

Original Article

Morphological and gene-expression characterization of viable heterogeneous circulating tumor cells size-captured and cultured from triple-negative breast cancer mouse models

Katarina Kolostova¹, Yong Zhang², Robert M Hoffman^{2,3}, Vladimir Bobek^{1,4,5}

¹Department of Laboratory Genetics, University Hospital, Kralovske Vinohrady, Srobarova 50, Prague, Czech Republic; ²AntiCancer, Inc., San Diego, CA 92111, USA; ³Department of Surgery, University of California, San Diego, CA 92103-8220, USA; ⁴3rd Department of Surgery, The First Faculty of Medicine Charles University and University Hospital Motol, Prague, Czech Republic; ⁵Department of Histology and Embryology, Wroclaw Medical University, Chalubinskiego 6a, 50-368 Wroclaw, Poland

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Abstract: The goal of the present study was to capture and characterize circulating tumor cells (CTC) from mouse models of triple-negative breast cancer (TNBC), a highly recalcitrant disease. CTCs were captured by size-based filtration from orthotopic nude-mouse models of the human MDA-MB-231-GFP cell line. Subsequently, CTCs were cultured for a 3 months period *in vitro*. Cyto-morphological analysis with confocal microscopy demonstrated heterogeneity among the CTCs, which alternated between adherent in culture. CTC gene-expression analysis was performed with qPCR for 20 tumor-associated markers from RNA derived from adherent/nonadherent CTCs in culture. Both Fox3p and TWIST1 were highly expressed in non-adherent CTCs. The present study demonstrates the power of size-based CTC isolation from orthotopic mouse models of TNBC and GFP imaging of viable CTC cells in culture, to characterize highly-heterogeneous TNBC CTC.

Keywords: Triple-negative, breast cancer, circulating tumor cells, CTC, heterogeneous cell culture, MetaCell®, orthotopic, nude mouse, GFP

Introduction

Worldwide, breast cancer (BC) is the most common invasive cancer in women. Approximately 15% of all breast cancers are triple-negative breast cancer (TNBC) [1, 2]. TNBCs are characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) [1, 3]. The prognosis of patients with this type of tumor is very poor, not only because hormonal therapy and treatment with trastuzumab are ruled out, but also because these tumors seem to be more aggressive than other breast carcinoma subtypes [4]. New treatment strategies are needed for TNBC. The present report focuses on developing mouse models of TNBC circulating tumor cells (CTC) that can be used to develop new treatment strategies.

We previously reported that hormone-refractory PC-3 human prostate cancer cells growing orthotopically efficiently produce CTC. This is in contrast to the ectopic tumors of the same cell line, which do not produce viable CTC. Green fluorescent protein (GFP)-labeled viable CTC had an increased metastatic propensity relative to red fluorescent protein (RFP)-labeled parental PC-3 cells upon orthotopic co-implantation [5].

Using the GFP-expressing PC-3 orthotopic model and immunomagnetic beads coated with anti-epithelial cell adhesion molecule (EpCAM) and anti-prostate specific membrane antigen (PSMA) in a previous experiment. GFP-expressing CTC were isolated within 15 minutes and were readily visualized by GFP fluorescence. It was possible to immediately place the

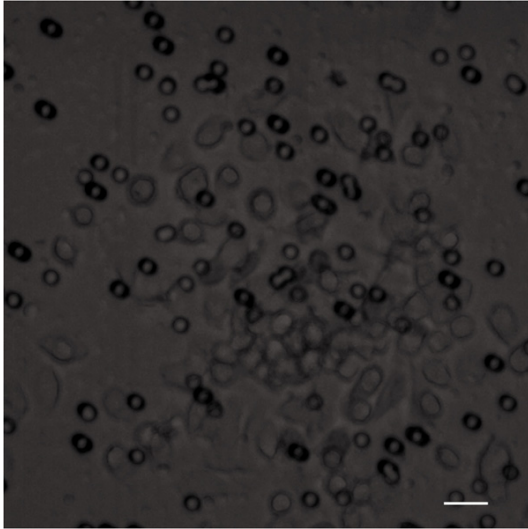


Figure 1. Size-based filtration-captured CTCs from the MDA-MB-231-GFP orthotopic model growing *in vitro* as adherent cells on a polycarbonate membrane. Bar represents 20 μ m.

immunomagnetic-bead-captured GFP-expressing PC-3 CTCs in 3-dimensional sponge-gel cell culture, where they proliferated [6].

In a subsequent study, using immunomagnetic beads, CTCs were rapidly isolated from the circulation of mice orthotopically implanted with human PC-3-GFP prostate cancer cells. The PC-3-GFP CTCs were then expanded in culture in parallel with the parental PC-3-GFP cell line. Both cell types were then inoculated onto the chorioallantoic membrane (CAM) of chick embryos. Inoculation of embryos with PC-3-GFP CTCs resulted in a 3 to 10-fold increase in brain metastasis when compared to those with the parental PC-3-GFP cells [7].

In another previous study, the chemosensitivity of circulating PC-3-GFP human prostate cancer cells, isolated from nude mice orthotopically implanted with PC-3-GFP, was compared to that of the parental PC-3 cells. The CTCs exhibited a significantly increased sensitivity to both cisplatin and docetaxel when compared to PC-3 parental cells, with docetaxel having the greater efficacy [8].

In another previous experiment, the captured RC-3 GFP CTCs were compared to PC-3-GFP cells isolated from various metastatic sites. Both types of cells were grown *in vitro* and examined under fluorescence microscopy. The

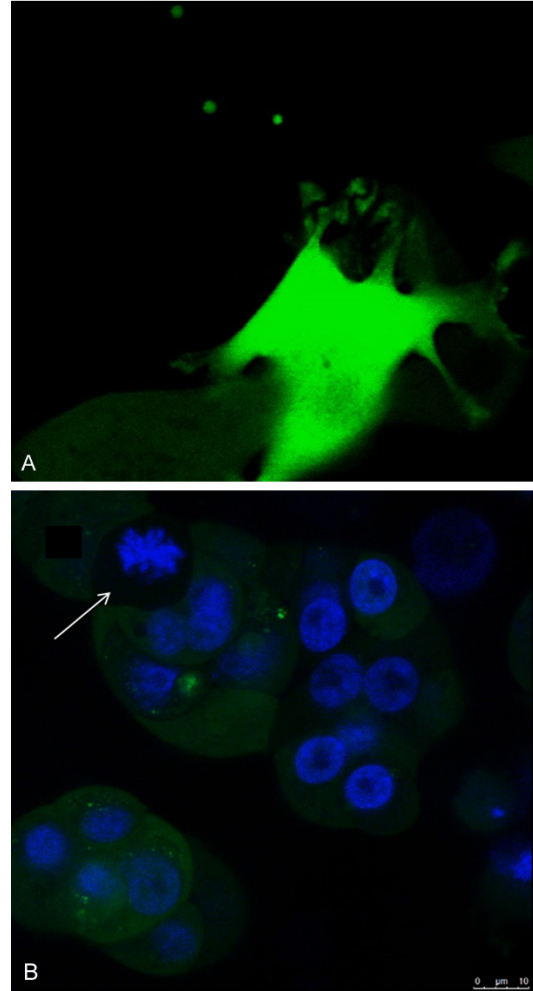


Figure 2. MDA-MB-231-GFP CTCs growing *in vitro* as adherent cells display a minimum of 2-3 phenotypes. A. The GFP-expressing MDA-MB-231 CTCs display an epithelial-like phenotype. B. NucBlue™ staining shows cell viability and proliferation of the cultured MDA-MB-231 cells. Mitosis is shown by an arrow.

differential morphology of primary tumor cells, CTCs and disseminated tumor cells (DTC) from multiple metastatic sites was analyzed. The cultured captured CTCs and DTC from various organs have distinctive morphologies. The distinct morphologies were maintained during *in vitro* culture. The results demonstrate extensive tumor heterogeneity that could account for the widely different behavior of cancer cells in a single tumor [9].

In another study, human lung cancer cell line H460, expressing red fluorescent protein (H460-RFP), was orthotopically implanted in nude mice. CTCs were isolated by a size-based filtration method and cultured *in vitro*. The cul-

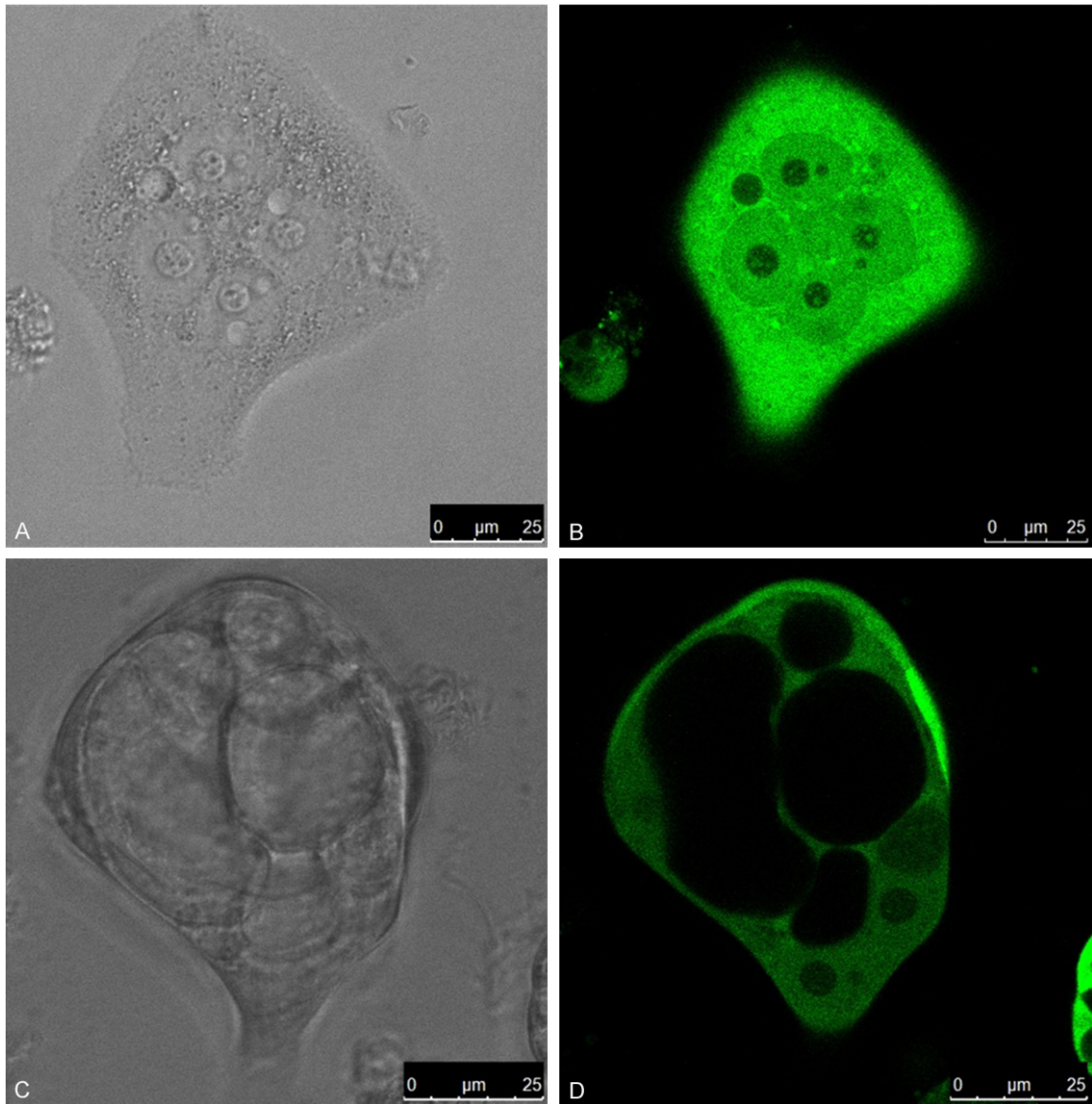


Figure 3. MDA-MB-231 GFP CTCs growing *in vitro* adherent to plastic. Multiple nuclei and nucleoli (A and B) are visualized by light and fluorescence microscopy. The CTCs are vacuolated as well (C and D).

tured CTCs were heterogeneous in size and morphology even though they originated from a single tumor [10].

The present study uses size-based isolation of CTCs from TNBC expressing GFP for culture and cytological as well as gene-expression analysis.

Methods and materials

Cell culture

Triple-negative breast cancer (TNBC) (MDA-MB-231-GFP cells) (AntiCancer, Inc., San Diego, CA)

were used. The cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum and gentamicin (Life Technologies, Carlsbad, CA) to 70-80% confluence as described previously [6].

Mice

Nude mice were from AntiCancer (San Diego). The mice were kept under isoflurane anaesthesia during surgery. Animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals under NIH Assurance No. A3873-01.

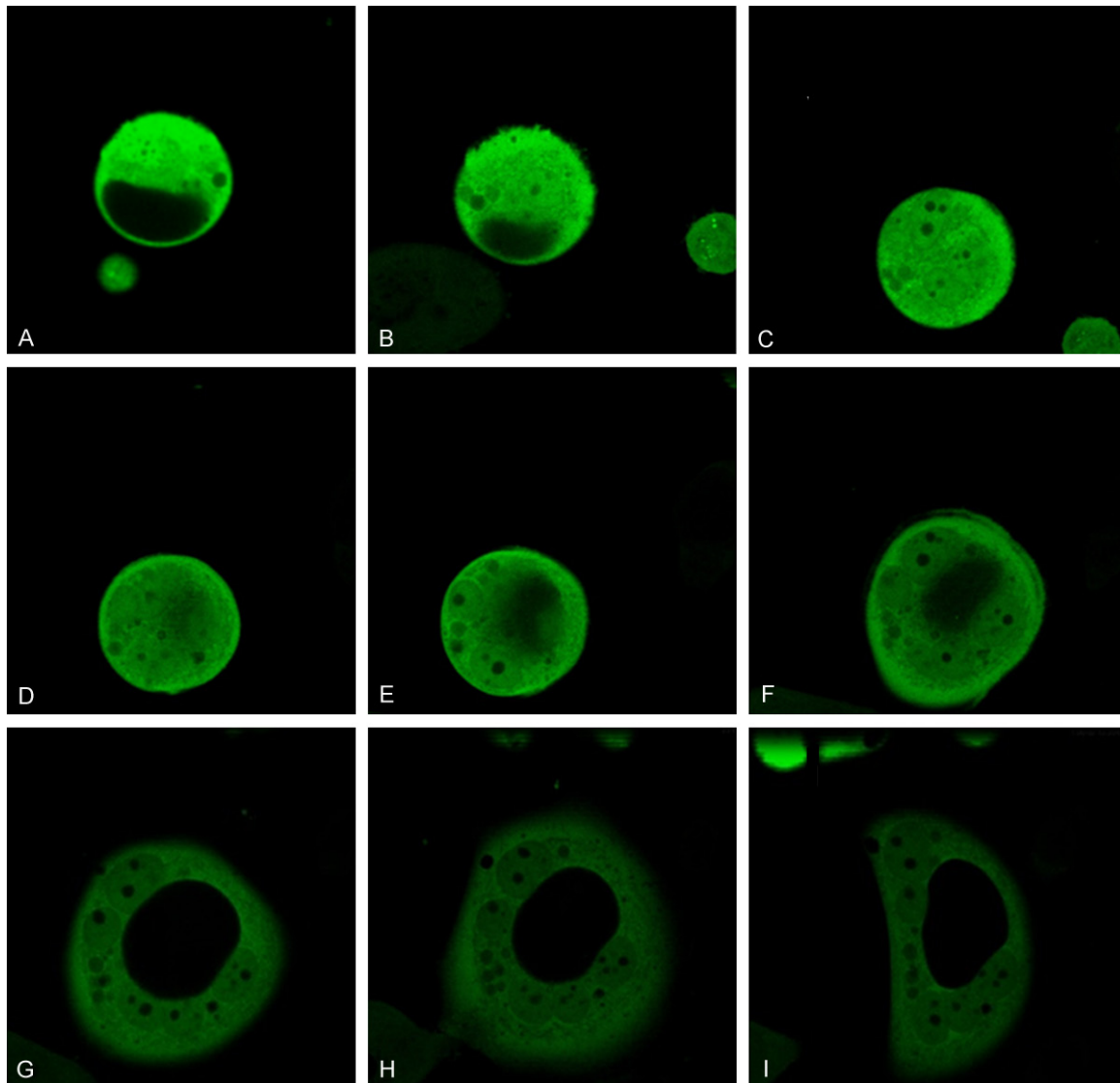


Figure 4. (A-I). Time-lapse imaging of MDA-MB-231-GFP CTCs cultured *in vitro* for 2 months with a large vacuole inside (A-E). The non-adherent cells also can adhere to the plastic. (See [Supplementary Video 1](#)).

Orthotopic models in nude mice

MDA-MB-231-GFP tumor fragments (1 mm³) from subcutaneously-growing tumors were implanted by surgical orthotopic implantation (SOI) into the mammary fat pad of female nude mice [11-14].

All procedures within the surgery were performed with a 7× magnification microscope (Olympus). Five weeks after orthotopic transplantation, blood was obtained by cardiac puncture, followed by peritoneal, pleural washing and examination of metastatic organs.

Isolation and culture of circulating tumor cells (CTCs)

Blood (0.5-1.0 ml) was obtained by cardiac puncture. The blood was put into an EDTA tube (BD) and put through a size-based filtration apparatus (MetaCell®). The size-based enrichment process is based on the filtration of peripheral blood (PB) through porous polycarbonate membrane (8 µm diameter pores). The PB filtration flow is supported by natural capillary action of the absorbent contacting the membrane filter. The membrane filter, in a plastic ring, is transferred into 6-well tissue-culture

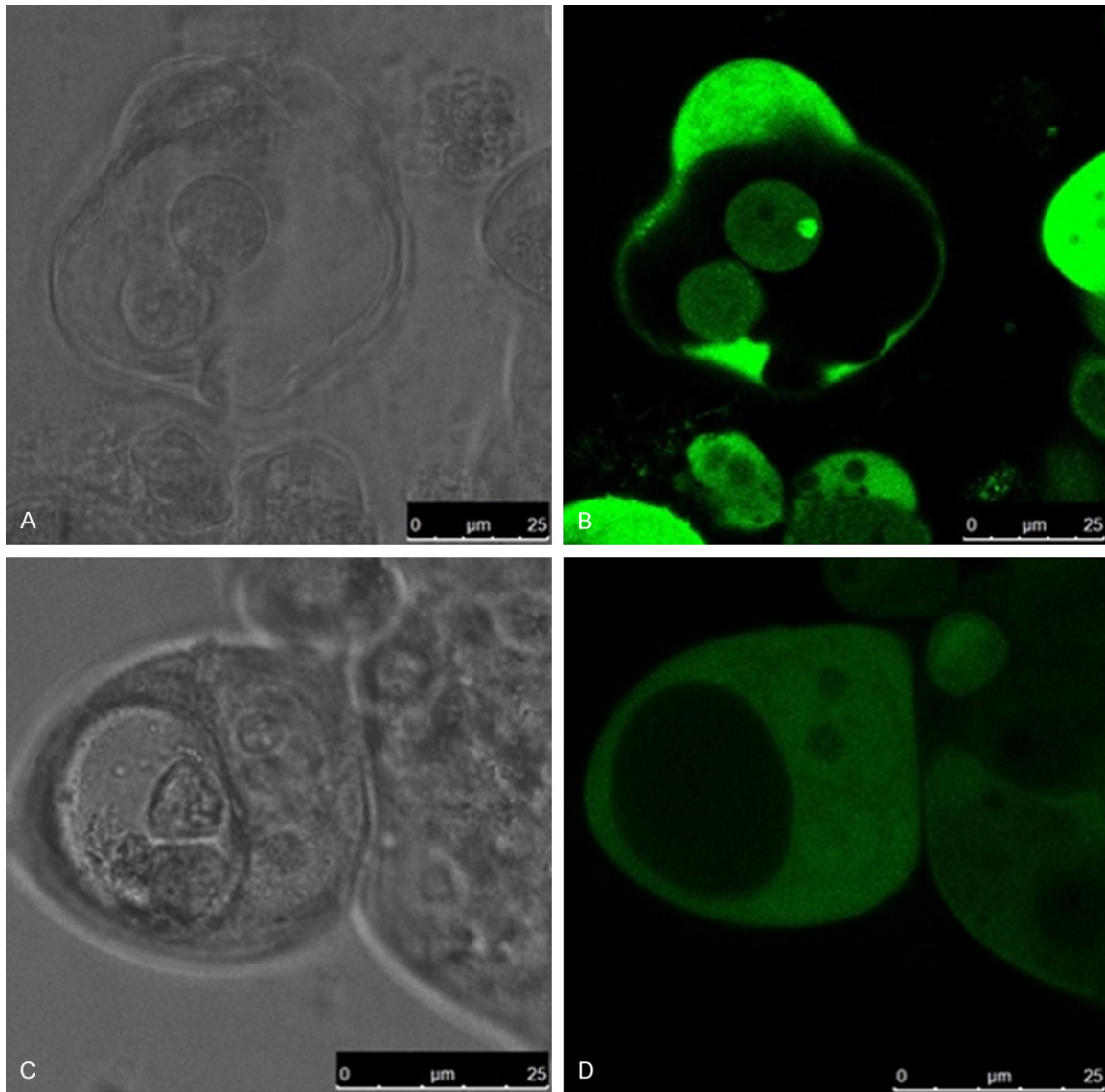


Figure 5. Vacuoles in MDA-MB-231 CTCs are shown in detail here. Two nuclei inside a vacuole were observed (A, B). Materials in vacuoles and eccentric nuclei were observed (C and D).

plates. RPMI-1640 medium with fetal bovine serum (10%) is added to the filter and cultured on the membrane *in vitro* at 37°C for at least 14 days.

Confocal imaging

For time-lapse imaging, a Leica TCS SP5 AOBS with a DFC350 FX digital camera was used [15].

Gene expression profiling

The quantitative polymerase chain reaction (qPCR) was used for 20 tumor-associated

markers in RNA from different adherent or non-adherent CTC in culture. RNA was obtained from CTCs attached on the membrane used for CTC isolation. After the cells were lysed with RLT-buffer with β -mercaptoethanol (Qiagen), RNA was isolated using an RNeasy Mini Kit (Qiagen). The quality/concentration of RNA was measured with NanoDrop (ThermoScientific). For cDNA production, the High Capacity cDNA Reverse Transcription Kit (Life Technologies) was used. For gene expression analysis, Taqman chemistry including Taqman MGB-probes and SYBR green chemistry (Life Technologies) were used.

Characterization of cultured CTCs in triple-negative breast cancer

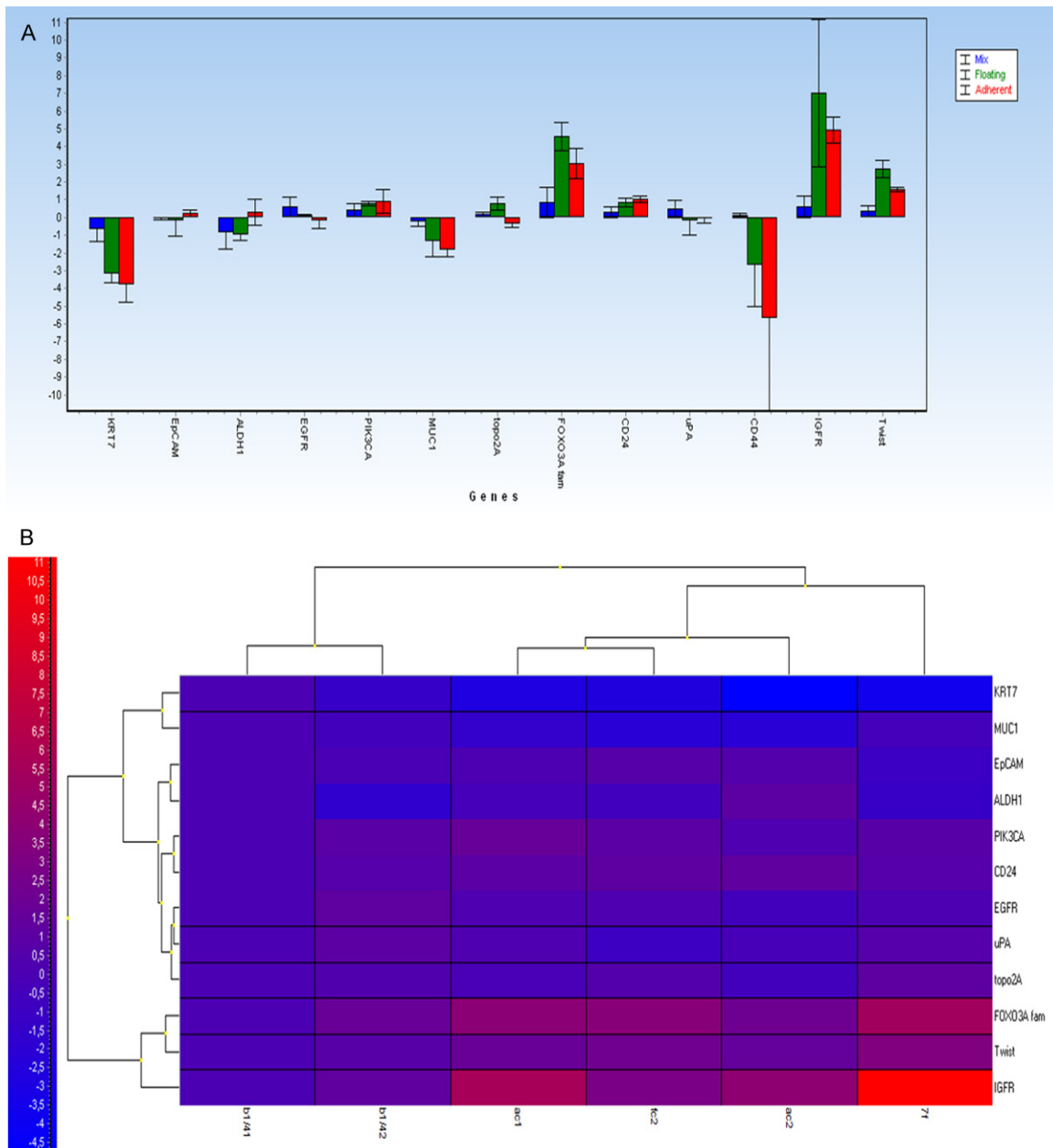


Figure 6. Gene expression analysis, RNA relative quantity shown for adherent, non-adherent, and mixed CTC cultures captured from the MDA-MB-231 orthotopic model. (A) Gene expression analysis demonstrates differences between the non-adherent CTCs (shown in green) and adherent CTCs (shown in red) derived from the MDA-MB-231-GFP model (A). Cluster analysis is shown in (B). Fox3p is clustered with TWIST1. Both of these genes are highly expressed in non-adherent CTCs (B). ALHD1, a stem cell marker, was detected as highly-abundant in adherent cells.

Results

We isolated viable CTCs from the orthotopic TNBC mouse models by size-based filtration. The captured viable CTCs were grown in culture. Proliferation of the CTCs on the filter membrane is shown in **Figures 1** and **2**. The confluent initial cultures on membranes were

transferred into culture flasks. The CTCs were cultured for 3 months. The cytomorphology was demonstrated with confocal microscopy. Time-lapse imaging is shown as [Supplementary Videos 1](#) and [2](#).

Cultured CTC-cells had different sizes and usually had several nuclei, and nucleoli (**Figure 3**),

large vacuoles (**Figures 3, 4**), and numerous vacuole inclusions (**Figure 5**). In general, vacuolization is seen as a marker of death [16]. However, our observations showed the opposite, the growing CTCs had large vacuoles (**Figure 4** and [Supplementary Video 1](#)). The cells grew as clusters and alternated between non-adherent and adherent. The clusters floated and then adhered to the growing plastic, where they persisted for a couple of minutes and reorganized their structure. Subsequently, they were detached again from the surface and floated (**Figure 5** and [Supplementary Video 1](#)). We observed non-adherent cells dividing in culture ([Supplementary Video 2](#)). Non-adherent cells appeared to be generated from adherent cells (**Figure 5**).

Gene-expression analysis

We have compared gene expression in the adherent and non-adherent (floating) CTC-fraction primarily to confirm the hypothesis, that there is a difference between the fractions. The difference could be significant for genes active in the epithelial-mesenchymal transition pathway.

The gene expression results are summarized on **Figure 6**. The tested genes which showed a difference between floating and an adherent CTCs in culture are marked by a star (**Figure 6A**). The most interesting observation was that Fox3p is clustering with TWIST1, both of which are highly expressed in floating cells. ALHD1, seen until now as a marker of stemness, was detected as highly abundant in adherent cells (cluster analysis **Figure 6B**).

Discussion

Current CTC-isolation protocols are not able to capture CTCs which do not express an epithelial surface marker (e.g. EpCAM). The size-based separation method used in the present study could have important clinical potential, since it should capture all the CTC.

CTC capture offers real-time access to cancer cells without the need of an invasive biopsy, while their phenotypic and molecular investigation can provide insight into the biological changes of the tumor that occur during treatment.

In order to represent clinical cancer, we have developed orthotopic mouse models [11, 12].

CTCs were observed in the MDA-MB-231 model to form non-adherent cell-clumps, which are multinucleic and vacuolized and similar to spheroids, as documented in videos ([Supplementary Videos 1](#) and [2](#)). The cells were able to proliferate even with vacuoles. Perhaps the vacuoles may help the cells to float in the circulation.

Current CTC capture technologies, based mostly on epithelial markers, might be unable to capture the potentially most dangerous cancer cells present in the circulation, mesenchymal CTCs, which have been correlated with disease progression and resistance to chemotherapy [17]. Size-based filtration enables all types of CTCs to be captured.

Conclusions

The combination of orthotopic mouse models of fluorescent-protein-expressing TNBC with a size-based filtration device (MetaCell®) provides a rapid means to capture, culture, and characterize CTCs from TNBC. The non-adherent CTCs appear multi-nucleated with multiple vacuoles and divide without attachment, suggesting their high degree of malignancy and should yield important information on the nature and therapy of TNBC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Vladimir Bobek, Department of Laboratory Genetics, University Hospital, Kralovske Vinohrady, Srobarova 50, 10034 Prague, Czech Republic. Tel: +420 26716 3578; E-mail: vbobek@centrum.cz; Dr. Robert M Hoffman, AntiCancer Inc., 7917 Ostrow Street, San Diego, CA 92111, USA. Tel: 858-654-2555; Fax: 858-268-4175; E-mail: all@anticancer.com

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