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ORIGINAL ARTICLE

Development and verification of a three-dimensional (3D) breast cancer tumor model composed of circulating tumor cell (CTC) subsets

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Abstract

Breast cancer is one of the most common cancer types among women in which early tumor invasion leads to metastases and death. EpCAM (epithelial cellular adhesion molecule) and HER2 (human epidermal growth factor receptor 2) are two main circulating tumor cell (CTC) subsets in HER2+ breast cancer patients. In this regard, the main aim of this study is to develop and characterize a three-dimensional (3D) breast cancer tumor model composed of CTC subsets to evaluate new therapeutic strategies and drugs. For this reason, EpCAM(+) and HER2(+) sub-populations were isolated from different cell lines to establish 3D tumor model that mimics in situ (in vivo) more closely than two-dimensional (2D) models. EpCAM(+)/HER2(+) cells had a high proliferation rate and low tendency to attach to the surface in comparison with parental MDA-MB-453 cells as CTC subsets. Aggressive breast cancer subpopulations cultured in 3D porous chitosan scaffold had enhanced cell–cell and cell–matrix interactions compared to 2D cultured cells and these 3D models showed more aggressive morphology and behavior, expressed higher levels of pluripotency marker genes, Nanog, Sox2 and Oct4. For the verification of the 3D model, the effects of doxorubicin which is a chemotherapeutic agent used in breast cancer treatment were examined and increased drug resistance was determined in 3D cultures. The 3D tumor model comprising EpCAM(+)/HER2(+) CTC subsets developed in this study has a promising potential to be used for investigation of an aggressive CTC microenvironment in vitro that mimics in vivo characteristics to test new drug candidates against CTCs.

Keywords HER2 · EpCAM · In vitro breast cancer model · CTCs · Circulating tumor cells · 3D tumor model

Introduction

Breast cancer is one of the most prevalent cancer type among women worldwide [1, 2], which is characterized by malignant proliferation of epithelial cells covering the ducts or lobules of the mammary gland [3]. The early spread of tumor cells and metastasis is one of the most common causes of cancer-related deaths. However, early diagnostics can decrease the mortality rate of the disease drastically [4].

Circulating tumor cells (CTCs) are known as one of the most considerable candidates to be used in characterizing sub-clonal tumor cell populations [5]. They detach from their primary site during the cancer metastasis process and infiltrate the circulatory system, migrate through the body and promote the formation of secondary tumors at distant sites [1, 6]. The peripheral presence of CTC was first reported in 1869 [7]. By detecting CTC, it is possible to detect cancer on early stage, especially with the early diagnosis of metastatic cells [8].

EpCAM is a cell surface antigen known as epithelial and cancer cell markers encoded by the GA733-2 gene with similar named constructs of this antigen (CD326, ESA, HEA125 and TACSTD1). EpCAM is expressed in more than 60% of metastatic breast cancer cells [9]. In breast carcinoma, higher EpCAM expression is observed in tumors with less differentiation, whereas larger tumors are associated with a decrease in metastasis and EpCAM expression [10]. EpCAM

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has been reported to be a potential marker of premature malignancy due to increased expression in tumor cells. Thus, EpCAM is a widely used marker for CTC isolation although there are certain limitations like EpCAM is not expressed by all CTC due to the epithelial–mesenchymal transition (EMT) [4, 11]. However, it is reported that EpCAM is detected in peripheral blood in metastatic breast cancer patients [12], it is still a promising candidate for CTC isolation from breast cancer patients.

Furthermore, several types of research have indicated that human epidermal growth factor 2 (HER2) is another proper target for isolation of CTC from the peripheral blood of HER2+ breast cancer patients compromising of the 25–30% of all breast cancer cases [13]. HER2 is the transmembrane glycoprotein which is expressed in many tissues and whose primary task is excessive and uncontrolled cell growth and facilitating tumor formation [14]. After treatment with standard or anti-HER2 agents in metastatic breast cancer, the change in peripheral CTC count- HER2+ cell count is essential for assessing response [15] as it has prognostic and predictive properties for breast cancer [16]. However, there are several pitfalls for usage of EpCAM and HER2 markers alone in breast cancer. For instance; EpCAM are downregulated during EMT [17], and thus its value is reduced for CTC isolation. Besides, a part of HER2(+) breast cancer patients had solely HER2-negative CTCs [18]. Although EpCAM and HER2 are candidate biomarkers for CTC selection with multiple markers, there is an unmet need for 2D and 3D characterization of cancer cells co-expressing EpCAM and HER2.

In this study, EpCAM and HER2 are selected as CTC markers to develop an aggressive 3D breast cancer tumor model. There are many ongoing in vivo researches for cancer therapies. However, in vitro studies also play a pivotal role in assessing the therapeutic potential of anticancer drugs. For this purpose, developing 3D in vitro tumor models has been recently become popular since monolayer cell cultures are unable to mimic in vivo accurately [19]. In addition, 3D structured models provide an environment for cell–cell and cell-ECM interaction, which imitates the physiological function and morphology of cells in a better way compared to 2D models. Also, cells in 3D models behave closely to the natural tumor structure in terms of polarity and gene expression [20–23].

The main goal of this study is to develop an aggressive breast cancer tumor model comprising of cells expressing EpCAM and HER2 as CTC subsets. In order to accomplish this, MDA-MB-453 breast cancer cell line was used for isolation of EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) (double positive) cells. These cells were isolated by magnetic activated cell sorting (MACS). Cell growth and attachment characteristics, the ability of micro tissue formation and sphere forming efficiencies in soft agar culture of these cells were investigated. As 3D model, cells were cultivated in chitosan scaffolds. 3D morphologies of cells were observed by scanning electron microscopy (SEM). These cell groups which were cultivated by 2D or 3D culture techniques were evaluated with regard to mRNA expression of pluripotency marker genes, Nanog, Sox2 and Oct4 by qPCR (quantitative polymerase chain reaction) technique. For the verification of the 3D model, the effects of doxorubicin which is a chemotherapeutic agent used in breast cancer treatment were examined and compared in 2D and 3D cultures. The results indicate that EpCAM(+)/HER2(+) cells had a high proliferation rate and low tendency to attach to the surface in comparison with MDA-MB-453 cells. According to results of the study; CTCs cultured in 3D expressed higher levels of pluripotency marker genes, Nanog, Sox2 and Oct4, than cells cultured in 2D. Furthermore, Sox2 and Oct4 expression levels of CTC subsets were higher than MDA-MB-453 cells. Aggressive breast cancer subpopulations grown in porous chitosan scaffold as 3D model exhibited enhanced cell–cell and cell–matrix interactions compared to 2D cultured cells and these 3D models showed more aggressive morphology and behavior; decreased flattened-morphology, increased expression of stemness genes and increased drug resistance. It is believed that the 3D aggressive tumor model developed in this study will be a promising alternative over in vivo studies for in vitro testing of new drug candidates developed against breast cancer, therapeutic DNA vaccines and monoclonal antibodies.

Results

Aggressiveness of breast cancer subpopulations expressing EpCAM and/or HER2 on the surface in vitro

In order to select the most appropriate breast cancer cell line for isolation of EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells, fluorescent immunostaining of MCF-7, MDA-MB 231 and MDA-MB-453 breast cancer cells was performed to visualize EpCAM(+) and HER2(+) cells (Fig. 1a) in the whole population and the positive cell ratios were quantified (Fig. 1b). It was determined that all tested cell groups contained EpCAM (+) cells, however MDA-MB-453 cells contained 3.2 times more EpCAM (+) cells (1,1% of whole population) than MDA-MB 231cells ($p = 0.0115$). When cells were examined in terms of HER (+) cell content, it was observed that no HER2 (+) cells were present in MDA-MB 231 cells and that MDA-MB-453 cells (0.51% of whole population) contained 28.3 times higher HER2 (+) cells than MCF-7 cells ($p = 0.0202$). Breast cancer subpopulations; EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells, were isolated from MDA-MB-453 cells,

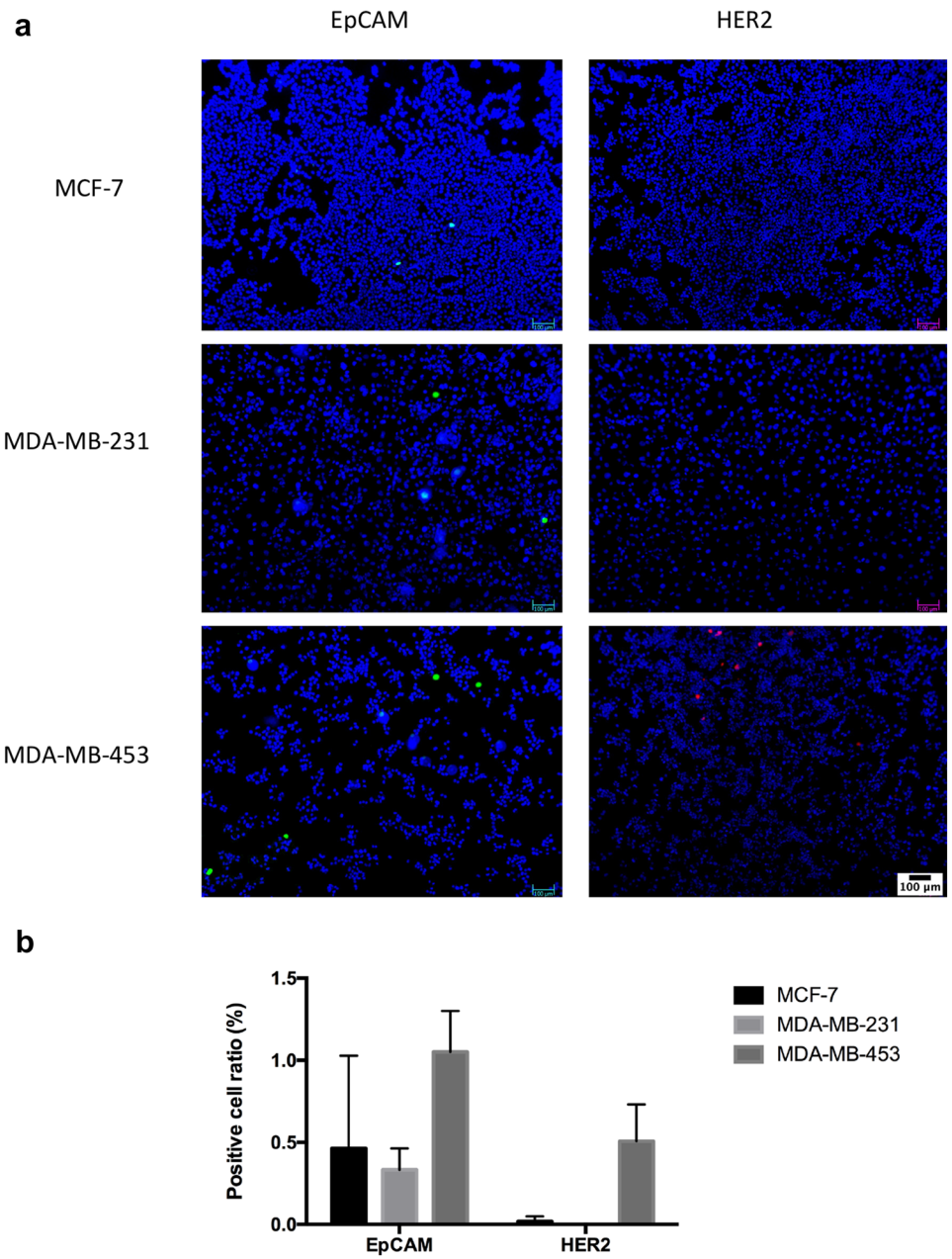


Fig. 1 **a** Fluorescent image of EpCAM (green) and HER2 (red) labeled MCF-7, MDA-MB 231 and MDA-MB-453 breast cancer cell lines. **b** Quantitative analysis of EpCAM(+) and HER2(+) cells in MCF-7, MDA-MB 231 and MDA-MB-453 cell populations. All cells were counterstained with DAPI (blue) for nucleus. Data are plotted as mean of replicates with error bars (±SD). Scale bar: 100 µm. (Color figure online)

containing EPCAM (+) and HER2 (+) cells at the highest rate among all tested cell lines, via MACS technique. Following the observation that the isolated cells expressed interested surface markers, HER2 or EpCAM markers, on their surfaces by immunohistochemical analysis, in order to investigate in vitro growth characteristics, viable cell number of the isolated breast cancer subpopulations was measured by trypan blue exclusion assay during 17 days (Fig. 2a). On the 7th day of culture (corresponding to the earlier logarithmic growth phase of cells), EpCAM(+)/HER2(+) subpopulation cells were 2.08, 1.79 and 1.77-fold higher than heterogeneous MDA-MB-453 ($p < 0.001$), EpCAM(+) ($p < 0.01$) and HER2(+) ($p < 0.01$) cells, respectively. EpCAM(+)/HER2(+) cells maintained their higher cell number compared to all other populations until the cells entered the death phase (between 9 and 13th day of culture) ($p < 0.0001$). Following the growth characteristics assessment, to investigate attachment tendencies of all cell populations to the culture surface, attached cells were trypsinized and counted by trypan blue exclusion assay during 5 h with 30 min interval (Fig. 2b). In the first 30 min, differences in the number of attached cells to the culture surface were began to observe between the experimental groups. At the 30th min of attachment, 12-fold higher MDA-MB-453 ($p < 0.001$) and EpCAM(+) ($p < 0.01$) cells were attached than HER2(+) cells and this statistical significance was maintained during

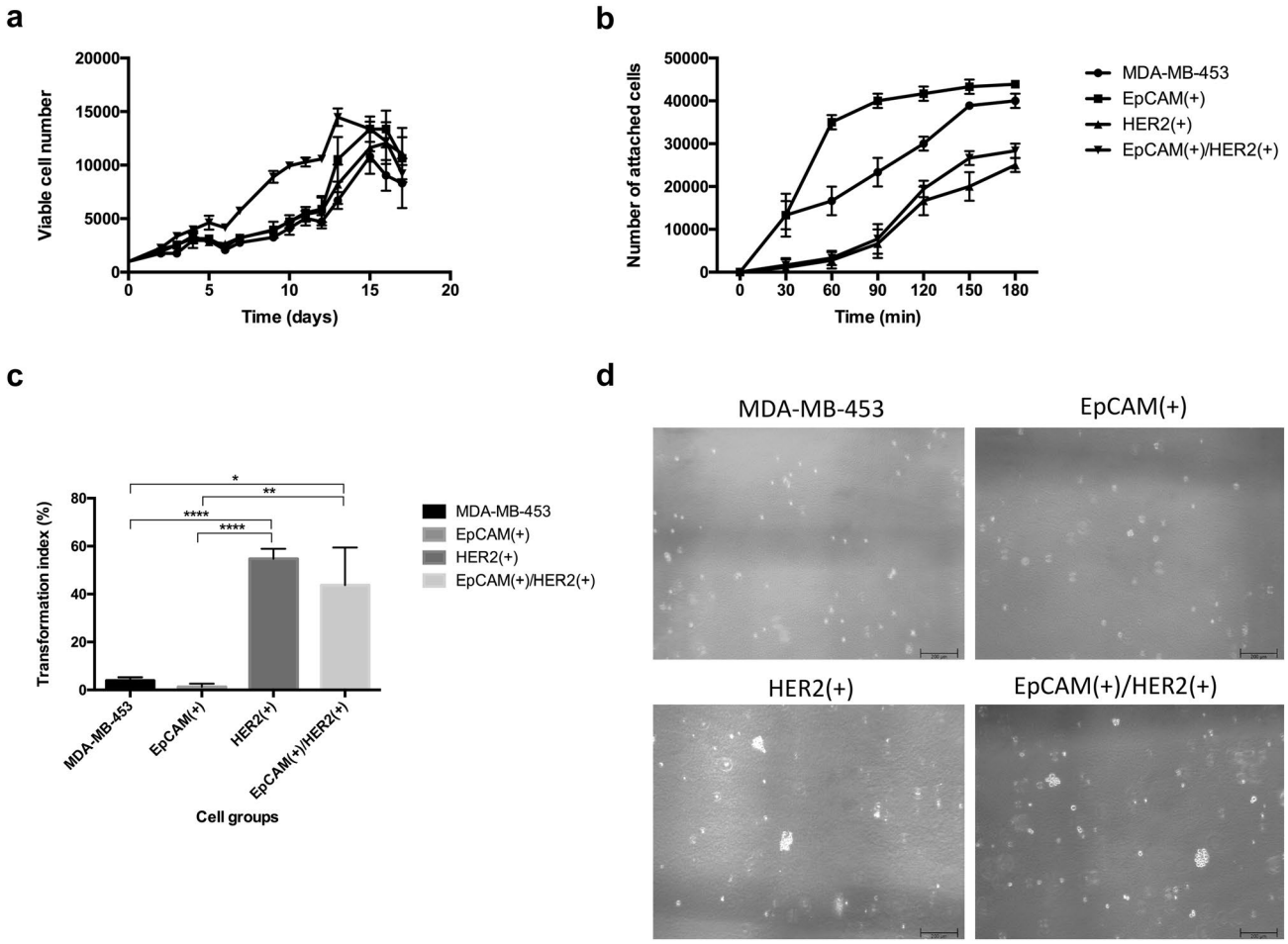


Fig. 2 Aggressiveness of MDA-MB-453 cells and subpopulations isolated from MDA-MB-453 in vitro. **a** Growth and **b** attachment curves, **c** transformation index (%) of MDA-MB-453, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells and **d** representative micrographs of cell spheres formed in soft agar. Data are plotted as mean of replicates with error bars (±SD) and statistically analyzed using a two-way ANOVA and Tukey's multiple comparisons test (**a**, **b**), and the unpaired Student's *t*-test (**c**). Statistical significance was defined as $P < 0.05$. Scale bar: 200 µm

the 180 min follow-up. At the end of 180 min, while ≥ 80% of MDA-MB-453 and EpCAM(+) cells were attached to the surface, only 50% and 57% of HER2(+) and EpCAM(+)/HER2(+) cells were attached. Similarly, lower number of EpCAM(+)/HER2(+) cells were attached than MDA-MB-453 ($p < 0.001$) and EpCAM(+) ($p < 0.01$) cells between 30 and 180th min of the attachment, there was no statistically significant difference between the numbers of attached EpCAM(+)/HER2(+) and HER2(+) cells during 180 min (except 150th min). Besides, soft agar colony formation assay was performed to evaluate the tumorigenicity of these cell populations in vitro (Fig. 2c, d). Statistically significant difference was not observed between HER2(+) and EpCAM(+)/HER2(+) cell groups ($p = 0.3076$) and between MDA-MB-453 and EpCAM(+) cell groups ($p = 0.0705$) in soft agar colony formation assay, however HER2(+) and EpCAM(+)/HER2(+) cells showed higher colony formation compared to the other two cell groups. Namely, HER2(+) cells (transformation index = 54.7%) formed 14 and 42-fold higher number of sphere, and EpCAM(+)/HER2(+) cells (transformation index = 43.8%) formed 11 and 33-fold higher number of sphere than MDA-MB-453 and EpCAM(+), respectively.

Micro-tissue assembly of breast cancer subpopulations

To examine scaffold-free 3D culture formation capacities of MDA-MB-453 cells and breast cancer cell subpopulations, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells were cultured in agar wells over 19 days (Fig. 3a, b). There was no statistically significant difference between the volumes of micro-tissues formed as a result of long-term culture (at the end of 19 days) except for MDA-MB-453 and

HER2(+) ($p < 0.05$). The volume of micro-tissue formed by MDA-MB-453 cells after 19 days of culture is 1.25 times higher than HER2(+) cells. Contrary to this similarity in the last volumes of micro-tissues formed by different cell subsets after 19 days, distinct differences were observed in volume changes of micro-tissues day by day, probably due to the nature and strength of cell to cell connections. The volume of micro-tissues formed by EpCAM (+) cells continuously increased day by day up to %94.8 increase compared to initial volume. The volume of micro-tissues formed by EpCAM(+)/HER2(+) cells did not steadily increase and even decreased by 27.5% compared to initial volume on the 9th day of culture possibly due to cellular loss in liquid flow during refreshment of medium. Despite this volume losses, it increased by 73.9% of the initial volume at the end of 19 days. Moreover, Live/Dead assay was performed to visualize the viability of the cells in the resulting 3D micro-tissues after 19 days of culture (Fig. 3c). The large majority of the cells were still alive on 19th day of the culture and only a small number of dead cells were observed. It has also been observed that dead cells are homogeneously distributed rather than in specifically internal regions of micro-tissues.

Scaffold based 3D culture of breast cancer subpopulations

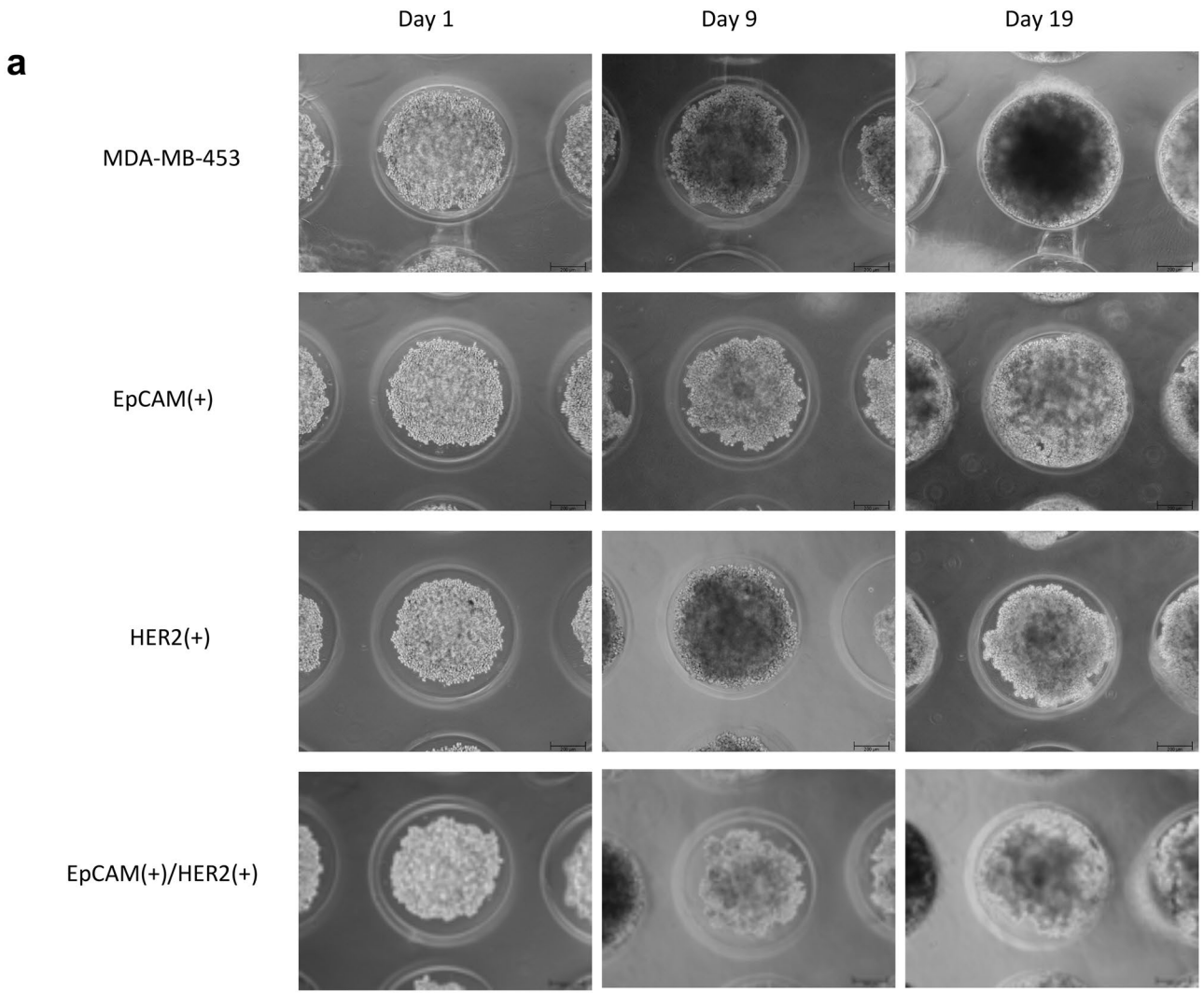
MDA-MB-453 cells and breast cancer cell subpopulations, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells were cultured for 1 week on sponge-like chitosan scaffolds in order to form 3D tissues and SEM analysis was performed to examine cell morphology (Fig. 4a). Irrespective of the cultured cell type, while the cells exhibit forced polarization with minimal cell–cell contact area in 2D culture as expected, they formed spherical structures with increased cell–cell interaction and reduced cell-material surface contact area in 3D culture. In 2D culture, EpCAM(+) cells exhibited more epithelial morphology, while HER2(+) and EpCAM(+)/HER2(+) cells contained cells that tended to remain spherical morphology without spreading on the surface. In 3D culture, all cell groups formed cellular clusters attached to the surface of the scaffold. Moreover, expression levels of Nanog, Sox2 and Oct4 in 2D and 3D cultured cells were analyzed by qPCR as markers in the evaluation of tumorigenesis (Fig. 4b). Nanog expression of cells were 6.3, 20.9, 4.2, 2.2-fold higher in 3D cultured cells compared to 2D cultured cells for MDA-MB-453 cells, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells respectively. 3D culture strategy similarly increased Sox2 expression in MDA-MB-453, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells (4.1, 12.5, 28.7 and 3.7-fold, respectively). Oct4 expression is also elevated in 3D cultured EpCAM(+) (2.7-fold), HER2(+) (3.7-fold) and EpCAM(+)/HER2(+) cells (1.9-fold) compared to 2D cultures, albeit less than for the other pluripotency genes. Furthermore, the results revealed that Sox2 and Oct4 expression of EpCAM(+) (3.1-fold for Sox2, 3.2-fold for Oct4), HER2(+) (8.4-fold for Sox2, 5.6-fold for Oct4) and EpCAM(+)/HER2(+) cells (1.6-fold for Sox2, 2.9-fold for Oct4) cultured in 3D culture were higher than MDA-MB-453 cells, while no significant difference was observed between Sox2 and Oct4 expression of the cell groups grown in 2D culture at the end of 10 days of culture. In contrast to the Sox2 and Oct4 genes, Nanog expression was found to be less in subpopulations compared to MDA-MB-453 cells both for 2D and 3D cultured cells.

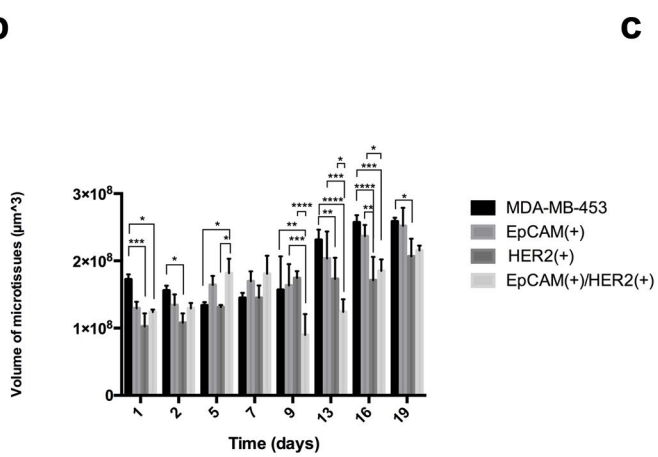
Drug sensitivity in 2D and 3D breast cancer subpopulation culture

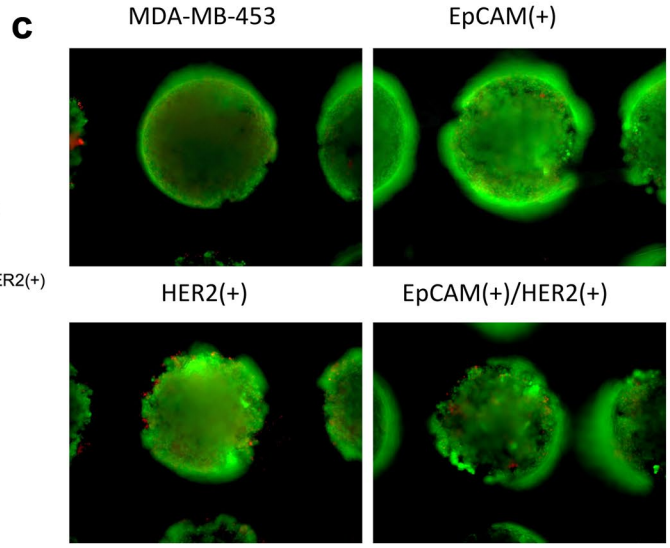
To further examine the 3D in vitro breast cancer models as in vivo representation, 2D and 3D cultured MDA-MB-453 cells, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells were exposed to a clinically-used anticancer drug, doxorubicin, for 3 days and cell viability was measured by MTT assay (Fig. 5). The cell viabilities after the drug treatment in the 3D culture of EpCAM(+) and EpCAM(+)/HER2(+) cells were 1.7 and 1.4 times higher than the 2D cultured cells, respectively ($p < 0.05$). Furthermore, cell viability values up to 57.8% were observed among 3D breast cancer model (EpCAM(+)/HER2(+) cells). Independently of the cell type, the average viability of 2D cultured cells was found to be 31.1%, while the average viability of 3D cultured cells was 43.1%.

Discussion

Breast cancers display considerable cellular heterogeneity as a result of several genetic and epigenetic mechanisms [24, 25]. These distinct breast cancer subpopulations differ in terms of various biological properties such as proliferative ability, metastatic potential and treatment susceptibility that influence clinical outcomes [26, 27]. Therefore, characterization of different breast cancer subpopulations and development of in vivo-like culture models of these cells are substantially crucial for studies in cancer biology and therapeutic drug testing in recent years. Here, firstly the most proper cell line was determined to isolate breast cancer subpopulations expressing EPCAM and/or HER2 surface markers that are frequently used to detect CTCs. Secondly, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells were isolated from the selected cell line, MDA-MB-453, and in vitro characteristics of these subpopulations were defined to evaluate aggressiveness of them. Lastly, the subpopulations, that have been shown to be more aggressive than the bulk population in 2D culture, were cultured on a 3D porous chitosan scaffold and a more aggressive 3D culture model


a
Day 1
Day 9
Day 19
MDA-MB-453
EpCAM(+)
HER2(+)
EpCAM(+)/HER2(+)


b
Volume of microtissues (μm^3)
3×10^8
2×10^8
1×10^8
0
1
2
5
7
9
13
16
19
Time (days)
MDA-MB-453
EpCAM(+)
HER2(+)
EpCAM(+)/HER2(+)


c
MDA-MB-453
EpCAM(+)
HER2(+)
EpCAM(+)/HER2(+)

◂**Fig. 3** Micro-tissue assembly of MDA-MB-453 cells and subpopulations isolated from MDA-MB-453 cells. **a** Representative micrographs and **b** volume changes of micro-tissues formed by MDA-MB-453, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells for 19 days. Data are analyzed using a two-way ANOVA and Tukey's multiple comparisons test, and statistical significance was defined as $P < 0.05$. **c** Live/dead image of micro-tissues after 19 days. Cell viability was visualized by live-dead staining (Calcein/ethidium homodimer-1; live: green, dead: red). Scale bar: 200 µm. (Color figure online)

has been demonstrated with altered phenotypic features, increased pluripotency and self-renewal characteristics, and enhanced drug resistance of cells.

Aggressive tumors mostly cause poor prognosis, resistance to treatment and thus higher mortality [28]. Due to this clinical importance of the aggressive nature of cancer, it has become critical to examine cellular aggressiveness. The aggressiveness of cancer on a cellular level is described with a faster growth rate, higher ability to metastasize and lesser response to drugs [29]. Expression of various characteristics hallmarks have been used to identify aggressive cancer subpopulations in breast cancer that is both clinically and biologically heterogeneous disease. EpCAM is one of the cell surface protein that is plentifully expressed in primary tumors. Expression of EpCAM mostly correlates with more aggressive tumor behavior [30] and its over-expression is considered as a poor prognostic marker in breast cancer [31]. Another common marker, HER2, is known to be associated with malignancy and a poor prognosis in case of overexpression [32]. In this study, cells expressing both of these common surface markers, EpCAM(+)/HER2(+) cells, showed higher proliferation rate and colony formation in soft agar, and lower attachment tendency to the surface compared to the bulk population, MDA-MB-453. These characteristics indicate that a more aggressive cell population has been obtained at the cellular level. Following the determination of an aggressive cell population that can be used to examine the cellular effects of therapeutics, it has been aimed to develop a realistic 3D model with these cells. We firstly have cultured cells in the 3D system using scaffold-free technology due to simple nature of the technique. However, especially EpCAM(+)/HER2(+) cells did not form a stable micro-tissue, suggesting loosely formed 3D structures. These micro-tissues, which are basically cellular clusters, did not form ideal structures for experiments due to their fragile structures. Therefore, 3D tumor models were constructed using a scaffold-based strategy that are in demand owing to its support for cell adhesion, proliferation and migration [33]. As scaffold, chitosan, that is widely used in diverse biomedical applications due to its non-toxicity, biocompatibility, biodegradability and antimicrobial property, was chosen to create 3D tumor model [34]. Furthermore, it has been reported that the chitosan support substrate itself or crosslinked with other molecules enhanced cancer cell stemness and tumor progression for multiple cancer types [35–37]. In the current study, culture on chitosan scaffold resulted in a more rounded and spherical cellular morphology and increased the expression of Nanog, Sox2 and Oct4, that are considered to serve as valuable markers for poorly differentiated aggressive tumors [38], in all tested breast cancer subpopulations. Moreover, aggressive breast cancer cells cultured within sponge-like chitosan scaffold displayed a higher drug resistance (i.e. doxorubicin) than 2D cultured cells. Taken together, breast cancer cells grown within porous chitosan scaffold as a 3D model had enhanced cell–cell and cell–matrix interactions compared to 2D cultures and this model exhibited in vivo-like characteristic, namely; decreased forced polarity and flattened cellular morphology, enhanced stemness and increased resistance to an anticancer drug.

Breast cancer displays diversity in terms of both biological and clinical outcomes between tumor subtypes [39] as well as substantial intra-tumor heterogeneity [24, 25]. Therefore, that the cancer subpopulations isolated in this study may not be applicable for all types of breast cancer, appears to be a limitation of this strategy [40]. Although more research is needed to fully elucidate entity, property and biological characteristic stability of EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) subpopulations in different breast cancer populations, the applicability of this isolation strategy has revealed in this work. Another limitation to be taken into consideration is that the ratio of these cancer subpopulations, especially of double positive cells, is substantially low in cell lines tested in the study and this increases the number of cells that must be used to isolate subpopulations and construct the model. However, it is thought that the effect of this limitation on the operation may be reduced by further reducing the size of the model and combined with microfluidic systems.

3D cancer models are used to create the closest example to the tumor microenvironment and to form a transitional form between the cancer cells isolated from the 2D culture medium and the developing human tumor in the xenogenic host [41]. 3D tumor models are indisputably superior to 2D models for anti-cancer drug testing and screening as well as drug delivery. However, there are several bottlenecks. First, most of the tumor models are using cell lines which are bearing distant characteristics rather than the original tumor. Thus, CTC enrichment from the tumor samples to mimic the real tumor characteristics gained importance. For functional assays, CTCs cultured on different 3D scaffold systems to mimic tumor microenvironment is elaboratively reviewed in [42]. Thus, the identification and isolation of aggressive cancer cell subpopulations mainly named as CTCs and the development of 3D tumor models are hot topics in cancer studies.

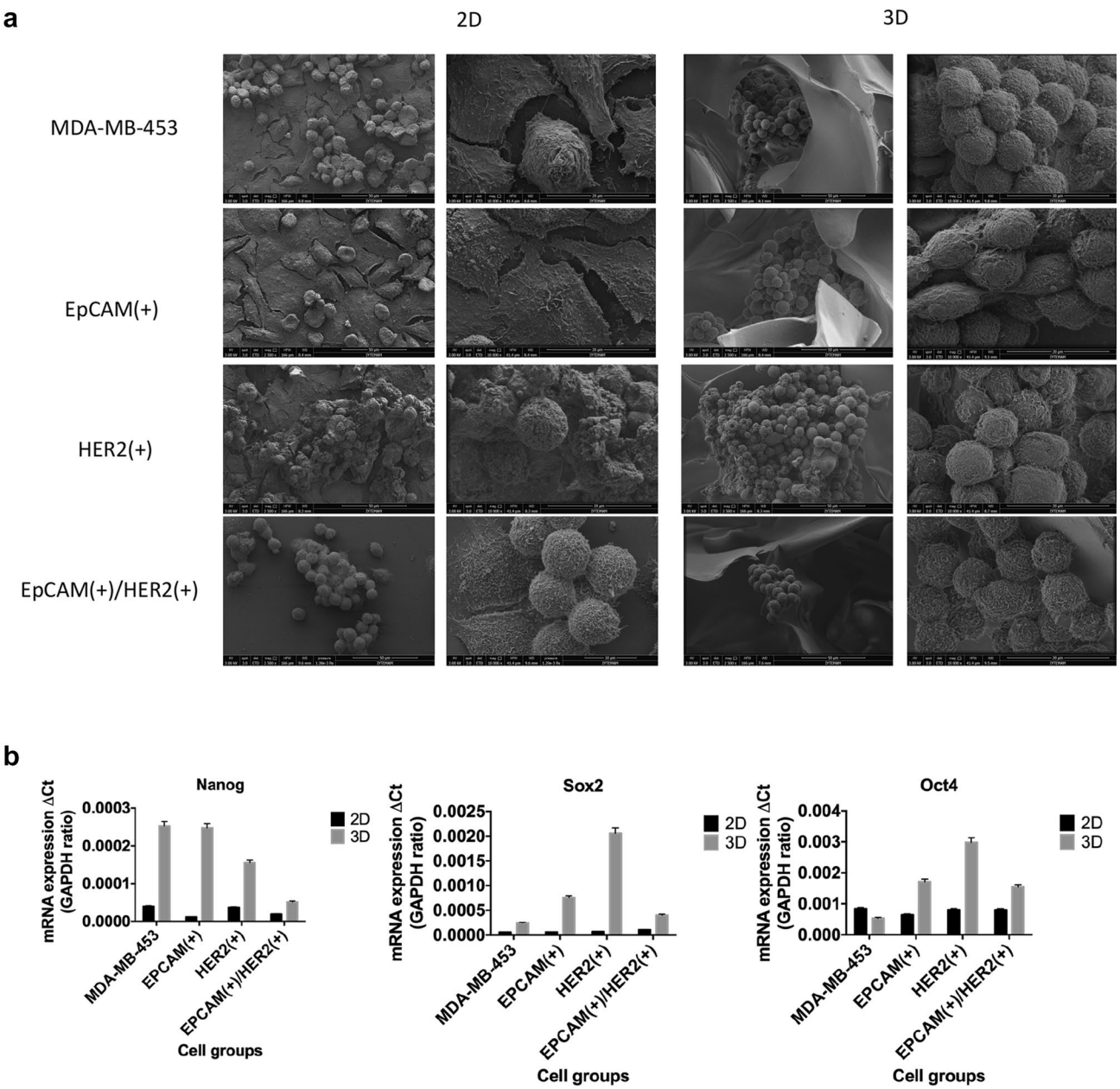


Fig. 4 **a** SEM images and **b** pluripotency gene expression levels of MDA-MB-453, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells cultured as monolayers and on chitosan scaffold for 10 days. Data are plotted as mean of replicates with error bars (±SD). Scale bars: 50 µm for left and 20 µm for right images (except for EpCAM(+)/HER2(+) cells, Scale bar: 10 µm) each 2D and 3D micrograph columns

However, there is still an unmet need for combined strategies to develop aggressive and realistic platforms. The 3D aggressive tumor model developed in our study presents several advantages, such as: (a) easy to conduct, (b) more cost-effective than the most of advanced 3D culture models, (c) providing a decreased flattened cellular shape and (d) mimicking aggressive nature of in vivo tumors with increased drug resistance and stemness. This model may provide a suitable platform for the research of cancer biology and screening of anticancer drugs.

Conclusions

In summary, the present work reveals that EpCAM and HER2 expressing breast cancer cells exhibit more aggressive behavior than bulk cancer population and 3D

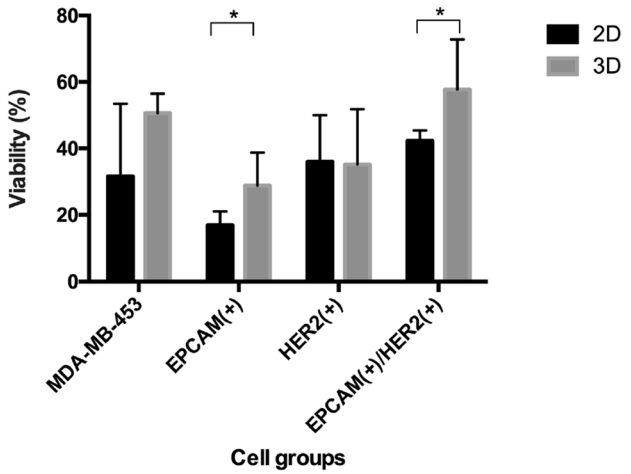


Fig. 5 Cell viability of 2D and 3D cultured MDA-MB-453, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells after 72 h of doxorubicin treatment. Cell viability was determined by MTT assay. Data are plotted as mean of replicates with error bars (±SD) and statistically analyzed using the unpaired Student's *t*-test. Statistical significance was defined as $P < 0.05$

culture of these cells within chitosan scaffold enhanced stemness property and anti-cancer therapeutic resistance. This novel, aggressive, inexpensive, easy and reproducible 3D breast cancer model would be a powerful tool to investigate cancer biology, efficiency of therapeutics and targeting specificity.

Materials and methods

Cell culture

Breast cancer cell lines; MCF-7 (human breast adenocarcinoma cell line), MDA-MB 231 (human breast adenocarcinoma *cell line*) and MDA-MB-453 (an androgen responsive human breast carcinoma cell line) were obtained from Ege University, Department of Bioengineering, Animal Cell and Tissue Engineering Laboratory, Turkey. Cells were seeded at a density of 1×10^5 cells/ml in 75 cm^2 cell culture flasks. MCF-7 cells were maintained in RPMI 1640 medium (Biochrom, Germany) supplemented with 10% fetal bovine serum (FBS) (Biochrom, Germany), 200 mM L-glutamine (Biochrom, Germany), 100U/ml penicillin and 200 µg/ml streptomycine (Biochrom, Germany). MDA-MB 231 and MDA-MB-453 cells were maintained in Leibovitz's L15 (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS), 200 mM L-glutamine, 100U/ml penicillin and 200 µg/ml streptomycine. Cells were cultured at 37 °C in a humidified CO_2 incubator. Cell lines were routinely tested for mycoplasma contamination on a regular basis.

Cell line selection by immunocytochemical analyses

MCF-7, MDA-MB 231 and MDA-MB-453 cell lines were seeded onto 3 separate coverslips (for HER2, EpCAM stain and negative control). Cells were fixed with 4% paraformaldehyde incubated for 30 min and washed with PBS. It was then blocked with blocking solution for 30 min (PBS containing 1% BSA, 22 mg/ml glycine, 0.1% Tween 20). Cells were stained with 1 µg/ml anti-HER2 or anti-EpCAM primer antibody (Abcam, USA) (diluted in PBS containing 1% BSA, 0.1% Tween 20) for 30 min at room temperature. Then, PBS washes were performed to remove unbound antibodies. 10 µg/ml Alexa Fluor® 647 Streptavidin secondary antibody (Life Technologies, USA) was used to label the Her-2 primer antibodies, and 10 µg/ml Alexa Fluor 488 Streptavidin secondary antibody (Life Technologies, USA) was used to label EpCAM primer antibodies. Following the incubation, cells were washed with PBS and cover slips were fixed on the slides. Observation under fluorescence microscope (Zeiss, Axio Vert.A1) was performed and labeled and unlabeled cell numbers were measured by ImageJ Software.

Isolation of EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cell population by MACS

EpCAM(+), HER2(+) and double positive cells, EpCAM(+)/HER2(+), were collected by MACS technique. Anti-EpCAM conjugated magnetic beads (MACS, Miltenyi, Biotec) were used for the isolation of EpCAM(+) cells. In summary, EpCAM(+) cells were labeled with anti-EpCAM conjugated magnetic beads (MACS, Miltenyi, Biotec) and the cell suspension was passed through the column in the magnetic separator (using the MS or LS column type according to the choice of MiniMACS or MidiMACS depending on the number of cells to be separated), then EpCAM (+) cells were separated. For isolation of HER2(+) and EpCAM(+)/HER2(+) cells, indirect labeling was performed using the biotin conjugated anti-HER2 antibody, anti-EpCAM conjugated magnetic beads (MACS, Miltenyi, Biotec) and Anti-Biotin MultiSort Kit (MACS, Miltenyi, Biotec). Isolation procedures were carried out according to the manufacturer's protocol. Briefly, HER2(+) cells were first labeled with biotin conjugated anti-HER2 antibody (Abcam, USA), then biotin-conjugated cells were labeled with anti-biotin magnetic beads (MACS, Miltenyi, Biotec) and the cell suspension was passed through the column in the magnetic separator, then HER2(+) cells were separated. Some of the isolated HER2(+) cells were collected in the HER2(+) cell group while the other was used for the isolation of the

subset of EpCAM(+)/HER2(+) cells. In this step, the magnetic beads bound to the HER2(+) cells were separated from the surface of cells by Multisort Release Reagent (MACS, Miltenyi, Biotec) and this reaction was stopped with MultiSort Stop Reagent (MACS, Miltenyi, Biotec). Later then, the HER2(+) cells from which the magnetic markers were removed, were labeled with anti-EpCAM conjugated magnetic beads (MACS, Miltenyi, Biotec) and the cell suspension was passed through the column in the magnetic separator to separate EpCAM(+)/HER2(+) cells.

Cell growth kinetic and attachment characteristics

The growth kinetic was determined with batch culture mode in a 6-well plate (Greiner, Germany). Briefly, MDA-MB-453 cells and breast cancer cell subpopulations; EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells were seeded at a starting concentration of 10^3 cells/well in a 96-well plate and cultured for 17 days. Cell viability were determined by MTT (methyl thiazol tetrazolium) (Sigma, ABD) day by day according to the manufacturer's protocol. In order to determine the ratio of live non-adhered cells, cell adhesion assay was performed. Cells were seeded at a concentration of 5×10^4 cell/well in a 6-well plate. In order to directly count the number of non-adherent cells, non-adherent cells were collected with cell culture medium from the plate every 30 min during 180 min and counted following the staining with 0.4% trypan blue dye.

Micro-tissue formation capacity

A 3D "Petri Dish®" (MicroTissues Inc. USA) mold wells (with a diameter and depth of 800 µm) was used to form micro-tissues. 330 µL of the sterilized agar (Sigma, USA) solution (2% w/v) was added to the mold reservoirs and allowed to gel for gelation. Following the gelation, the mold was inverted, and 3D non-adhesive agar gels were taken in a 24-well plate to form micro-tissues. MDA-MB-453, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells were added to conditioned wells (5×10^5 cell/well) in defined volumes and cultured for 19 days. The micro-tissues were observed daily under an inverted microscope (Olympus CK-40, Japan). Diameters of micro-tissues were measured by using ImageJ Software and volumes of micro-tissues were calculated.

Live/dead assay

The viability of MDA-MB-453, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) micro-tissues formed for 19 days was visualized by live/dead assay (Thermo Fisher Scientific, USA). Briefly, micro-tissues were washed with PBS and incubated with dye mixture for 30 min at room temperature. Samples were observed under an inverted microscope (Olympus CK-40, Japan).

Sphere formation capacity in soft agar

Colony formation activities of MDA MB453, EpCAM(+), HER2(+) and double positive cells were determined by the soft agar test to assess in vitro tumorigenic activity of cells. 6 ml of agarose (Sigma, USA) solution (approximately 0.51% w/v) supplemented with cell culture medium (1× final concentration), 5% fetal bovine serum (FBS) and 200 mM L-glutamine, was poured into a 2 mm grided tissue culture petri-dish of dimensions 60×15 mm (Greiner, Germany) and allowed to solidify. Cell suspension (5×10^5/ml, 0.4 ml/petri) were mixed with the top layer agarose medium (1 mL). This mixture was added to the petri dishes following the gelation of the lower layer and incubation was started (37 °C, 5% CO_2). At the end of the 10–15 days of incubation, Cell colonies (consisting of at least 5 cells) formed in 25 small squares (0.04 cm^2 area of each square) was were counted in gridded petri dishes under the microscope. The number of all colonies in a petri dish (9.6 cm^2) was calculated taking account of the colony number in a small square (0.04 cm^2) and the transformation index was calculated with the following formula:

$$\%Transformation\ index = \frac{Number\ of\ colonies\ on\ a\ petridish}{Number\ of\ cells\ inoculated\ to\ a\ petridish}\times 100$$

Formation of 3-D tumor model by using chitosan scaffold

For the 3D model, chitosan scaffolds were produced by using lyophilization technique subsequent to freezing of chitosan (Sigma, USA) in a container which allows − 1 °C/min cooling rate [43]. First, chitosan powder was dissolved in 0.2 M acetic acid solution (2% w/v) at room temperature on a shaker. The chitosan solution added into the syringe was frozen at − 86° C. The solvent was sublimed by lyophilizing overnight in the lyophilizer. Chitosan scaffolds (volume of each scaffold: 0.48 cm^3) were sterilized with ethylene oxide gas and left to stand for 2 days to eliminate toxic effects of gas. Then scaffolds were dehydrated through a graded series of ethanol for 10 min at each ethanol concentration (98%, 90%, 80%, 70% and 50%) and washed 5–6 times with sterile PBS. Chitosan scaffolds were transferred into well plates and left to be conditioned by overnight incubation with cell culture medium. MDA-MB-453 cells and EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells isolated from MDA-MB-453 cells were seeded on chitosan scaffold (1×10^6 cells/cm^3).

SEM analysis

MDA-MB-453 cells, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells were cultured on conventional 2D culture plates or in chitosan scaffolds and morphologies of 2D and 3D cultured cells were observed using scanning electron microscope (SEM; FEI QUANTA 250 FEG). After the culture period, living samples were fixed with 5% glutaraldehyde (Merck Millipore, Darmstadt, Germany) prepared in 0.1 M sodium cacodylate (Sigma Aldrich St. Louis, MO, USA) at 4 °C for 30 min. Following the treatment of cells with 7% sucrose (in 0.1 M sodium cacodylate) (Merck Millipore, Darmstadt, Germany) at 4 °C for 30 min, post-fixation was performed for 30 min with 1% aqueous osmium tetroxide (Sigma Aldrich St. Louis, MO, USA) in 0.1 M cacodylate. The samples were gradually dehydrated with ethanol and treated with hexamethyldisilazan (HMDS, Sigma Aldrich St. Louis, MO, USA) for 5 min. The samples sputter-coated with 200 Å gold/palladium (Au/Pd) examined with a scanning electron microscope (LEoL JSM-5200, Japan).

Quantitative polymerase chain reaction (qPCR) analysis

MDA-MB-453 cells, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells that were cultivated in 2D or 3D culture (in chitosan scaffold) conditions for 10 days were evaluated with regard to mRNA expression of pluripotency marker genes Nanog, Sox2 and Oct4 by qPCR (quantitative polymerase chain reaction) technique. Total mRNA was isolated from 2D to 3D cultured cells. To isolate RNA, the samples (approximately 1 million cells per sample) were lysed by a homogenizer, suspended in 1 ml tripure reagent (Life Science, 11667165001, USA) and vortexed. 200 µl chloroform was added and the samples were centrifuged at 12,000×*g* for 20 min at 4 °C. RNA was transferred into a fresh tube and 500 µl isopropanol was added. Following an incubation step for 10 min at room temperature, the samples were centrifuged at 12,000×g for 10 min at 4 °C. The supernatant was removed and pellets were washed with 1 ml 75% EtOH and centrifuged at 7500×*g* for 5 min at 4 °C. Following removal of the supernatant again, EtOH was evaporated at 57 °C and pellets were resuspended in 50 µl RNAse-free water. RNA concentrations and ratios were determined by spectrophotometric measurement. cDNA synthesis was performed with "Transcriptor First Strand cDNA Synthesis Kit" (Roche; 04379012001, USA). The primers (Table 1) were designed using Clustal Waling and Oligo7 software. For qPCR (LightCycler 480II), GAPDH was used as the house-keeping gene.

Table 1 Primers designed for the gene expression analysis of pluripotency markers (Nanog, Sox2 and Oct4) for human breast cancer cells

Gene	Direction	Sequence
Nanog	F	ACAGGTGAAGACCTGGTTCC
	R	TTGCTATTCTTCGGCCAGTT
Sox2	F	ATGGGTTCGGTGGTCAAGT
	R	CTGATCATGTCCCGGAGGT
Oct4	F	CTTCGGATTTCGCCTTCTC
	R	CTTAGCCAGGTCCGAGGAT
GAPDH	F	AGCCACATCGCTCAGACAC
	R	AATACGACCAAATCCGTTGACT

GAPDH was used as the housekeeping gene for all groups

Determination of doxorubicin activity by using MTT assay

MDA MB 543, EpCAM(+), HER2(+) and EpCAM(+)/HER2(+) cells were cultured in both 2D culture and chitosan scaffolds during 5 days. 2D and 3D cultured cells were exposed to doxorubicin (10 µM) for 72 h and cell viability was assessed by MTT assay.

Statistical analysis

All experiments were repeated at least three times and data are presented as mean ± standard deviation (SD). Statistical significance was determined by Student's *t* test (two-tail) or two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test, through GraphPad Prism version 6.0 (GraphPad Software). $P < 0.05$ was considered statistically significant.

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References

1. Maltoni R et al (2015) Circulating tumor cells in early breast cancer: a connection with vascular invasion. Cancer Lett 367:43–48. https://doi.org/10.1016/j.canlet.2015.06.020
2. Wang HY et al (2015) Detection of circulating tumor cell-specific markers in breast cancer patients using the quantitative RT-PCR assay. Int J Clin Oncol 20:878–890. https://doi.org/10.1007/s10147-015-0798-3
3. Fauci AS, Braunwald E, Kasper DL (2008) Breast cancer Harrison's principles of internal Medicine, 17th edn. McGraw-Hill Companies, Inc, New York
4. Joosse SA, Gorges TM, Pantel K (2015) Biology, detection, and clinical implications of circulating tumor cells. EMBO Mol Med 7:1–11. https://doi.org/10.15252/emmm.201303698
5. Zeune L et al (2017) Quantifying HER-2 expression on circulating tumor cells by ACCEPT. PLoS ONE 12:e0186562. https://doi.org/10.1371/journal.pone.0186562
6. Andree KC, van Dalum G, Terstappen LW (2016) Challenges in circulating tumor cell detection by the cell search system. Mol Oncol 10:395–407. https://doi.org/10.1016/j.molonc.2015.12.002
7. Ashworth TRA (1869) A case of cancer in which cells similar to those in the tumours were seen in the blood after death. Med J Aust 14:146–147
8. Esmaeilsabzali H, Beischlag TV, Cox ME, Parameswaran AM, Park EJ (2013) Detection and isolation of circulating tumor cells: principles and methods. Biotechnol Adv 31:1063–1084. https://doi.org/10.1016/j.biotechadv.2013.08.016
9. Gadalla SE, Ojemalm K, Vasquez PL, Nilsson I, Ericsson C, Zhao J, Nister M (2013) EpCAM associates with endoplasmic reticulum aminopeptidase 2 (ERAP2) in breast cancer cells. Biochem Biophys Res Commun 439:203–208. https://doi.org/10.1016/j.bbrc.2013.08.059
10. Zieglschmid V, Hollmann C, Gutierrez B, Albert W, Strothoff D, Gross E, Bocher O (2005) Combination of immunomagnetic enrichment with multiplex RT-PCR analysis for the detection of disseminated tumor cells. Anticancer Res 25:1803–1810
11. Yeo SK, Guan JL (2017) Breast cancer: multiple subtypes within a tumor? Trends Cancer 3:753–760. https://doi.org/10.1016/j.trecan.2017.09.001
12. Riethdorf S et al (2007) Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the cell search system. Clin Cancer Res 13:920
13. Pestrin M et al (2009) Correlation of HER2 status between primary tumors and corresponding circulating tumor cells in advanced breast cancer patients. Breast Cancer Res Treat 118:523–530. https://doi.org/10.1007/s10549-009-0461-7
14. Iqbal N, Iqbal N (2014) Human epidermal growth factor receptor 2 (HER2) in cancers: overexpression and therapeutic ımplications. Mol Biol Int 2014:852748. https://doi.org/10.1155/2014/852748
15. Witton CJ, Reeves JR, Going JJ, Cooke TG, Bartlett JM (2003) Expression of the HER1-4 family of receptor tyrosine kinases in breast cancer. J Pathol 200:290–297. https://doi.org/10.1002/path.1370
16. Fehm T, Maimonis P, Katalinic A, Jäger WH (1998) The prognostic significance of c-erbB-2 serum protein in metastatic. Breast Cancer Oncol 55:33–38. https://doi.org/10.1159/000011832
17. Pantel K, Alix-Panabieres C (2010) Circulating tumour cells in cancer patients: challenges and perspectives. Trends Mol Med 16:398–406. https://doi.org/10.1016/j.molmed.2010.07.001
18. Man Y, Wang Q, Kemmner W (2011) Currently used markers for CTC isolation—advantages, limitations and ımpact on cancer prognosis. J Clin Exp Pathol. https://doi.org/10.4172/2161-0681.1000102
19. Caballero D, Kaushik S, Correlo VM, Oliveira JM, Reis RL, Kundu SC (2017) Organ-on-chip models of cancer metastasis for future personalized medicine: from chip to the patient. Biomaterials 149:98–115. https://doi.org/10.1016/j.biomaterials.2017.10.005
20. Balalaeva IV, Sokolova EA, Puzhikhina AD, Brilkina AA, Deyev SM (2017) Spheroids of HER2-positive breast adenocarcinoma for studying anticancer ımmunotoxins ın vitro. Acta Naturae 9:38–43
21. Mahmoudzadeh A, Mohammadpour H (2016) Tumor cell culture on collagen-chitosan scaffolds as three-dimensional tumor model: a suitable model for tumor studies. J Food Drug Anal 24:620–626. https://doi.org/10.1016/j.jfda.2016.02.008
22. Ozcelikkale A et al (2017) Differential response to doxorubicin in breast cancer subtypes simulated by a microfluidic tumor model. J Control Release 266:129–139. https://doi.org/10.1016/j.jconrel.2017.09.024
23. Sant S, Johnston PA (2017) The production of 3D tumor spheroids for cancer drug discovery. Drug Discov Today Technol 23:27–36. https://doi.org/10.1016/j.ddtec.2017.03.002
24. Martelotto LG, Ng CK, Piscuoglio S, Weigelt B, Reis-Filho JS (2014) Breast cancer intra-tumor heterogeneity. Breast Cancer Res 16:210
25. Marusyk A, Almendro V, Polyak K (2012) Intra-tumour heterogeneity: a looking glass for cancer? Nat Rev Cancer 12:323
26. Brooks MD, Burness ML, Wicha MS (2015) Therapeutic implications of cellular heterogeneity and plasticity in breast cancer. Cell Stem Cell 17:260–271
27. McGranahan N, Swanton C (2015) Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. Cancer Cell 27:15–26
28. Arpino G, Milano M, De Placido S (2015) Features of aggressive breast cancer. Breast 24:594–600
29. Parekh A, Das D, Das S, Dhara S, Biswas K, Mandal M, Das S (2018) Bioimpedimetric analysis in conjunction with growth dynamics to differentiate aggressiveness of cancer cells. Sci Rep 8:783
30. Spizzo G et al (2011) EpCAM expression in primary tumour tissues and metastases: an immunohistochemical analysis. J Clin Pathol 64:415–420
31. Gastl G, Spizzo G, Obrist P, Dünser M, Mikuz G (2000) Ep-CAM overexpression in breast cancer as a predictor of survival. Lancet 356:1981–1982
32. Yarden Y (2001) Biology of HER2 and its importance in breast cancer. Oncology 61:1–13
33. Rijal G, Li W (2016) 3D scaffolds in breast cancer research. Biomaterials 81:135–156
34. Cheung RCF, Ng TB, Wong JH, Chan WY (2015) Chitosan: an update on potential biomedical and pharmaceutical applications. Mar Drugs 13:5156–5186
35. Chang P-H, Sekine K, Chao H-M, Hsu S-h, Chern E (2017) Chitosan promotes cancer progression and stem cell properties in association with Wnt signaling in colon and hepatocellular carcinoma cells. Sci Rep 7:45751
36. Kievit FM et al (2014) Proliferation and enrichment of CD133 + glioblastoma cancer stem cells on 3D chitosan-alginate scaffolds. Biomaterials 35:9137–9143
37. Sims-Mourtada J, Niamat RA, Samuel S, Eskridge C, Kmiec EB (2014) Enrichment of breast cancer stem-like cells by growth on electrospun polycaprolactone-chitosan nanofiber scaffolds. Int J Nanomed 9:995
38. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA (2008) An embryonic stem cell–like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 40:499

39. Savci-Heijink CD, Halfwerk H, Hooijer GK, Horlings HM, Wesseling J, van de Vijver MJ (2015) Retrospective analysis of metastatic behaviour of breast cancer subtypes. Breast Cancer Res Treat 150:547–557
40. Gabriel MT, Calleja LR, Chalopin A, Ory B, Heymann D (2016) Circulating tumor cells: a review of non–EpCAM-based approaches for cell enrichment and isolation. Clin Chem 62:571–581
41. Elliott NT, Yuan F (2011) A review of three-dimensional in vitro tissue models for drug discovery and transport studies. J Pharm Sci 100:59–74. https://doi.org/10.1002/jps.22257
42. Luo YT et al (2018) The viable circulating tumor cells with cancer stem cells feature, where is the way out? J Exp Clin Cancer Res 37:38. https://doi.org/10.1186/s13046-018-0685-7
43. Madihally SV, Matthew HW (1999) Porous chitosan scaffolds for tissue engineering. Biomaterials 20:1133–1142