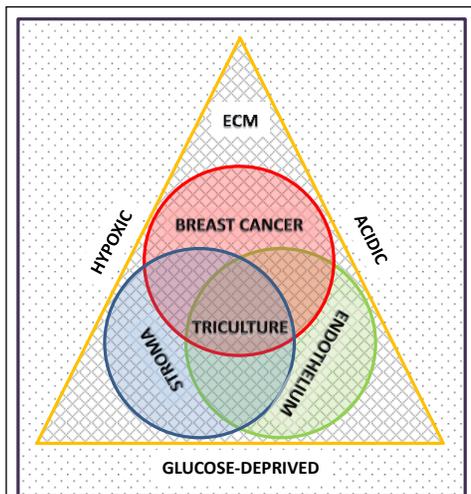


Figure 1. The essential elements of the triculture model are breast cancer cells, endothelial cells, stromal cells, ECM, and tumor-aligned medium.

To recreate the tumor microenvironment, breast cancer cells and stromal cells are assembled into tumor microtissues that are seeded on preformed vascular networks and embedded in a collagen-containing ECM under tumor-aligned conditions which is acidic, hypoxic and glucose-deprived (Figure 2). 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 [11, 12]. 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 [13]. The tissue vasculature provides a critical component for tumor progression given the metabolic requirements of a growing tumor and known tumor-vascular interactions [9], 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 [14-16]. By using ECM 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 (excitation 540 nm/ 587 nm).

Tricultures provide a physiologically relevant model for assessing cancer cell growth and invasion. Breast cancer spheroids develop tumor physiology for MCF-7 (Figure 3A) and MDA-MB-231 (Figure 3B) breast cancer models. Similar to xenograft models, MCF-7 spheroids develop multi-lobular structures [17] and MDA-MB-231 cells develop wavy protrusions [8]. Preformed vascular networks composed of endothelial and stromal cells are stable over extended culture periods, and these contractile vascular networks are

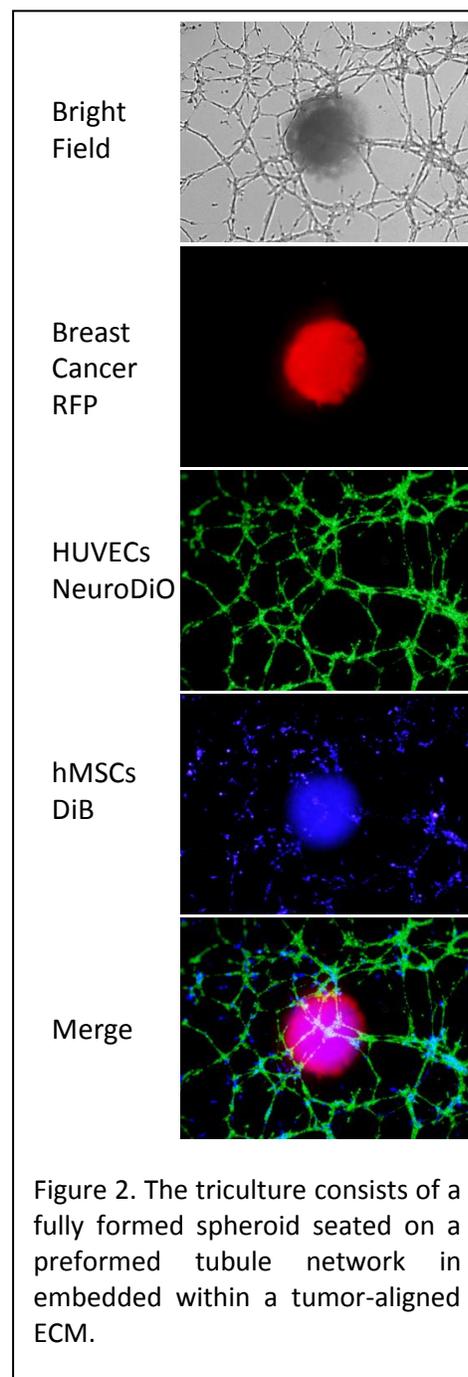


Figure 2. The triculture consists of a fully formed spheroid seated on a preformed tubule network in embedded within a tumor-aligned ECM.

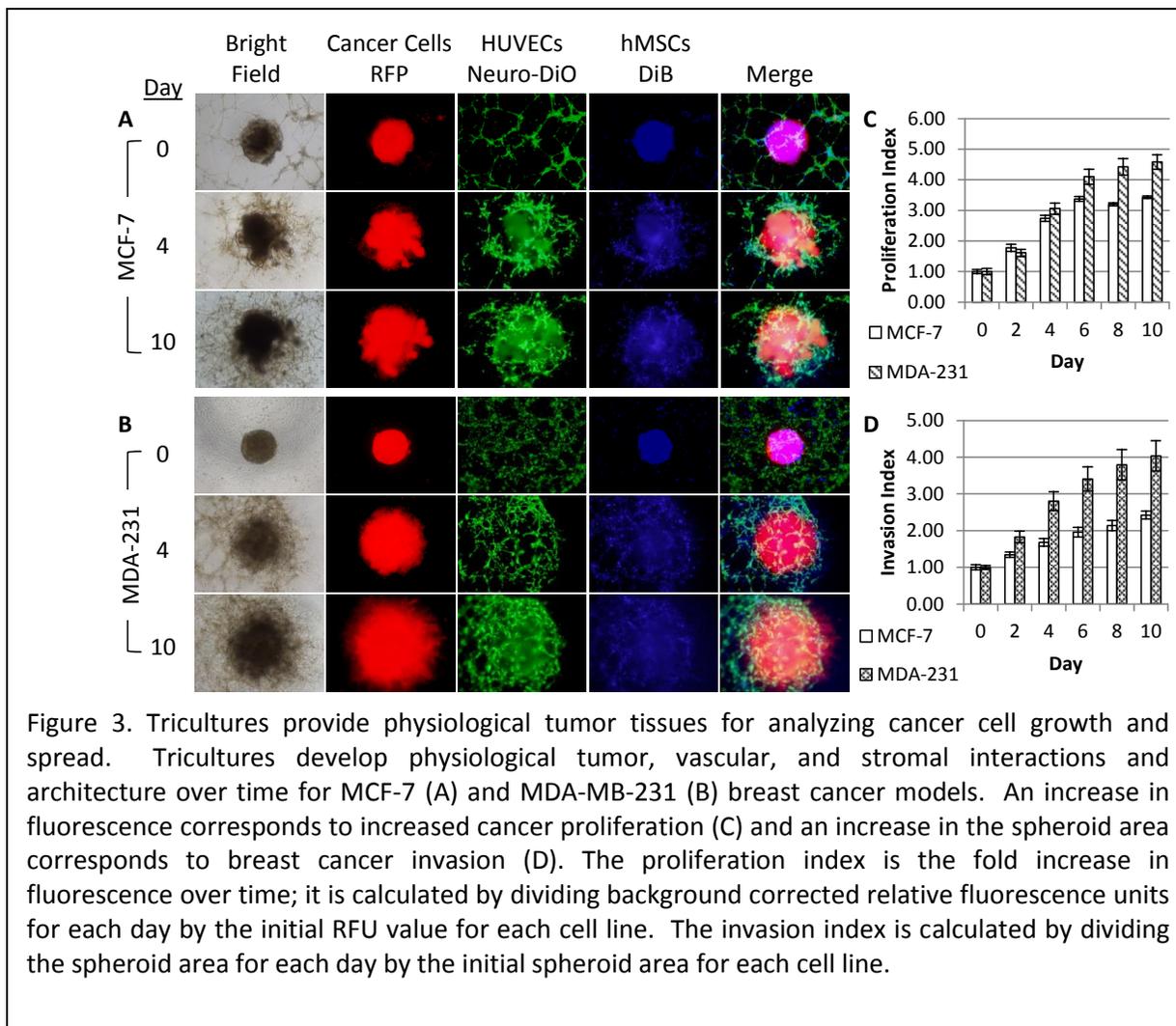


Figure 3. Tricultures provide physiological tumor tissues for analyzing cancer cell growth and spread. Tricultures develop physiological tumor, vascular, and stromal interactions and architecture over time for MCF-7 (A) and MDA-MB-231 (B) breast cancer models. An increase in fluorescence corresponds to increased cancer proliferation (C) and an increase in the spheroid area corresponds to breast cancer invasion (D). The proliferation index is the fold increase in fluorescence over time; it is calculated by dividing background corrected relative fluorescence units for each day by the initial RFU value for each cell line. The invasion index is calculated by dividing the spheroid area for each day by the initial spheroid area for each cell line.

recruited to the expanding tumor microtissue. Stromal cells are recruited to and incorporated into both tumor microtissues and vascular networks. By measuring the relative fluorescent units (RFU) initially, and every 24 hours thereafter, breast cancer microtissue growth can be evaluated (Figure 3C). Doubling times for breast cancer cells within the tricultures are approximately 72 hours, compared to a normal doubling period of 24 hours in 2D culture. The proliferation rates appear to decrease as the tumor microtissue grows; these slower rates are more representative of *in vivo* tumor growth. Similar rates for breast cancer microtissue spreading are observed (Figure 3D).

Tricultures provide phenotypic and quantitative drug response similar to what has been reported *in vivo*. The maximum serum concentrations (C_{max}) achievable for fluorouracil are between 38 μM and 384 μM [18], and the C_{max} values for paclitaxel are between 0.228 μM and 4.27 μM [19]. We treated breast cancer tricultures with 100 μM fluorouracil and 1 μM paclitaxel to represent doses that patient tumors would be exposed to *in vivo*. It has been reported in xenograft models that MCF-7 tumors were resistant to fluorouracil [20], and MDA-MB-231 tumors exhibited moderate sensitivity to fluorouracil [21]. Similarly, MCF-7 xenografts were resistant while MDA-MB-231 xenografts

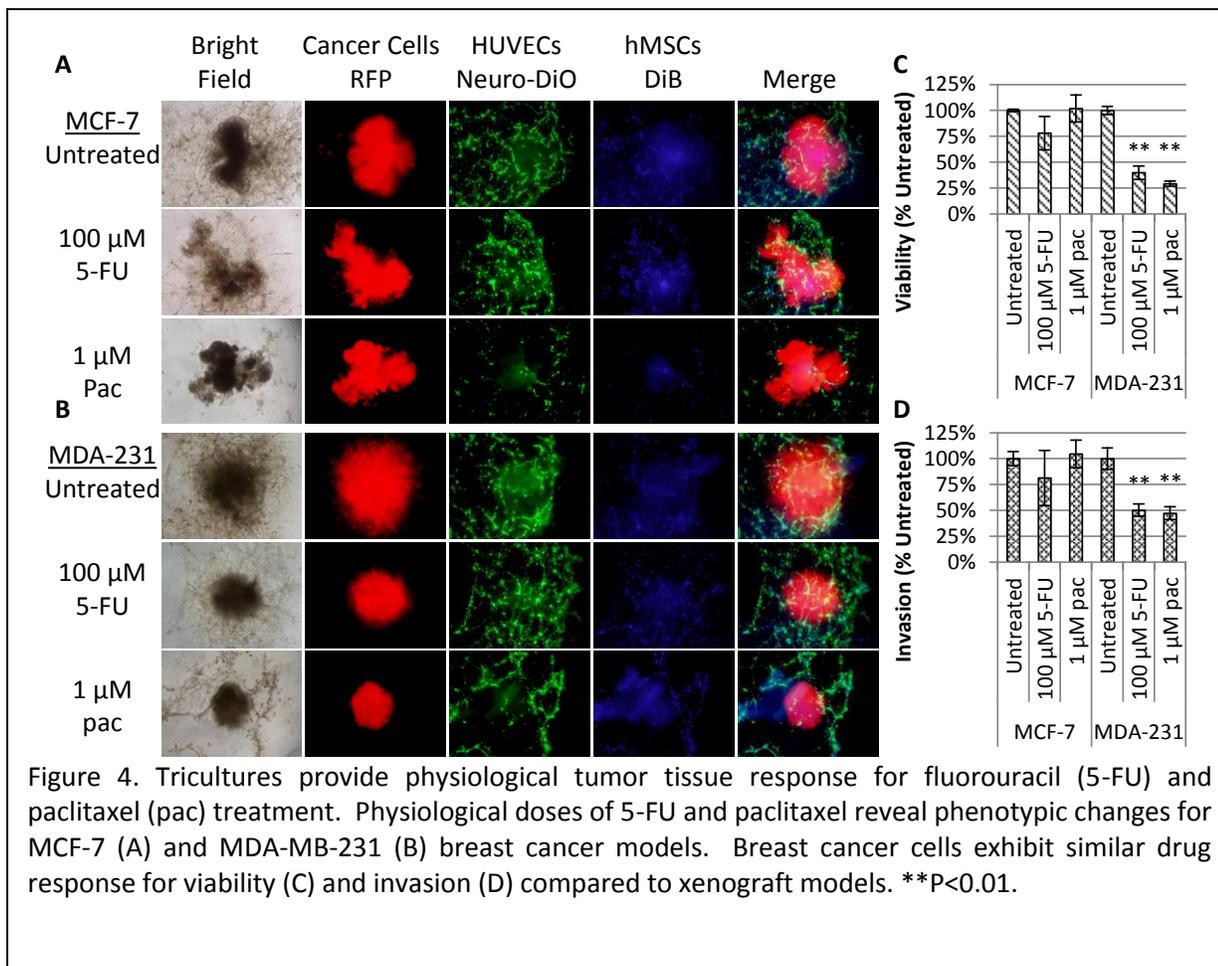


Figure 4. Tricultures provide physiological tumor tissue response for fluorouracil (5-FU) and paclitaxel (pac) treatment. Physiological doses of 5-FU and paclitaxel reveal phenotypic changes for MCF-7 (A) and MDA-MB-231 (B) breast cancer models. Breast cancer cells exhibit similar drug response for viability (C) and invasion (D) compared to xenograft models. **P<0.01.

remained sensitive to paclitaxel treatment [22]. The phenotypic data was similar for MCF-7 tricultures which exhibited no significant alteration in breast cancer microtissue morphology as a result of drug treatment (Figure 4A); whereas, a noticeable decrease in size and extent of protrusion was obvious for MDA-MB-231 tricultures (Figure 4B). Furthermore, it is evident that paclitaxel inhibited recruitment of the tubule networks, and this is the result of microtubule stabilization where reorientation of the centrosome is critical for directed cell migration [23]. Viability of breast cancer cells based on fluorescence intensity of RFP-expression confirms the phenotypic data for tricultures (Figure 4C), and similar values were generated for the invasion values based on measurement of the area of the breast cancer microtissues (Figure 4D). Thus, the triculture was consistent and predictive for morphology, viability and invasion.

In summary, current *in vitro* models for assessing tumor cell response do not correlate with clinical data *in vivo*, and this is due to the fact that these models do not accurately represent the complexity of tumors. Tumor microenvironments are acid, hypoxic, and glucose-deprived, and the tumor ECM exhibits collagen deposition. Tumor cells also interface with vascular and stromal cells within the tumor microenvironment through cell-cell and soluble interactions, and these interactions are instrumental during cancer progression. The triculture consists of a microtissue composed of breast cancer cells and stromal cells which is seeded on a preformed tubule network; together, these structures are embedded in tumor-aligned Invasion Matrix (Figure 5). Over time, the breast cancer microtissue develops tumor morphology and expands into the matrix. Simultaneously, the tubule network is recruited to the microtissue. These microtissues can be treated with pharmacological compounds for drug screening, or the cancer cells may be genetically modified to identify molecular targets. The tricultures may then be evaluated using a fluorescence plate reader or microscope. Changes in the tumor microtissue morphology, viability, and invasion can be easily identified, and these changes are physiologically predictive of *in vivo* tumor response.

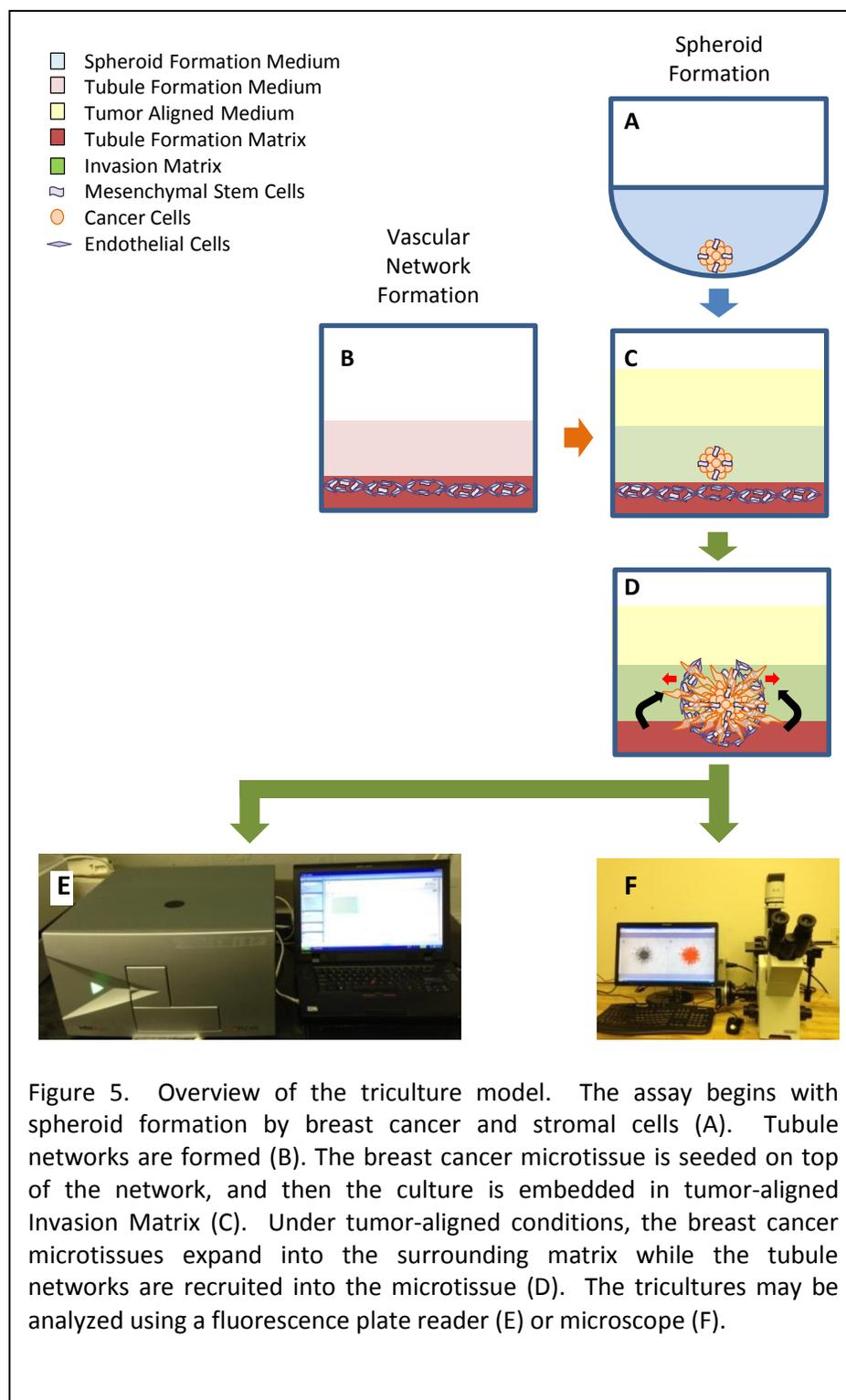


Figure 5. Overview of the triculture model. The assay begins with spheroid formation by breast cancer and stromal cells (A). Tubule networks are formed (B). The breast cancer microtissue is seeded on top of the network, and then the culture is embedded in tumor-aligned Invasion Matrix (C). Under tumor-aligned conditions, the breast cancer microtissues expand into the surrounding matrix while the tubule networks are recruited into the microtissue (D). The tricultures may be analyzed using a fluorescence plate reader (E) or microscope (F).

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