Heterogeneous Breast Tumoroids: An In Vitro Assay for Investigating Cellular Heterogeneity and Drug Delivery

ALEXANDRA P. VAMVAKIDOU,[1] MARK J. MONDRINOS,[2] SOKOL P. PETUSHI,[3] FERNANDO U. GARCIA,[3] PETER I. LELKES,[2] and AYDIN TOZEREN[1]

Breast tumors are typically heterogeneous and contain diverse subpopulations of tumor cells with differing phenotypic properties. Planar cultures of cancer cell lines are not viable models of investigation of cell-cell and cell-matrix interactions during tumor development. This article presents an in vitro coculture-based 3-dimensional heterogeneous breast tumor model that can be used in drug resistance and drug delivery investigations. Breast cancer cell lines of different phenotypes (MDAMB231, MCF7, and ZR751) were cocultured in a rotating wall vessel bioreactor to form a large number of heterogeneous tumoroids in a single cell culture experiment. Cells in the rotating vessels were labeled with CellTracker fluorescent probes to allow for time course fluorescence microscopy to monitor cell aggregation. Histological sections of tumoroids were stained with hematoxylin and eosin, progesterone receptor, E-cadherin (E-cad), and proliferation marker ki67. In vitro tumoroids developed in this study recapture important features of the temporal-spatial organization of solid tumors, including the presence of necrotic areas at the center and higher levels of cell division at the tumor periphery. E-cad-positive MCF7 cells form larger tumoroids than E-cad-negative MDAMB231 cells. In heterogeneous tumors, the irregular surface roughness was mainly due to the presence of MDAMB231 cells, whereas MCF7 cells formed smooth surfaces. Moreover, when heterogeneous tumoroids were placed onto collagen gels, highly invasive MDAMB231 cell-rich surface regions produced extensions into the matrix, whereas poorly invasive MCF7 cells did not. The fact that one can form a large number of 1-mm tumoroids in 1 coculture attests to the potential use of this system at high-throughput investigations of cancer drug development and drug delivery into the tumor. (*Journal of Biomolecular Screening* 2007:13-20)

Key words: heterogeneous, 3-dimensional, tumoroids, coculture, rotating wall vessel bioreactor

INTRODUCTION

BREAST TUMORS ARE BIOLOGICALLY HETEROGENEOUS in that they are composed of numerous tumor cell subpopulations.[1] In the present study, we have cocultured breast cell lines of different invasive potentials and developed large numbers of heterogeneous tumoroids of about 1 mm in diameter in a rotating wall vessel (RWV) bioreactor. Breast cell lines have varying phenotypic characteristics, including morphology, growth rate, invasive potential, and drug sensitivity.[2] As such, they provide a model system to study the molecular and genetic basis of tumor growth, but when cell lines are used in monocultures, they fail to capture the presence of cancer cells of different phenotypes in a breast tumor. Tumor heterogeneity is evident from distinct cellular and molecular properties of primary tumor subpopulations such as receptor status, ploidy, tumor cell growth, and hormone dependency.[3]

Tumor malignancies progress through variable stages and, in some cases, with time acquire an invasive potential.[4] Tumor progression has been associated with decreased genomic stability, oncogene and tumor suppressor gene expression changes, and loss of DNA repair mechanisms within the cells.[5-8] Existence of multiple cell subpopulations was shown to be correlated with the process of tumor growth and sensitivity to chemotherapy,[9] radiation therapy,[10] immunotherapy,[11] and hormone therapy.[12]

Tumor heterogeneity is modulated by the microenvironment with regard to hypoxia, metabolism, and nutrient supply limitations.[13,14] Interactions of subpopulations are expected to play a significant role in growth and behavior within a tumor's architecture.[14] As time progresses, certain subpopulations of a breast tumor may dominate other cells in heterogeneous

[1]Center for Integrated Bioinformatics, School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA.
[2]Cellular Tissue Engineering, School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA.
[3]Department of Pathology and Laboratory Medicine, College of Medicine, Drexel University, Philadelphia, PA.

Received Aug 7, 2006, and in revised form Aug 7, 2006. Accepted for publication Oct 1, 2006.

Journal of Biomolecular Screening 12(1); 2007
DOI: 10.1177/1087057106296482

tumors.[15] This phenomenon, also referred to as clonal dominance, has been observed in many studies and is thought to result from altered growth kinetics of subpopulations in solid tumors.[16-20] An in vitro tumor model capable of sustaining long-term culture of heterogeneous cell populations could be a powerful tool for investigating the influence of subpopulation diversity on tumor-invasive potential.

Three-dimensional monoculture and coculture models have been used in studies with varying applications such as angiogenesis, differentiation, and metastasis.[21-30] A 3-dimensional coculture model combined prostate cancer cell lines with bone cells to investigate metastatic potential, progression, and metastasis to bone.[31] Using these publications as a foundation, we have developed a high-throughput heterogeneous tumor model that has the potential to represent the behavior of early stage tumors in vivo. The 3-dimensional tumoroid model presented in this study recaptures important features of the temporal-spatial organization of solid tumors, including the presence of necrotic areas in the center[32] and the preferential location of invasive cell types close to the tumoroid surface. The high-throughput production of in vitro tumor models will accelerate early stage drug development and drug delivery studies.

MATERIALS AND METHODS

Cell culture

The human breast cell lines used in this study (MDAMB231, MCF7, and ZR751) were obtained from American Type Culture Collection (ATCC). These cell lines were maintained at 37 °C, 95% relative humidity, and 5% CO_2 and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Biowhittaker, Walkersville, MD), supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). The cancer research literature describes MCF7 and ZR751 as noninvasive[3] estrogen receptor (ER), progesterone receptor (PR), and E-cadherin (E-cad) positive,[33-36] whereas the MDAMB231 cell line has been characterized as a highly invasive,[3] ER, PR, and E-cad negative.[37]

Development of tumoroids

Aggregation experiments described here were performed using an RWV bioreactor (10-mL disposable High Aspect Ratio Vessel [HARV], Synthecon, Houston, TX). The RWV was sterilized with 0.1N NaOH and exposed to UV for 20 min on each side.[38] The RWV was rinsed 3 times with Ca^{2+}/Mg^{2+} free phosphate-buffered saline (PBS; Cellgro, Herndon, VA) and filled with fresh medium.[38] Cell cultures were initiated by seeding 0.2×10^6 cells per mL in a 10-mL RWV. Labeled cells of the 3 cell types were seeded in the vessel at predetermined cell densities to assess the extent of heterogeneity in the resulting tumoroids. Prior to each experiment, cells were trypsinized using trypsin/EDTA, counted, and labeled. Cell labeling was performed using CellTracker Probes (Molecular Probes, Eugene, OR), CellTracker Green CMFDA (C2925), CellTracker Blue CMAC (C2110), and CellTracker Red CMTPX (C34452), which are fluorescent probes that are retained in living cells through several generations. CellTracker reagents were loaded into cells by adding the reagent to serum-free RPMI-1640 culture medium and then washed briefly with fresh medium. The vessels were routinely checked for possible contamination, air bubbles, and vessel leakage. Initially, the vessels were set to rotate at 12 rpm in a 37 °C incubator with 5% CO_2. This rpm setting allowed us to avoid frequent crashes of the cell aggregates with the vessel walls.[32]

Determination of the size distribution of aggregates

Images of in vitro tumoroids on the planar surface of the vessel were captured at discrete time points using a phase contrast microscope and a fluorescent microscope (Leica DMRX, Wetzlar, Germany). The size distribution of tumoroids in the RWV was computed from these images using standard imaging software (ImageJ, National Institutes of Health, Bethesda, MD).

Determination of cellular composition

Following culture in the RWV, the cell suspension was passed through a 40-µm mesh (BD Falcon, San Jose, CA) to separate single cells from aggregates. Aggregates were then collected and subsequently dispersed by treatment with trypsin/EDTA. The resulting single-cell suspension was counted by time course fluorescent microscopy using red and green CellTracker fluorescent probes.

Histology evaluation

A representative sample of tumoroids per cell line composition was processed for routine histology by fixing them in 10% neutral-buffered formalin (Formalde-Fresh, Fisher, Fairlawn, NJ) and embedding them in paraffin. The embedded tumoroids were sectioned at 10-µm intervals, deparaffinized in xylene, and processed through a graded series of alcohol concentrations. Serial sections were prepared and stained using hematoxylin and eosin (H&E) (Fisher), as well as progesterone receptor (PR), E-cadherin (E-cad), and proliferating marker ki67, and samples were examined using the Coolscope scanner VS Digital Microscope (Nikon Corporation, Kanagawa, Japan).

Implantation and evaluation of migration potential

For studies involving tumoroid-extracellular matrix interactions, coculture tumoroids were implanted into 3-dimensional collagen I gels after being cultured in the RWV for 10 days. A collagen solution was prepared consisting of 1 mg/mL rat tail collagen type I solution (BD Biosciences, Rockville, MD) and was added to 12-well plates. The tumoroids were mixed into the

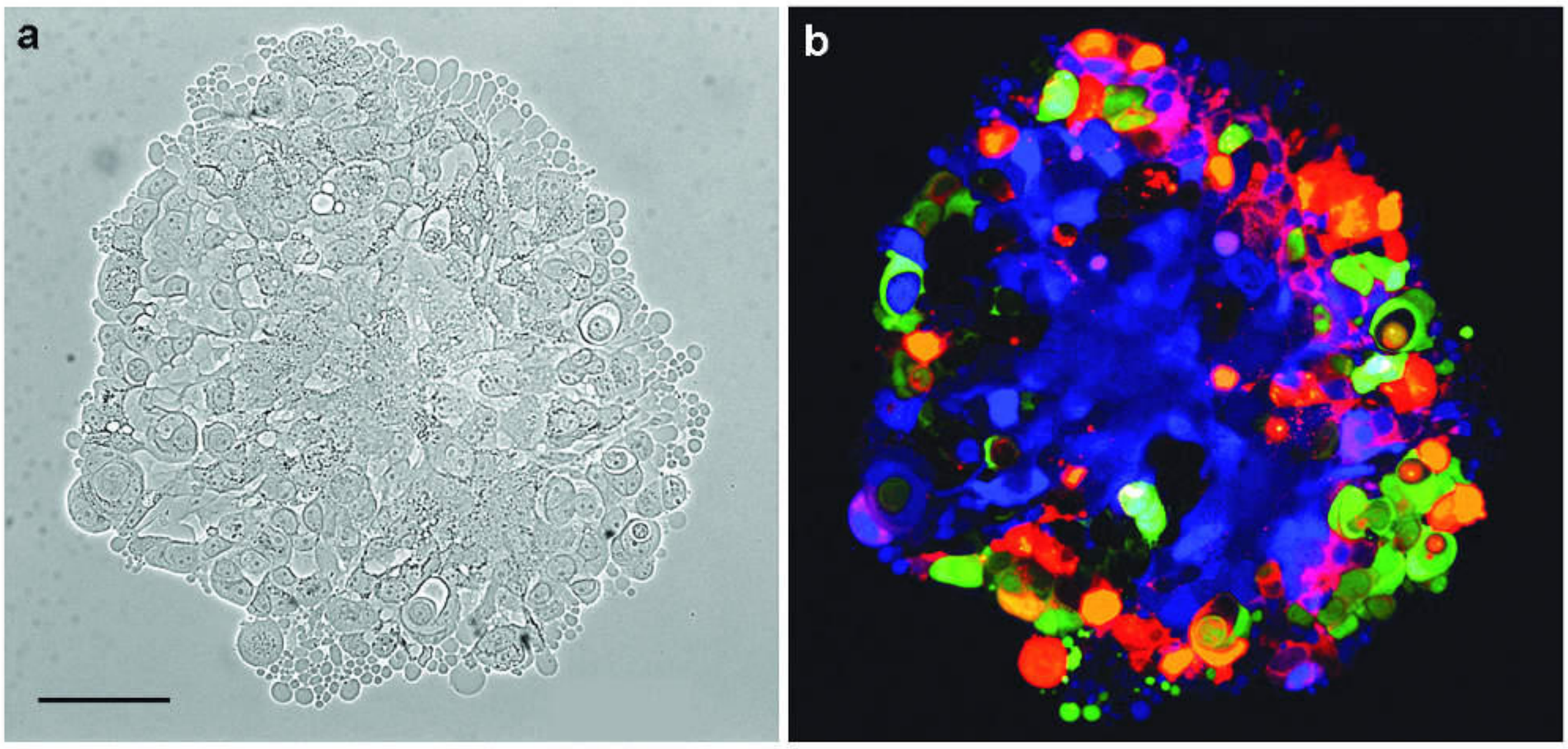

FIG. 1. Day 1 cell aggregate of 1:1:1 coculture of MDAMB231, ZR751, and MCF7 breast cancer cells formed in the rotating wall vessel (RWV). (**a**) Image taken using phase contrast microscopy. (**b**) Fluorescent labeling of the 3 cell lines reveals spatial distribution, where the least aggressive cell line (blue: MCF7) is located in the center and the most aggressive (red: MDAMB231) at the periphery. Bar: 30 μm.

gel using a pipette. Cell invasion into the matrix was recorded after 24 h at 37 °C in a humidified incubator of 5% CO_2, using an inverted phase contrast light and fluorescent microscope.

Statistical analysis

All values are reported for triplicate cultures and expressed as means and standard deviation (SD) of the mean. Statistical significance was assessed using the Student *t*-test, and $p < 0.05$ was considered statistically significant. Uncertainties in measurement of cell aggregates must be considered. Sources of uncertainty include loss of the spherical shape of the cellular aggregates and the effects of periodic changes in the culture medium, among others.

RESULTS

Coculture model

MCF7, MDAMB231, and ZR751 cells aggregated together in the RWV to form heterogeneous tumoroids that were similar in shape and size to homogeneous tumors reported in the literature.[39] Shown in **Figure 1a** is a 24-h postseeding coculture aggregate composed of 1:1:1 concentration ratio of the 3 cancer cell lines. Fluorescent microscopy clearly shows the coculture of multiple cell types aggregated in groups or singles. In particular, MDAMB231 cells, labeled red, appear to be closer to the periphery of the in vitro cell aggregate (**Fig. 1b**). These results indicate that it is feasible to develop heterogeneous breast tumoroid models using a combination of breast cancer cell lines.

Time course of tumoroid evolution

The following experiments focus on homogeneous tumoroids and heterogeneous tumoroids composed of 2 cell lines of highly different invasive potential: MCF7 and MDAMB231 cells. Homogeneous tumoroids composed of MDAMB231 cells form small aggregates of 50 to 100 μm, 24 h postseeding (**Figs. 2a**, **3a**). Subsets of these aggregates develop into steadily growing tumoroids by doubling their size by day 6 (**Fig. 2a**). Tumoroids composed of only MCF7 cells exhibit a rapid aggregation phase, particularly after day 2 (**Figs. 2b**, **3a**). Phase contrast images of MCF7 cell aggregates show that these tumoroids are more compact and dense than the MDAMB231 cell aggregates (data not shown). The development of MCF7 aggregates into tumoroids exhibits a steady exponential growth, reaching a plateau of about 1 mm in diameter by day 6 (**Figs. 2b**, **3a**). The time course of tumoroid formation depends on the composition of the tumoroids. The growth rate of MCF7 tumoroids is much greater than either MDAMB231 or coculture tumoroids (**Fig. 2c**).

Results also show that at day 1, the number of MDAMB231 aggregates is significantly higher than the MCF7 monoculture and 1:1 concentration ratio coculture (**Fig. 3b**). Only a fraction of these initial aggregates, however, develops to become larger tumoroids at later time points. A viable rim of cells approximately 100 μm is visible in the tumoroid periphery. Necrotic cores in all

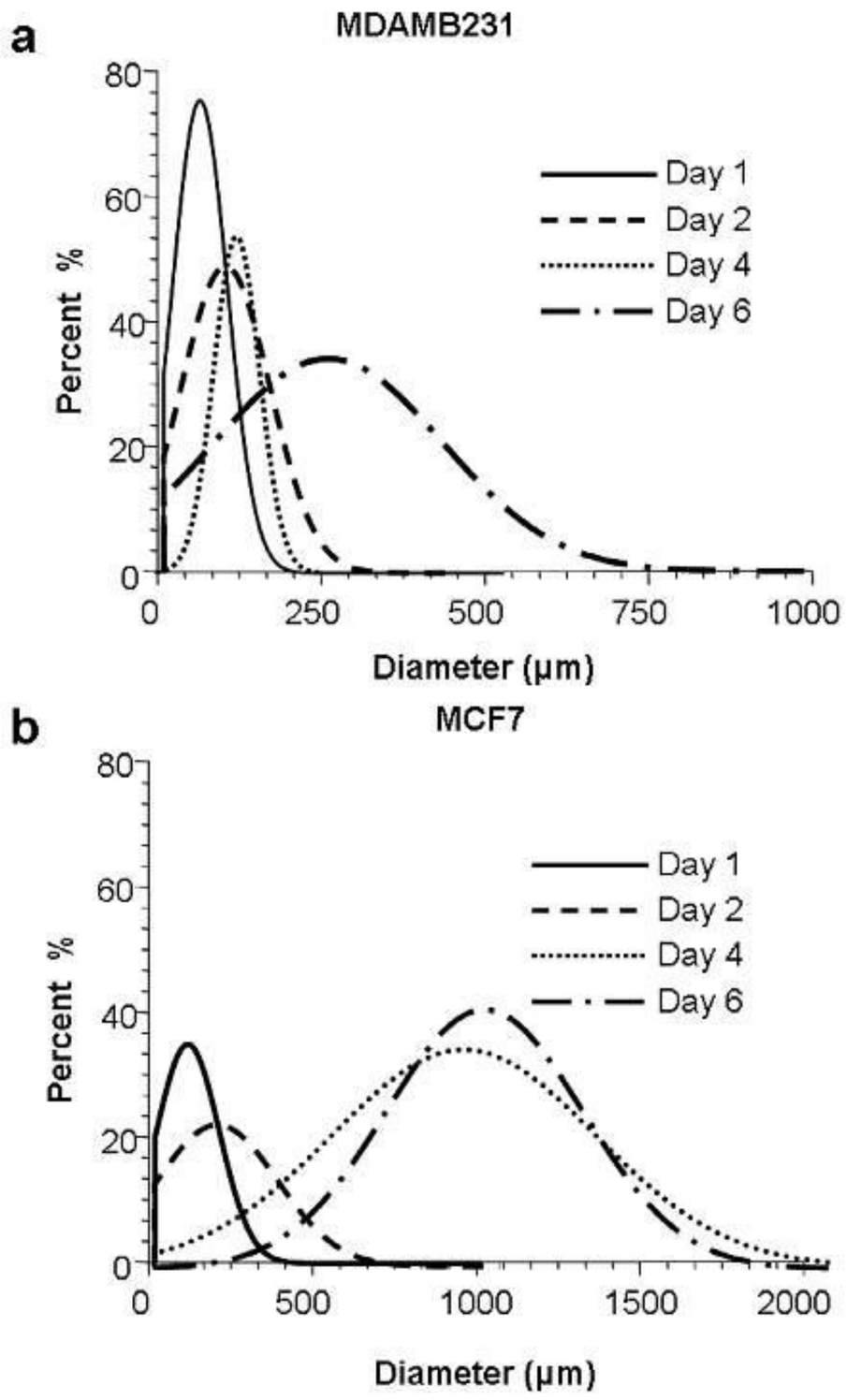

FIG. 2. Size distribution for cell aggregates cultured in the rotating wall vessel (RWV). (**a**) MDAMB231 cell aggregates seeded initially at 0.2×10^6 cells per mL in 10 mL Roswell Park Memorial Institute (RPMI)–1640 medium for days 1, 2, 4, and 6. (**b**) MCF7 cell aggregates seeded initially at 0.2×10^6 cells per mL in 10 ml RPMI-1640 medium for days 1, 2, 4, and 6. (**c**) 1:1 coculture cell aggregates seeded initially at 0.1×10^6 cells per mL in 10 mL RPMI-1640 medium for days 1, 2, 4, and 6.

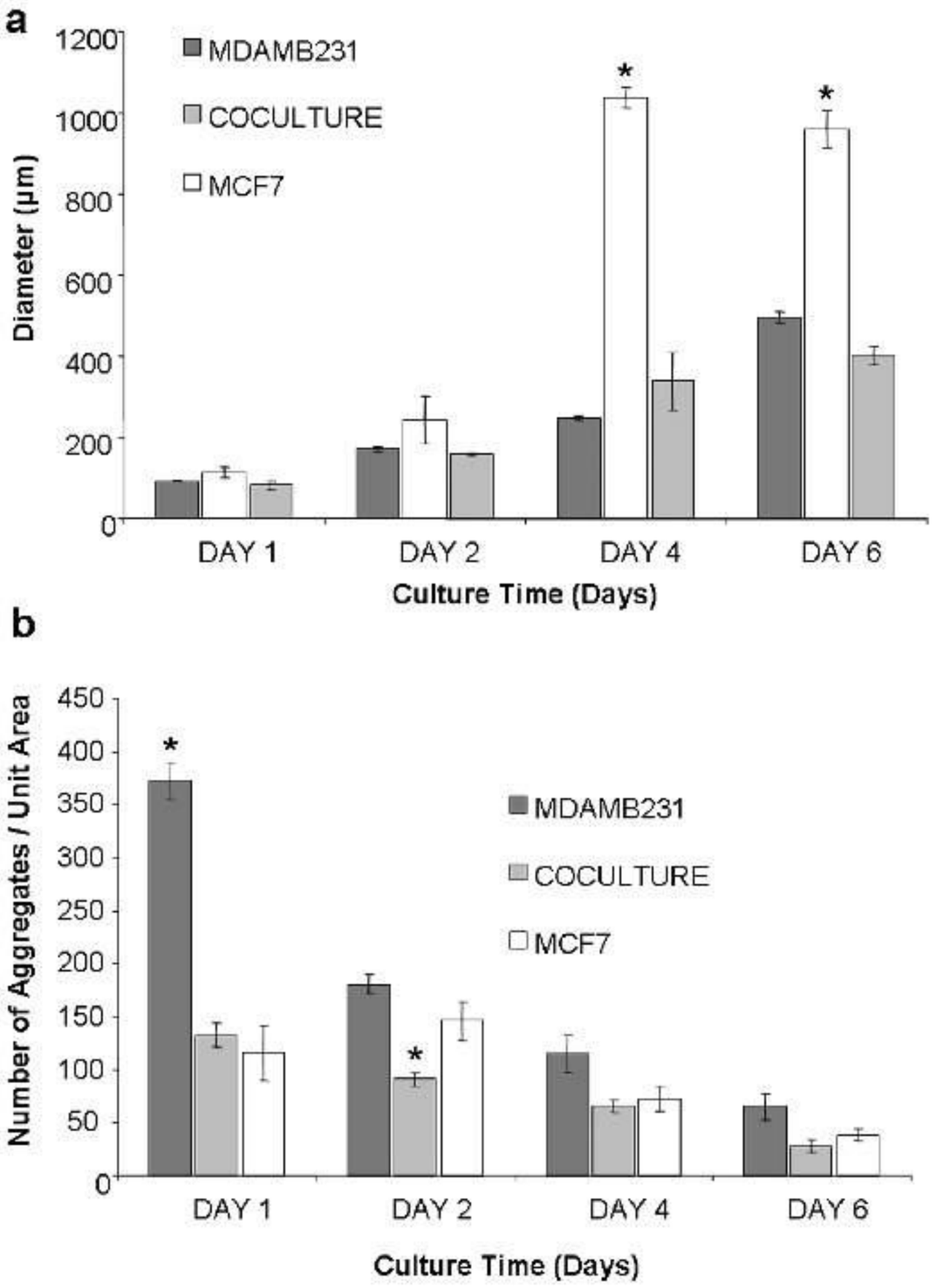

FIG. 3. (**a**) Average diameter of the cell aggregates cultured in the rotating wall vessel (RWV) follows an exponential growth pattern. Tumoroids display the typical solid tumor growth pattern of an exponential growth period followed by a plateau with little or no growth. (**b**) Number of aggregates per unit area cultured in RWV and obtained from phase contrast images. The figure shows the mean of 3 different experiments ($^*p < 0.05$).

tumoroids begin to appear once the aggregate diameter is greater than 300 µm (**Fig. 5**). Saturation and stabilization of tumoroid size in E-cad-positive MCF7 cell tumors may be partly due to depletion of nutrient content caused by diffusion limitations, particularly glucose and oxygen.

Cellular composition of heterogeneous tumoroids

Tumoroids composed of these cell lines at the 1:1 initial concentration ratio show that 24 h postseeding, aggregates are composed of both cell lines at equal concentrations (**Fig. 4**). During the following days, however, the concentration of MCF7 cells increases over MDAMB231 (**Fig. 4**), suggesting that either a larger proportion of MCF7 cells underwent cell

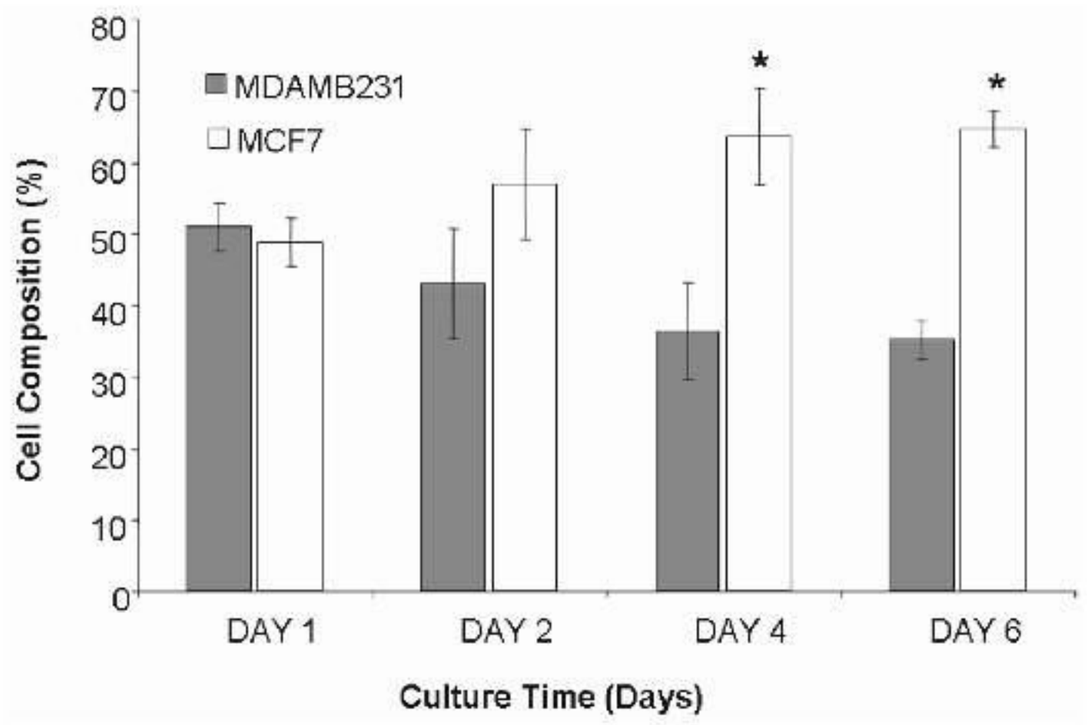

FIG. 4. Cellular composition of tumoroids. Initially, cells were seeded at the 1:1 ratio at 0.2×10^6 cells per mL. MDAMB231 and MCF7 cell lines were relabeled red and green, respectively, prior to mixing. At each time point, aggregates were collected, and cells were counted using time course fluorescent microscopy. The figure shows the mean of 3 different experiments ($*p < 0.05$).

division compared to MDAMB231 cells or that MCF7 singlets have a higher affinity to aggregate than MDAMB231 cells (**Fig. 3a**).

Cellular spatial distribution of heterogeneous tumoroids

The coculture of breast cancer cell lines into tumoroids displays morphological and developmental distinctions. Because MCF7 cells are E-cad and PR positive and MDAMB231 cells are not, it is possible to gain information on the spatial distribution of these cell types in tumoroids using biomarkers in histology slides. Cell organization and thickness of the viable rim differ from tumoroid to tumoroid, even at identical initial cell-type composition. However, most abundant cell types in tumoroids typically appeared preferentially at the outer layers of heterogeneous tumoroids (**Fig. 5a-i**, **b-i**). Moreover, intensity of the proliferation marker ki67 staining decreases from the tumoroid surface toward the tumoroid core (**Fig. 5a-iv**, **b-iv**), indicating that tumoroids grew fundamentally by surface growth.

Cell migration potential of heterogeneous tumoroids

MDAMB231 cells on the surface of heterogeneous tumoroids form protrusions on the periphery, resulting in the rougher surface in MDAMB231-rich heterogeneous tumoroids (**Fig. 5b-i**, **iii**). This observation prompted us to investigate the morphology of tumoroid-extracellular matrix interactions. Heterogeneous tumoroids were placed onto collagen gels and cultured for 24 h. Phase contrast and fluorescence microscopy images of tumoroids on collagen gel show frequent formation of footholds into the surrounding matrix (**Fig. 6a**) by MDAMB231 cells on the surfaces of heterogeneous tumoroids, whereas MCF7 cells do not appear to migrate into the surrounding matrix (**Fig. 6b**).

DISCUSSION

Tissue engineering literature suggests that 3-dimensional structures formed by cultured cells in vitro are better models of in vivo tissue than planar cultures of cell lines, particularly in mimicking some of the cell-cell, cell-matrix interactions found in vivo.[38] Cancer research studies performed on monolayer culture conditions cannot account for limitations caused by diffusion, cell-cell interactions, and factors such as hypoxia, apoptosis, and proliferation. In the present study, we used rotating wall vessel chambers for high-throughput production of heterogeneous tumoroids using combinations of breast cell lines. We showed that a multitude of breast cell lines could be cocultured to form heterogeneous tumoroids of about 1 mm in diameter. Tumoroids reached their steady-state size in approximately 6 days and were found to be viable even after 14 days of culture in RWV. MCF7-rich tumoroids developed faster into larger aggregates than MDAMB231-rich tumoroids. This finding is consistent with earlier in vitro studies of cells expressing E-cadherin that demonstrate an increased potential to aggregate.[40] According to Ramsey et al.,[41] motile subpopulations in a tumor will spontaneously replace weaker intercellular adhesions with stronger ones until they approach that configuration in which adhesion bonding is maximized. When different cell populations are mixed, as we did in this study, cells expressing similar adhesion molecules aggregate to each other with homophilic bonds.[42] Tumoroids formed in this study exhibited morphological features observed in the in vivo tumors of similar size, such as the presence of a necrotic core and viable rim. Moreover, MDAMB231 cells on the surface of heterogeneous tumoroids often formed footholds of linear extensions into the surrounding collagen-rich matrix, mimicking breast cancer cell invasion into the stroma. Thus, the 3-dimensional tumor model developed in this study allows for study of cell-cell interactions in tumor development.

The coculture model presented here corresponds to early stages of tumor growth in the absence of vasculature.[39] As such, it provides a high-throughput in vitro tumor model that could be potentially valuable for investigating early stages of tumor development. The ability to monitor the growth and spatial distribution of cancer cells in a high-throughput in vitro system will aid our understanding of the early stages of cancer development. The model system developed here can be used to investigate the impact of cell phenotype heterogeneity on global gene expression profiles (microarray data), as well as develop in vitro drug testing and drug delivery systems. Comparing gene expression profiles of heterogeneous tumors against those of in vivo tumors is subject to future study. Effective therapeutic agents and trials for the treatment of malignant tumors should target the

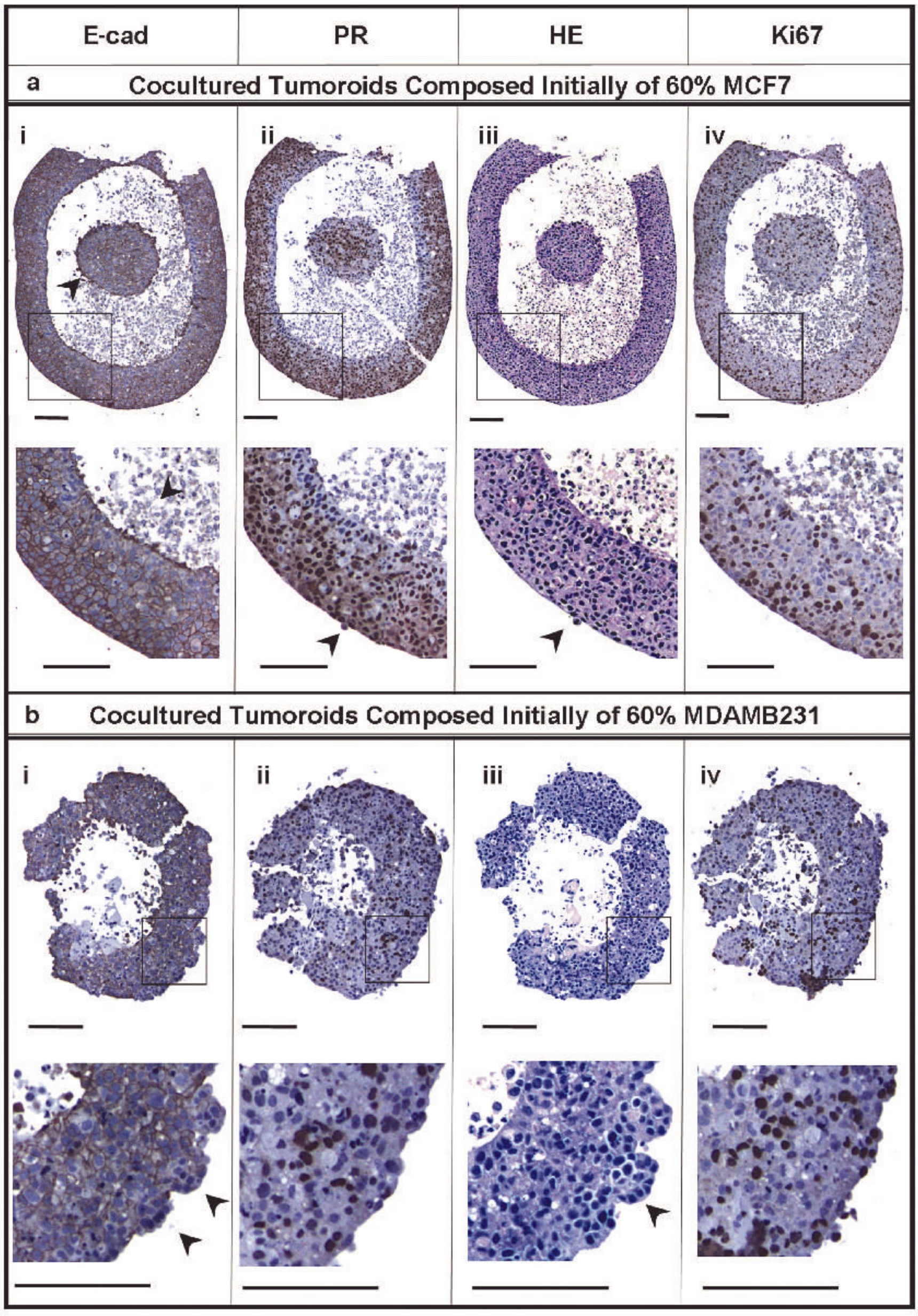

FIG. 5. Hematoxylin-eosin (HE) and immunohistochemical staining of progesterone receptor (PR), E-cadherin (E-cad), and proliferation marker ki67 are shown of day 8–stained 10-μm thick paraffin sections through spheroid cocoltures of 3 breast cancer cell lines formed in the rotating wall vessel (RWV); they are composed of different cell concentrations at an initial density of 2×10^6 cells per mL and show a viable cell layer and necrotic core. Cell concentrations are (**a**) 1×10^6 cells per mL MCF7, 0.5×10^6 cells per mL MDAMB231, and 0.5×10^6 cells per mL ZR751 and (**b**) 0.5×10^6 cells per mL MCF7, 1×10^6 cells per mL MDAMB231, and 0.5×10^6 cells per mL ZR751. Bar: 100 μm.

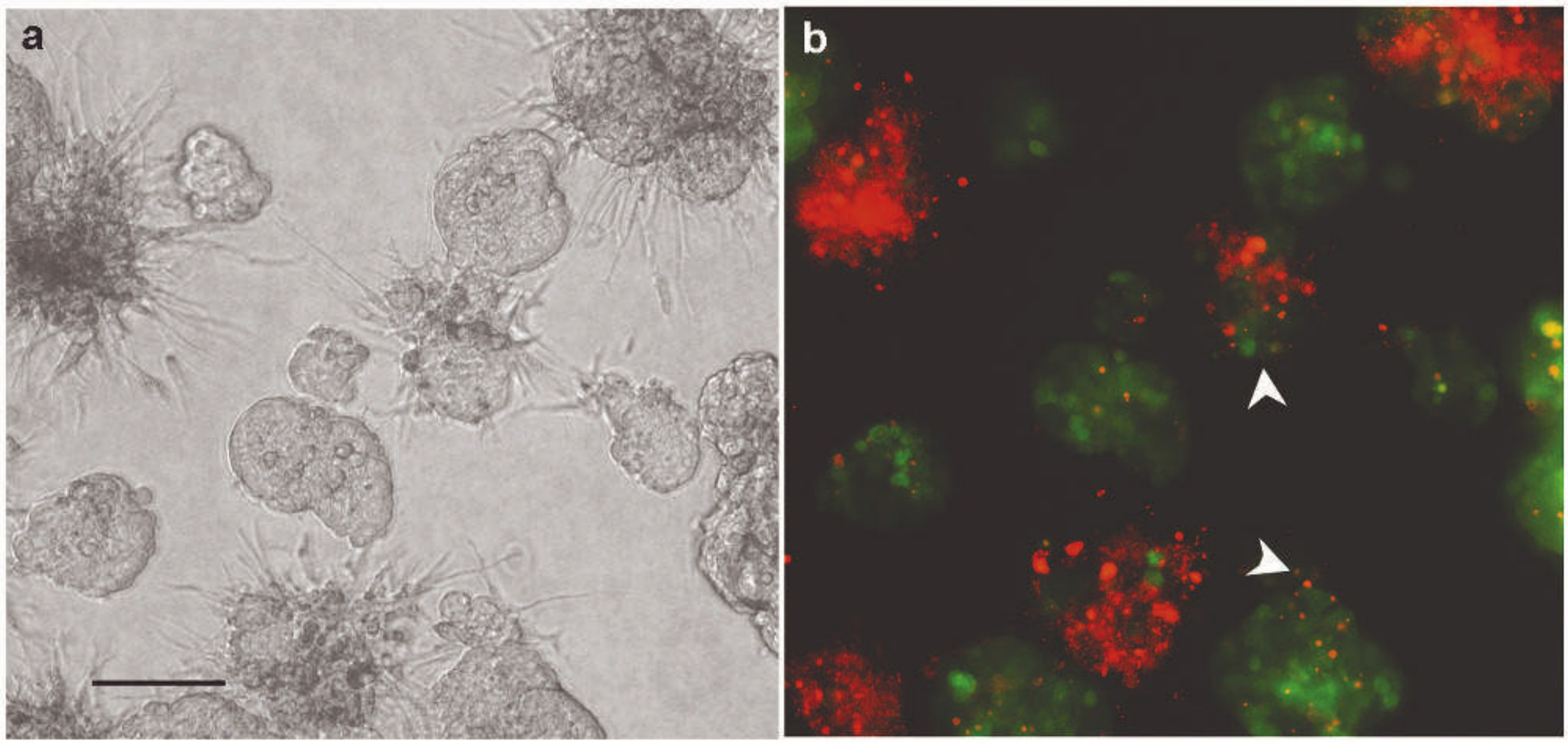

FIG. 6. Aggressiveness and metastatic potential of heterogeneous tumors highly depends on the cellular composition. Heterogeneous tumors composed primarily of MDAMB231 (red) show invasion of the surrounding collagen matrix, in contrast to tumors that are primarily composed of MCF7 (green). (**a**) Phase contrast image of tumor spheroids composed of MDAMB231 and MCF7 cells and (**b**) fluorescent micrograph of the same field demonstrating the identity of the cells comprising the morphologically different tumor spheroids. Bar: 100 μm.

metastatic subpopulations of cells. Heterogeneous in vitro models could account for the mixed responses to detection, treatment, and evaluation protocols as a result of tumor heterogeneity. Moreover, metastatis potential into different tissue types can be considered with this system by infusing heterogeneous tumoroids into microfluidic channels coated with cultured cells of other tissue types.

ACKNOWLEDGMENTS

This study was supported by a National Institutes of Health (NIH) grant (High Throughput Biology No. 232240) and by National Science Foundation (NSF) grant no. 235327.

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Address correspondence to:
Aydin Tozeren, PhD
Distinguished Professor of Biomedical Engineering and Director Center for Integrated Bioinformatics
Bossone 714, Drexel University
3143 Chestnut Street
Philadelphia, PA 19104
E-mail: aydin.tozeren@drexel.edu