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# Tissue-engineered human 3D model of bladder cancer for invasion study and drug discovery

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ABSTRACT

The tumour microenvironment is critical to both the initiation and maintenance of tumorigenesis. Reconstitution of the microenvironment is a major challenge for *in vitro* cancer models. Indeed, conventional 2D culture systems cannot replicate the complexity, diversity and dynamic nature of the tumour microenvironment. In this study, we have developed a 3D endotheliazed vesical equivalent by using tissue engineering from primary human cells in which non-invasive or invasive bladder cancer (BCa) cell lines, cultured as compact spheroids, were incorporated. Invasive BCa cells cross the basement membrane and invade the stromal compartment whereas non-invasive BCa cells are confined to the urothelium. Our 3D BCa model could be used as a reliable model for assessing drug responses, potentially reducing or partially replacing animal experiments, and thus should have applications in the identification of novel targets as well as toxicological evaluation of anti-cancer therapies.

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## 1. Introduction

Bladder cancer (BCa) has the highest recurrence rate (31–78% within five years) of all solid cancers. It is responsible for nearly 150,000 deaths worldwide each year and therefore represents a major public health problem [1–3]. BCa develops along two major tracks, non-muscle invasive cancer (NMIBC) or muscle-invasive cancer (MIBC) that require two different clinical management interventions. Although treated effectively by transurethral resection paired with intravesical immunotherapy or chemotherapy, patients with NMIBC frequently have a recurrence of their cancer and 45% are at risk to progress to MIBC, leading to a bad prognosis with a reported 33–73% risk of death within 5 years following surgery [1,4]. On the other hand, the management of MIBC is one of the most difficult issues in urologic oncology [5]. Despite radical cystectomy with neoadjuvant chemotherapy, up to 32–52% of patients will eventually develop a recurrence and they have a high risk of metastatic dissemination [6,7]. This overview of BCa highlights the need for robust preclinical models to improve anti-cancer drug development.

Research towards BCa drug discovery has been particularly hampered by models that do not accurately recapitulate many critical aspects of BCa biology, leading to 95% of new anti-cancer molecular entities failure in clinical trials [8–10]. Although simple, 2D cell culture systems do not recapitulate the tumour-stroma organization and the complex cellular interactions, normally found within the native tumour microenvironment while xenograft or transgenic animal models do not generate authentic human BCa pathobiology and have major limitations regarding microscopic observations at tumour site [11–14]. Moreover, genetic and molecular fidelity is often a concern both *in vitro* and *in vivo*, making difficult an appropriate translation of the results to patients [15].

Interaction between cancer cells and stromal components in the tumour microenvironment is increasingly recognized to play significant roles in tumour development, progression, metastasis and response to therapeutic regimens, including therapeutic resistance

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[16,17]. However, the generation of a representative tumour microenvironment is often overlooked when developing *in vitro* models. Tumour microenvironment cells, such as macrophages, endothelial cells or cancer-associated fibroblasts (CAF), are involved in a dynamic crosstalk with cancer cells, sustaining their proliferation and migration [18–20]. Non-cellular component of the tumour microenvironment—including extracellular matrix (ECM), secreted growth factors, exosomes and cytokines—also plays an important role in key processes involved in metastasis as well as response to therapy [21,22].

By encouraging reciprocal communication between cancer cells and microenvironment, 3D models support angiogenesis, enhance aggressiveness and metastatic potential, slower proliferation, increased resistance to anti-cancer drugs and allow physiological gene-expression profiles, all of which are characteristics of tumour development *in vivo* [23–26]. Compared to other types of cancer, few 3D models have been developed for the study of BCa. Most of 3D BCa models have been established using gel-embedding strategy. Smith and coworkers illustrated that cell-matrix interactions can influence gene expression in bladder cancer cell lines grown on Matrigel and have a potential role in malignant disease progression [27]. Several studies have demonstrated that the growth of bladder cancer cell lines in hydrogels suppresses some malignant functions seen in 2D [28,29]. Boxberger and colleagues have successfully recapitulated *in vivo* characteristics of bladder cancer cells and their phenotypes through 3D culture using alginate [30]. Despite the extensive uses of the natural hydrogel materials as 3D microenvironments for tumor growth and angiogenesis, they still have critical drawbacks to be utilized as well-defined tumor microenvironments due to relatively narrow range of physical properties, limited ability to control the matrix rigidity and cell adhesion peptide density independently and inherent batch-to-batch variability.

Tissue engineering offers a unique toolset to build cancer models that incorporate relevant features of the tumour microenvironment in a precisely controlled design experiment [31–33]. In this study, we have developed a novel human 3D BCa model by tissue engineering; a full-thickness vesical equivalent from primary human cells in which NMIBC and MIBC spheroids were implemented. The 3D BCa model was designed to reproduce a physiologically relevant microenvironment by providing to BCa spheroids a 3D stromal compartment surrounded by a fully differentiated urothelium laid on a stroma composed of organ-specific ECM, fibroblasts and endothelial cells. Using this new model, we are now able to investigate BCa cells breaching basement membrane and invading surrounding stroma in a physiologically relevant setting. We also demonstrate that our model allows drug response to be investigated. Overall, this model allows real-time tracking of BCa spheroids growth and invasion within vesical tissue and provides a useful platform for *in vitro* therapeutic screening.

## 2. Materials and methods

### 2.1. Ethics statement

Bladder biopsies from paediatric patients undergoing urologic surgery and umbilical cords from healthy full-term and naturally delivered newborns were obtained at CHU de Québec –Laval University in accordance with the local ethics committee. All patients provided their informed formal written consent, agreeing to supply biopsy for this study.

### 2.2. Cell isolation and culture

The fibroblasts and urothelial cells were isolated from a human bladder biopsy while umbilical vein endothelial cells (HUVEC) were obtained from an umbilical cord. Briefly, the stroma was separated from the urothelium by incubation in thermolysin O/N at 4 °C. The fibroblasts were enzymatically dissociated from the stroma using collagenase H and then, cultured in Dulbecco-Vogt modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS; Saint-Jean-Baptiste, QC, Canada) and antibiotics (100 U/ml penicillin and 25 μg/mL gentamicin; Sigma-Aldrich, Saint-Louis, MO). Urothelial cells were isolated from urothelium using trypsin for 30 min at 37 °C and were cultured in DMEM:Ham's F12 (DH; ratio 3:1; Invitrogen, Oakville, ON, Canada) supplemented with 10% FBS, 0.4 μg/mL hydrocortisone (Calbiochem, La Jolla, USA), 5 μg/mL bovine insulin (Sigma), 10 ng/mL human epidermal growth factor (EGF) (Austral, San Ramon, USA), $10^{-10}$ M cholera toxin (ICN, St-Laurent, Canada). HUVEC were isolated from the umbilical cord by enzymatic digestion with collagenase H and were cultured on gelatin-coated culture flasks in EGM-2 media (Lonza, Walkersville, MD) containing 5% FBS and antibiotics.

### 2.3. Production of 3D vesical equivalents

Self-assembly technique was used as previously described for the production of 3D vesical equivalent [34–36]. Briefly, the fibroblasts and HUVEC were seeded at a ratio of 3:1 for a final concentration of $3 \times 10^4$ cells/cm$^2$ in 6-well plates and culture medium was supplemented with 50 μg/mL ascorbate for 21 days until their neosynthesized ECM proteins were sufficiently assembled to form a sheet (Fig. 1). Three stromal sheets were superimposed and urothelial cells were seeded on top of the 3D tissue at a concentration of $3 \times 10^4$ cells/cm$^2$ in DH:EGM-2 media (ratio 1:1). The vesical equivalents were then elevated to the air/liquid interface for 21 days until mature urothelium differentiation.

### 2.4. Spheroid production

RT4 (NMIBC) and T24 (MIBC) cell lines were obtained from ATCC® (HTB-2™, HTB-4™) and cultured in DMEM media containing 10% FBS and antibiotics. Cells were stably transduced with lentivirus encoding the fluorescent DsRed proteins under the CMV promoter. The hanging drop technique was used to agglomerate cancer cells into spheroid shape. Briefly, 50 μL containing $2.5 \times 10^4$ cells were pipetted onto the lid of a 100 mm Petri dish. Droplets were incubated for 4 days to form compact spheroids. They were then harvested and dropped on 3D vesical equivalents for 21 days to form the 3D BCa model.

### 2.5. Spheroids collagen invasion assay

Rat tail type I collagen (Thermo Fisher Scientific, Waltham, MA) was diluted to 2 mg/mL in DMEM. RT4 or T24 spheroids were added to 100 μL of unpolymerized collagen solution in each well of a 96-well plate. Collagen self-polymerizes into a gel after 1 h at 37 °C. Spheroids were imaged every 24 h for the duration of the experiment. Phase contrast images were thresholded and then spheroids invasion potential was examined by measuring the area and circularity index for n = 12 spheroids from three independent experiments using ImageJ (NIH, Bethesda, MD, USA). The circularity describes the roundness of a spheroid and is defined as ($4\pi$ Area/Perimeter$^2$).

### 2.6. Histological analysis

3D BCa model and human native bladder tissue were embedded in paraffin, sectioned and stained with Masson's trichrome. Pictures were taken using a Zeiss Axio Imager M2 microscope equipped

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