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Multi-parametric hydrogels support 3D *in vitro* bioengineered microenvironment models of tumour angiogenesis

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ABSTRACT

Tumour microenvironment greatly influences the development and metastasis of cancer progression. The development of three dimensional (3D) culture models which mimic that displayed *in vivo* can improve cancer biology studies and accelerate novel anticancer drug screening. Inspired by a systems biology approach, we have formed 3D *in vitro* bioengineered tumour angiogenesis microenvironments within a glycosaminoglycan-based hydrogel culture system. This microenvironment model can routinely recreate breast and prostate tumour vascularisation. The multiple cell types cultured within this model were less sensitive to chemotherapy when compared with two dimensional (2D) cultures, and displayed comparative tumour regression to that displayed *in vivo*. These features highlight the use of our *in vitro* culture model as a complementary testing platform in conjunction with animal models, addressing key reduction and replacement goals of the future. We anticipate that this biomimetic model will provide a platform for the in-depth analysis of cancer development and the discovery of novel therapeutic targets.

1. Introduction

Innovative medical and materials research has led to novel technologies in the quest for improved cell culture methods. Pioneering research in three dimensional (3D) culture models from the Bissell laboratory [1] has opened the way for more sophisticated and relevant culture models than the traditional two dimensional (2D) methods. Although 2D methods have contributed unsurmountable medical breakthroughs in cancer research and drug discovery, the progression of cancer to an advanced state cannot be recapitulated in 2D and moreover cannot be completely understood from small animal models. Therefore it is imperative that we close the gap between poorly instructive 2D cultures and the often less descriptive and already too complex small animal models. This will lead towards a more detailed understanding of cancer progression using a far more adaptable and relevant physiological systems biology approach [2].

Tumour growth and development is dependent on the surrounding microenvironment, including the extracellular matrix (ECM), cell–cell contacts and environmental cues [3,4]. Cellular interactions and growth factor signalling are known to regulate cancer development, including tumour angiogenesis [5,6]. However, little information is available to study the tumour microenvironment with respect to the vascularisation *in vitro*. Attempts to mimic tumour angiogenesis *in vitro* by means of co-cultivation or using layered synthetic materials have had limited success, as current methodologies are insufficient to support and maintain all cellular types in culture [7], and the outcomes of the culture model are most likely dependent on how much of the microenvironment is actually recapitulated within the bioengineered construct.

Only a few research groups have attempted breast or prostate tumour-vasculature co-culture, and show varying results. A recent report utilised MDA-MB-231 breast cancer cells co-cultured with human umbilical vein endothelial cells (HUVECs) and normal human dermal fibroblasts to mimic *in vivo* tumour-vasculature interactions within collagen type I gels [8]. In another report, collagen

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I and fibrinogen gels were utilised for a co-culture model with PC3 prostate cancer cells, as well as HUVECs and human foreskin fibroblasts [9]. One problem arising from these approaches is that such natural matrices can result in batch-to-batch variability, inherent cellular signalling molecules, degradation, shrinkage, difficulties in the ability to monitor the culture progression and a general lack of mechanical stability which can have unknown effects on cancer cell biology and development.

To address these challenges, we have developed matrix metalloproteinase (MMP)-sensitive four-arm star-shaped poly(ethylene glycol) (starPEG)-heparin hydrogels [10,11] in which RGD motifs are incorporated at a defined density. The RGD motifs provide binding sites for cells via integrins, and the MMP-responsive sequences allow cells to locally remodel the matrix for the purposes of proliferation and migration. The materials can be readily fashioned into a hydrogel structure and can be tuned to the particular requirements of the tumour microenvironment via the precise adjustment of stiffness and mesh size. Furthermore, decoration with various growth factors and ECM protein-based cues is possible, utilising the signal molecule affine glycosaminoglycan heparin as a bioactive building block. We have previously shown that these starPEG-heparin hydrogels provide an effective matrix for the co-culture of hepatocarcinoma cells with HUVECs [12].

Herein, we established a highly sophisticated and complex 3D microenvironment of human cancer angiogenesis by culturing breast or prostate epithelial carcinoma cells (MCF-7, MDA-MB-231, LNCaP, PC3) with HUVECs and mesenchymal stromal cells (MSCs) within matrices fabricated from synthetic starPEG and maleimide-functionalised heparin (Fig. 1). This study is conducted in three stages: comparative evaluation of cancer tumour growth within starPEG-heparin hydrogels and within Matrigel™, evaluation of the bioengineered tumour angiogenesis microenvironment, and evaluation of the responsiveness of the culture model to chemotherapeutics and angiogenesis inhibitors.

2. Materials and methods

2.1. Cell culture

MCF-7, MDA-MB-231 and LNCaP cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and used within 10 passages. The PC3 cell line was obtained from the American Type Culture Collection (ATCC; LGC Standards GmbH, Wesel, Germany), authenticated by the DSMZ and used for experiments within 10 passages. MCF-7 cells were cultured in medium consisting of RPMI medium supplemented with GlutaMax (Life Technologies, Darmstadt, Germany), 10% fetal bovine serum (FBS; Hyclone Thermo Scientific, Schwerte, Germany), 1X MEM non-essential amino acids (Life Technologies), 1 mM Sodium Pyruvate (Sigma Aldrich, Munich, Germany), 0.1% human insulin (Life Technologies) and 1% penicillin/streptomycin solution (PS; Life Technologies). MDA-MB-231 cells were cultivated in DMEM supplemented with 10% FBS and 1% PS. LNCaP and PC3 cells were propagated in RPMI medium supplemented with 10% FBS and 1% PS. HUVECs were isolated as previously described [13] and cultured in Endothelial Cell Growth Medium (ECGM; Promocell, Heidelberg, Germany). Bone marrow-derived MSC were isolated as previously described [14] and cultured in DMEM supplemented with 10% FBS and 1% PS. HUVECs and MSCs were utilised for experiments between passages 1 and 6.

2.2. Preparation of tumour constructs

Hydrogels were prepared as described previously [11]. The heparin batch was modified with an average of 6 maleimide groups per molecule of heparin. MCF-7, MDA-MD-231, LNCaP or PC3 cells at a density of 5×10^5/mL were seeded separately into non-functionalised starPEG-heparin hydrogels for tumour only experiments utilising gels at a crosslinking degree of γ0.75, γ1.0 or γ1.5 (molar ratio of starPEG to heparin-maleimide), which vary in stiffness from 500 to 3000 Pa. For tumour angiogenesis cultures, the heparin-maleimide fraction of the hydrogel was first functionalised with 2 mol of RGD-SP (H_2N-GCWGGRGDSP-$CONH_2$; MW 990; synthesised within our laboratory) per mole of heparin and vortexed thoroughly. 5 μg/mL of each vascular endothelial growth factor (VEGF; Peprotech, Hamburg,

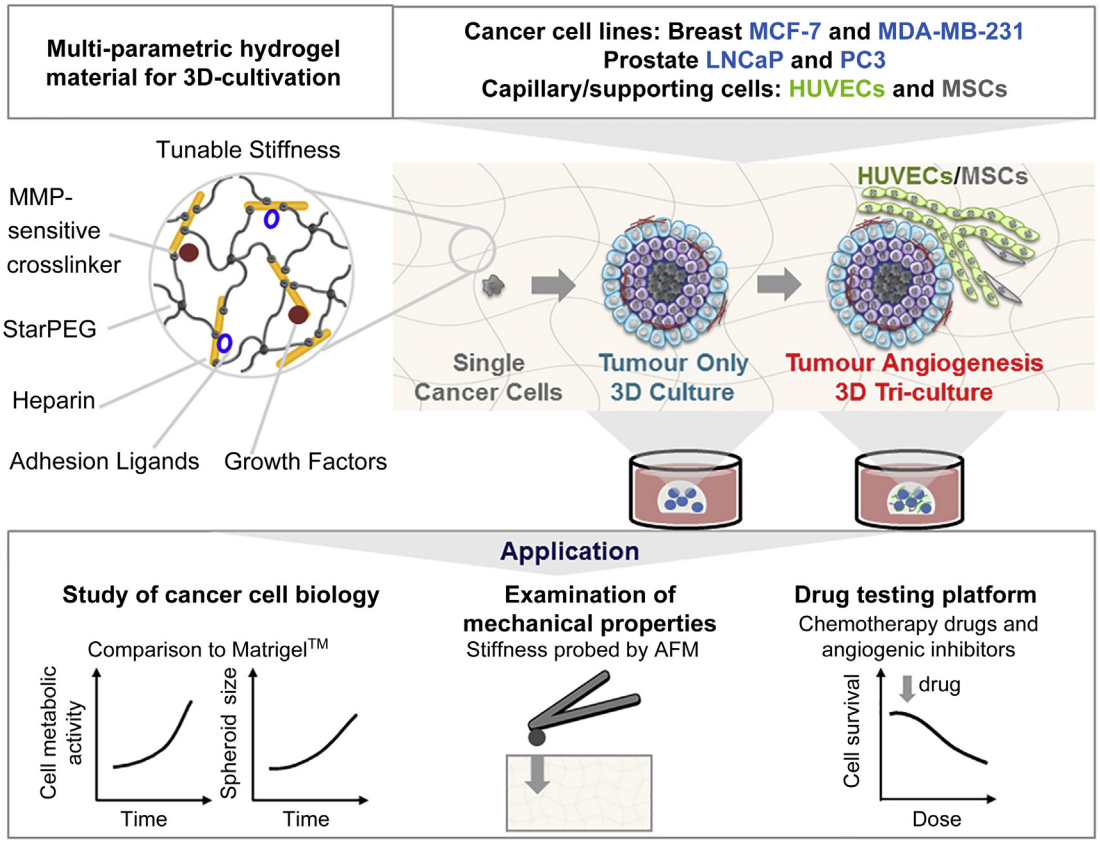

Fig. 1. Multi-parametric hydrogels for the development of tumour angiogenesis microenvironments.