H2020-ITN THERACAT (765497)

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Overview/Abstract

We have successfully established several clinically relevant breast cancer models that can be used for an accurate pre-clinical evaluation of therapeutics. Once the various prodrugs and prodyes will be synthesized and characterized (WP 3-4), these models will be exploited to evaluate their *ex vivo* and *in vivo* biological activity, including tumor accumulation, biodistribution in healthy organs, safety profile (WBC count, neurotoxicity, cardiotoxicity, blood chemistry), and antitumor activity (WP 5-6).

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1. Set of mCherry-labelled orthotopic models of cancer in mice

Introduction

Clinically relevant cancer models are crucial for the discovery and evaluation of novel therapeutic strategies. We will exploit both established *in vivo* mouse models currently used in pre-clinical practice as well as novel 3D *ex vivo* models. These pre-clinical breast cancer models will enable the biological evaluation of polymers of various compositions in this project.

Objectives

To establish pre-clinical models of breast cancer: (i) *In vivo* syngeneic and xenogeneic mCherry-labeled mouse models (murine, human and patient-derived xenografts); (ii) *Ex vivo* models (3D spheroids and 3D-bioprinted models).

Methodology

Cell lines. MDA-MB-231 human breast cancer cells and 4T1 and EMT6 murine mammary adenocarcinoma were obtained from the American Type Culture Collection (ATCC). MDA-MB-231 and EMT6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL Penicillin, 100 U/mL Streptomycin, 12.5 U/mL Nystatin (PSN), and 2 mM L-glutamine (L-Glu). 4T1 cells were grown in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% FBS, 100 mg/mL penicillin, 100 U/mL streptomycin, 12.5 U/mL nystatin, and 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer and 2.5 g/l D-glucose. Cells were grown at 37°C; 5% CO2.

Patient-derived xenografts (PDX). Fresh human breast cancer tissues were obtained from The Chaim Sheba Medical Center (Tel HaShomer, Israel) in accordance with a protocol approved by the IRB committee (5727-18-SMC). Tumor tissues were obtained during surgical resection, kept in cold PBS and processed within 40 min. In order to isolate tumor cells and generate cells monolayer, tumor specimens were dissected to 0.5 mm pieces, plated in 6 cm plates and cultured with 1 mL DMEM supplemented with 10% FBS, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 2 mM L-glutamine. Following continuous media replacement, viable cancer cells remained attached to culture plates and kept growing in culture, while stroma and cell debris were washed. Cells were routinely tested for mycoplasma contamination with a mycoplasma detection kit (Biological Industries, Israel). All cells were grown at 37°C in 5% CO2. Furthermore, fresh tumor tissues were used to generate patient-derived xenogtrafts by implanting small tissue fragments subcutaneously (sc) into SCID mice, allowing them to grow and then excise them. Additionally, patient-derived samples were used to generate tumorspheres. Tumorspheres were grown in ultra-low attachment surface flasks and then implanted into SCID mice.

Generation of mCherry-infected human and murine breast cancer cell lines. MCherry-labeled cells were established as previously described[1]. Briefly, human embryonic kidney 293T (HEK 293T) cells were cotransfected with pQC-mCherry and the compatible packaging plasmids (pMD.G.VSVG and pGag-pol.gpt). Forty-eight hours following transfection, the pQC-mCherry retroviral particles containing supernatant was collected. MDA-MB-231, 4T1 and EMT6 cells were infected with the retroviral particles media, and 48 h following the infection, mCherry positive cells were selected by puromycin resistance.

Human and murine breast cancer mouse models. Nu/nu female mice were inoculated to the mammary fat pad with $1x10^6$ MDA-MB-231 mCherry-labeled mammary adenocarcinoma cells. Balb/C female mice were inoculated to the mammary fat pad with $0.5x10^6$ mCherry-labeled 4T1 mammary adenocarcinoma cells. Tumor growth was continuously followed by non-invasive intravital imaging systems (Maestro Cri™, IVIS SpectrumCT).

3D tumor spheroids. We created a 3D spheroid co-culture system of tumor cells with stromal cells in thick matrigel as we previously described for our unique hanging-drop spheroids[2]. Briefly, cells suspension of mCherry-labeled tumor cells, alone or in co-culture with with stromal cells (80,000 cells/ml) was prepared DMEM supplemented with 0.24 w/v% methyl cellulose. Cells were deposited in 25 µl droplets on the inner side of a 20 mm dish and incubated for 48 h at 37°c when the plate is facing upside down to allow for spheroid formation. Spheroids were then embedded in matrigel, seeded in a 96-well plate and monitored for cells' invasion using EVOS FL Auto cell imaging system (ThermoFisher Scientific).

3D bio-printing. For the bio-printing process, cells were resuspended and mixed with our tailor-made polymer-based biocompatible hydrogels (gelatin methacrylate, fibrin, pluronic, hyaluronic acid), which provide oxygen and other nutrients to keep the cells viable. The resulting 'bio-ink' was then fed into a 3D printer cartridge (3D-Bioplotter®, EnvisionTEC) that dispenses the cells layer by layer, forming the patient-specific 3D tissue. The printed product was analyzed by confocal microscopy to evaluate cell-growth and blood vessels formation.

Results

Murine, human and PDX mouse models. We established several human and murine models of orthotopic breast cancer from mCherry-labeled[1] MDA-MB-231 and 4T1 cells (**Fig. 1A**). mCherry-labeled mammary tumors were visualized by non-invasive fluorescence imaging (Maestro CRi™) and by an integrated fluorescence and computed tomography imaging (IVIS SpectrumCT) (**Fig 1B**). Furthermore, using fresh tissue samples, we generated PDX models and established tumorspheres that were implanted into SCID mice (**Fig. 1A**).

Ex vivo 3D tumor models. We created 3D spheroids from mCherry-labeled EMT6 or 4T1 murine cells co-cultured with stromal cells and monitored cells invasion into Matrigel for 48-72 h (**Fig. 1C-D**). The addition of tumor stroma to 4T1 spheres resulted in enhanced tumor cells invasion (**Fig. 1D**). The spheroids will be further used for invasion assays in matrigel following treatments and for internalization kinetics studies of our nanomedicines. Exploiting our fibrin hydrogel as bio-ink for tumor cells and stroma and the sacrificable pluronic hydrogel to print vascular network, we created a 3D-printed tumor model composed of tumor cells, stroma, and a functional vessels connected to a microfluidic pump (**Fig 1E**). The inner surface of the resulting vascular network was coated with endothelial cells, which successfully formed a vascular lumen (**Fig F-G**).

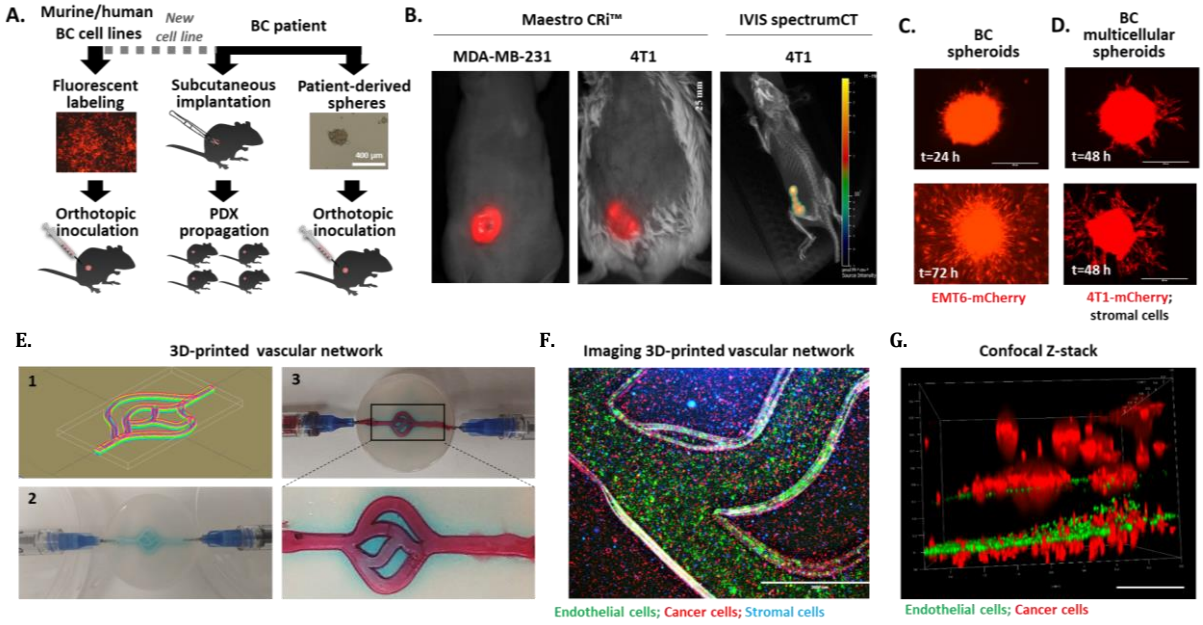

Figure 1. Models of breast cancer (BC). A. Human, murine and patient-derived xenograft (PDX) mouse models for BC. **B.** Non-invasive imaging of mice bearing BC. Right panel: Fluorescence-imaging of mCherry-labeled MD-MB-231 and 4T1 mammary tumors, performed by Maestro CRi™; Left panel: 3D reconstruction combining fluorescence and µCT imaging of mice bearing mCherry-labeled 4T1 tumors, performed by IVIS SpectrumCT Imaging System. **C-D.** 3D spheroids composed of mCherry-labeled EMT6 cells (C) or mCherry-labeled 4T1 cells co-cultured with tumor stroma. Spheroids were embedded in matrigel and monitored for cells' invasion. **E.** 3D-printed microfluidics system. Vascular network was designed by computer modeling (1) then printed inside fibrin hydrogel using the thermoreversible pluronic hydrogel (2). Once the pluronic hydrogel was sacrificed, flow of blood-like fluid was allowed (3). **F.** The 3D-printed blood vessels are composed of GFP-labeled endothelial cells and surrounded by mCherry-labeled cancer cells and iRFP-labeled stromal cells. **G.** 3D projection showing the lumen of the 3D-printed vessels. Z-stack was acquired by confocal microscopy and generated using manual and automated processes in LAS-AF and Imaris software programs.

Summary

We have successfully established several clinically relevant breast cancer models that can be used for an accurate pre-clinical evaluation of therapeutics. Once the various prodrugs and prodyes will be synthesized and characterized (WP 3-4), these models will be

exploited to evaluate their *ex vivo* and *in vivo* biological activity, including tumor accumulation, biodistribution in healthy organs, safety profile (WBC count, neurotoxicity, cardiotoxicity, blood chemistry), and antitumor activity (WP 5-6).

2. References

1. Segal, E., et al., *Targeting angiogenesis-dependent calcified neoplasms using combined polymer therapeutics.* PLoS One, 2009. **4**(4): p. e5233.
2. Ferber, S., et al., *Co-targeting the tumor endothelium and P-selectin-expressing glioblastoma cells leads to a remarkable therapeutic outcome.* Elife, 2017. **6**.