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Engineered microenvironments provide new insights into ovarian and prostate cancer progression and drug responses

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Abstract

Tissue engineering technologies, which have originally been designed to reconstitute damaged tissue structure and function, can mimic not only tissue regeneration processes but also cancer development and progression. Bioengineered approaches allow cell biologists to develop sophisticated experimentally and physiologically relevant cancer models to recapitulate the complexity of the disease seen in patients. Tissue engineering tools enable three-dimensionality based on the design of biomaterials and scaffolds that re-create the geometry, chemistry, function and signalling milieu of the native tumour microenvironment. Three-dimensional (3D) microenvironments, including cell-derived matrices, biomaterial-based cell culture models and integrated co-cultures with engineered stromal components, are powerful tools to study dynamic processes like proteolytic functions associated with cancer progression, metastasis and resistance to therapeutics. In this review, we discuss how biomimetic strategies can reproduce a humanised niche for human cancer cells, such as peritoneal or bone-like microenvironments, addressing specific aspects of ovarian and prostate cancer progression and therapy response.

1. Introduction

Since the collective vision of its early pioneers Robert Langer and Joseph Vacanti, the main purpose of tissue engineering was to restore, maintain and improve the function of a wide range of human tissues by combining principles of biology and engineering [1-3]. The concept of tumour tissue engineering on the other hand is still in its infancy but holds great promise. Over the last decade, advances in molecular and cellular biology have shown that the mutual interactions between extracellular matrix (ECM) components and cancer cells are bidirectional and signals from the local microenvironment, or niche, are critical for tumour progression and metastasis [4]. Although a series of genetic and epigenetic alterations of single cells may be necessary for carcinogenesis, the paradigm that cancer is a cellular disease defined only by these alterations has been left in favour to one in which the disease harbours a dynamic multistep process initiated and maintained by interactions between malignant and non-malignant cells.

This niche concept is not new and has already been postulated in a similar, although metaphorical way, by Stephen Paget in 1889: '... Every single cancer cell must be regarded as an organism, alive and capable of development. When a plant goes to seed, its seeds are carried in all directions, but can only live and grow if they fall on congenial soil ...' [5, 6]. *A fortiori*, it is remarkable that the vast majority of experimental studies, particularly in the cancer community, still apply conventional two-dimensional (2D) approaches for drug screening. Such suboptimal systems might result in misleading observations and hypotheses [7-9]. Although it is well known that structural variables and signalling from the three-dimensional (3D) tumour microenvironment alter the phenotype, invasiveness and drug resistance of cancer cells, this fact is mostly ignored in traditional cell monolayer (2D) experiments [10-12].

Tissue engineering strategies can overcome these limitations and provide a potent tool box for cancer research by *in vitro* or *in vivo* modelling of the 3D tumour microenvironment [13, 14]. It is still a long road to re-create the molecular architecture of the human cancer cell niche one to one and the dynamic mechanisms of the signalling milieu between ECM components and cancer cells. Nevertheless, mimicking these complex physiological phenomena under reproducible conditions allows a more reliable preclinical evaluation of anti-cancer drug candidates (Figure 1).

In the following review, we will provide an overview of bioengineered *in vitro* and *in vivo* models that are suitable to recapitulate the interactions between the tumour and its microenvironment. The main focus will lie on ovarian and prostate cancer progression as our group is currently developing several engineered models for both tumour entities [15-24].

2. Clinical features of ovarian and prostate cancer

Ovarian cancer accounts only for 3% of all incident cancer cases among women per year, while prostate cancer is the most common cancer with 29% of all incident cancer cases in men, with an estimated cancer death rate of 5% and 10% respectively in the United States. Ovarian cancer has the highest mortality rate of all gynaecological malignancies of the reproductive system, with a 5-year relative survival rate of 30%. Prostate cancer is the most commonly diagnosed malignancy in males and the second leading cause of male cancer deaths [25]. Epidemiological studies focusing on the initiation of prostate cancer and its

potential for progression have revealed a high dependency on racial disparity and nationality, suggesting the importance of early lifestyle, environmental and exogenous carcinogenic parameters [26]. Initially, most patients with ovarian or prostate cancer respond to therapy (*e.g.* platinum- or taxane-based chemotherapy, hormone therapy), but eventually relapse occurs accompanied with resistance to therapy and re-initiation of tumour growth and formation of secondary lesions [27, 28]. There is still a strong need for more effective and targeted treatments for metastatic disease and to bypass mechanisms underlying drug resistance, which has been reviewed elsewhere [29, 30].
Within the next two sections the histological and molecular characteristics of the heterogeneity of ovarian and prostate cancer will be described leading to the design of various engineered experimental models to study their specific path of disease progression and therapy response.

2.1. Ovarian cancer

Ovarian cancer should not be regarded as a single entity, although its only unifying clinical feature is the loco-regional dissemination of cancer cells to the ovaries and related pelvic organs [31, 32]. This malignant disease comprises epithelial (>85%), sex cord-stromal (>5%) and germ cell ovarian tumours (>3%). Epithelial ovarian carcinoma are characterised by four morphological subtypes: serous, endometrioid, mucinous and clear cell ovarian adenocarcinoma. These are defined by their differentiation status, with high-grade serous ovarian carcinoma being the most frequent and lethal worldwide [33, 34]. The high mortality rate is primarily due to difficulties in sensitive screening of the early disease stages. This female malignancy is often described as a ‘silent killer’ due to its asymptomatic early disease stages, and hence, most patients are diagnosed at an advanced stage, with evidence of intraperitoneal tumour outgrowth beyond the ovary, presence of tumour fluid (ascites) and poor prognosis [35]. Initially, patients respond to a cytoreductive and debulking surgical approach and platinum- and taxane-based chemotherapy, but eventually chemoresistance occurs leading to a median progression-free survival rate of only 18 months [36, 37]. Developing improved therapeutic approaches in terms of a long-term cure has remained challenging as little is known about the underlying events promoting ovarian cancer progression, the tumour-stroma network and how chemoresistance occurs [37]. Early postoperative intraperitoneal therapy of patients with advanced disease has resulted in improved progression-free survival and overall survival compared to intravenous administration; however, toxicity associated with intraperitoneal treatment was considerably increased [38]. Drug-loaded tumour-penetrating micro-particles can provide an alternative intraperitoneal treatment regime for tumour-selective delivery and reduction of toxicity due to fast drug release as demonstrated in an ovarian cancer animal model [39]. Estrogen and progesterone receptor signalling is also partially involved in ovarian cancer progression, and both receptors are prognostic biomarkers for ovarian cancer. Whether the hormone receptor status can predict response to endocrine therapy, needs to be further studied [40].

2.2. Prostate cancer

During prostate cancer development, one of the indispensable factors is androgen and androgen receptor (AR)-mediated signalling. In fact, anti-androgen therapy (*e.g.*

Bicalutamide, Enzalutamide) has become a standard care for advanced prostate cancer [28]. The management of localised adenocarcinoma of the prostate involves primarily two other options, external-beam radiation and radical prostatectomy [41]. These therapeutic strategies are highly successful and have resulted in improved survival rates of patients with localised disease. The prognosis of these patients is excellent with an overall 5-year survival rate of >90% [42]. However, after a median time of 12-33 months, a relapse is observed despite anti-androgen therapy as noted above with castration-like levels of serum testosterone [43]. This hormone-refractory/androgen-insensitive status is referred to as castrate-resistant prostate cancer and is associated with disease progression subsequently leading to multiple metastases, most frequently involving the axial skeleton. To date, treatment options for patients with prostate cancer bone metastases are at best palliative resulting in a 5-year relative survival rate of <25% [44-46]. Metastatic bone disease causes some of the most distressing symptoms and complications in advanced prostate cancer, such as pathological fracture or spinal cord compression, which are also referred to as skeletal-related events, and are associated with poor prognosis [47]. Taxane-based chemotherapy in combination with corticosteroids became the standard of care in these patients but most of them develop a relapse within the first year of therapy [48]. This resistance to therapy might occur through genetic alterations and changes in the bone microenvironment leading to increased cancer cell survival and proliferation [49]. Therefore, several combinatorial microenvironment- and bone-targeted therapeutic strategies have been developed [50]. While agents like Denosumab or Bisphosphonates reduce the number of skeletal-related events, there are still no drug candidates available that significantly increase the overall survival of patients with metastatic disease. To better understand the molecular and cellular mechanisms of prostate cancer bone metastasis, novel engineered models are needed to mimic the disease seen in patients. A tissue-engineered *in vitro* bone model, using for example a 3D silk fibroin scaffold modified with a bone morphogenic protein (*e.g.* BMP2), can help to decipher the osteoblastic and osteolytic processes occurring during prostate cancer metastasis to bone [51].

3. Molecular characteristics of ovarian and prostate cancer

The molecular biomarkers cancer antigen 125 (CA125, also known as mucin 16/MUC16) and prostate-specific antigen (PSA, also known as kallikrein-related peptidase 3/KLK3) are used to detect ovarian and prostate cancer respectively and to monitor response to therapy clinically. However, CA125 lacks specificity for screening of early stage, persistent and recurrent disease and results in one third of all cases in false negatives. The tumour-associated expression of KLKs, in particular KLK5, and the human epididymis protein 4 (HE4, WFDC2) are promising complementary biomarkers for ovarian cancer-expressed CA125 [52-56]. The presence of KLK5, KLK6 and KLK7 in ovarian cancer tissues showed a closer association with a large remaining tumour mass following surgery, high-grade and late stage disease than CA125 [57]. KLK6 expression, as per immunohistochemical and multivariate analyses, was correlated with the presence of stromal cells in cancerous tissue and shorter overall and progression-free survival of ovarian cancer patients [58]. KLK7 levels, as determined by ELISA and multivariate analyses, were correlated with prolonged overall and progression-free survival of ovarian cancer patients [59]. Screening or rigorous clinical testing for circulating PSA has helped to achieve an overall 5-year survival rate for

prostate cancer of 99%; however, benign pathologies can also cause a non-specific release of PSA into the blood [60, 61]. PSA testing has also been shown to have a greater influence on incidence than on mortality [62]. This has generated considerable discussion about the benefits of early disease detection and aggressive therapeutic approaches in early localised prostate cancer [63, 64]. Other non-invasive diagnostic tools, such as disease-related circulating microRNAs (miRNA), are being currently clinically evaluated for the detection of ovarian and prostate cancer and predictors of therapy outcome [65, 66].

3.1. Molecular fingerprint of ovarian cancer

Ovarian cancer develops *de novo* due to multiple genetic alterations, without identifiable precursor lesions, from different tissues of origin, such as the ovarian surface epithelium, abdominal peritoneum or distal fallopian tube [36, 67, 68]. Evidence for a potential fallopian tube origin has been provided [33], for example by complete sectioning of the fallopian tubes of patients with serous carcinoma [69, 70] and a double knockout animal model with a reproductive tract-specific deletion in the miRNA processing enzyme Dicer and the tumour suppressor *PTEN* [71]. As such, secretory epithelial cells from the fimbrial end can be used experimentally to study fallopian tube transformation during ovarian cancer development [32, 72]. Another *de novo* animal model was genetically engineered to recapitulate the early disease stages by targeting commonly altered genes in secretory epithelial cells [73].

Epithelial ovarian cancer is not a single disease but is composed of a diverse group of tumours that can be classified based on their distinctive clinico-pathological heterogeneity. A two-pathway model of ovarian cancer has been postulated [74, 75]. Type I tumours are serous, endometrioid, mucinous, clear cell and borderline tumours displaying low-grade nuclear and architectural features, including slow growth and no *TP53* mutations. Type II tumours comprise highly aggressive, rapidly growing carcinomas discovered at a late clinical stage, including high-grade serous carcinoma, undifferentiated carcinoma and malignant mixed mesodermal tumours, with *TP53* mutations [69, 75-77]. The most common and malignant type of ovarian cancer, high-grade serous carcinoma (type II tumour), is characterised by frequent *TP53* (>80%) and *CCNE1* (30%) amplifications, while low-grade serous carcinoma (type I tumour) show *KRAS* (30%), *BRAF* (30%) or *ERBB2* mutations. Most type I tumours harbour *CTNNB1* (endometroid), *PTEN* (endometroid), *PIK3CA* (endometroid, clear cell) or *KRAS* (mucinous) mutations and are genetically more stable than type II tumours. Germline mutations of the tumour suppressor genes *BRCA1* or *BRCA2* are causative for the majority (>90%) of hereditary ovarian cancers [78, 79]. Additionally, a protein signature, including AR and members of the EGFR and MEK/ERK signalling cascade, for high-grade serous ovarian cancer has been developed and is correlated with the risk of disease recurrence, progression-free survival and overall survival time, and can, upon integration of genetic mutations, predict patient's outcomes and opportunities for early therapeutic intervention [80]. These scientific advances on the molecular characterisation of ovarian cancer provide the translational foundation for such signatures to be integrated into clinical practice to improve patient care and to design personalised therapies for individual patients with greater selectivity and lower toxicity [81]. For example, patients with *BRCA* mutations respond better to platinum-based chemotherapy and may be highly susceptible to poly(adenosine diphosphate-ribose) polymerase (PARP) inhibitors that target the *BRCA* gene

[82-85]. A molecular signature of therapy resistance of patients diagnosed with advanced serous ovarian cancer has been postulated by comparing chemo-naive with chemo-resistant ascites-derived cells. Chemo-resistant cells had a more epithelial and cancer stem cell-like phenotype (*e.g.* high levels of the epithelial cell adhesion molecule (EpCaM), CA125, Oct4, STAT3) and were non-adherent in contrast to chemo-naive cells [86]. Platinum-based treatment induced a transition from an epithelial to mesenchymal expression profile in residual cancer cells accompanied with MAPK signalling, suggesting that phenotypical plasticity drives a cellular protection mechanism to escape the cytotoxic effects of chemotherapy [87].

3.2. Molecular fingerprint of prostate cancer

Although it is widely accepted that prostatic intraepithelial neoplasia may represent a precursor lesion of prostate cancer [88], the processes that promote prostate carcinogenesis are still poorly understood. However, the development of prostate cancer is causally linked to gene expression changes associated with cellular senescence and inflammatory responses [89]. The vast majority (>95%) of malignant prostatic lesions are pathologically classified as adenocarcinomas [90], while other types, such as mucinous, neuroendocrine or signet ring carcinomas are extremely rare [91]. In contrast to ovarian cancer, there is a lack of pathologically defined subtypes that differ in their prognosis or response to therapeutic regimes. Nevertheless, recent studies have shown that molecular changes are associated with different stages of the disease which can help to determine the prognosis of patients with these markers in the future [92]. Down-regulation of the *NKX3.1* homebox gene is a critical event in prostate cancer development and increases in frequency with cancer grade [93]. Despite the loss of the *NKX3.1* locus, no mutations in the remaining allele have been detected [94]. Therefore, *NKX3.1* seems to be a haplo-insufficient tumour suppressor gene that functions as 'gatekeeper gene' for prostate cancer development [95]. The *MYC* oncogene also plays an important role in prostate cancer initiation as it is over-expressed in prostatic intraepithelial neoplasias and carcinomas [96]. Several studies have identified chromosomal rearrangements that lead to an activation of transcription factors of the ETS family [97, 98]. One of these rearrangements results in the development of the *TMPRSS2-ERG* fusion gene in approximately 15% of intraepithelial neoplasias and 50% of prostate cancer, suggesting an early effect of the fusion gene on cancer development [99, 100]. A step towards cancer progression is the loss of *PTEN* which is correlated with the development of castration resistance [101, 102]. Simultaneous activation of the AKT/mTOR and MAPK signalling pathways also contributes to cancer progression, while inhibition of these pathways results in an inhibition of castration resistance in a transgenic animal model [103]. Furthermore, PI3K/Akt/mTOR-mediated signalling plays a role in resistance to chemotherapy and radiation involving EpCAM [104]. Inside-out activation of β1 integrins by talin1 promotes prostate cancer bone metastasis using an *in vivo* metastasis assay, which is abrogated by silencing of the involved talin1 phosphorylation site [105]. A molecular signature based on tissue-specific 3D architecture gene expression has been developed to predict diagnosis and therapy outcome. Among others, *PDCD4* and *KLF6* were less prominent in poorly differentiated tissues and can be used as prognostic markers in prostate cancer [106]. Most

importantly, the molecular bidirectional interplay between tumour cells and osteoblasts enhances skeletal metastasis in castration-resistant prostate cancer [107].

4. Recapitulation of the tumour's 3D extracellular microenvironment

Advances in tissue engineering have produced a versatile tool box to design tissue-like microenvironments representing specific aspects of the human disease. Bioengineered platforms allow a 3D matrix or tissue reconstruction within scaffold-based and organotypic cultures and animal models [8]. Awareness of this third dimension has risen dramatically over the last decade which has led to a plethora of physiologically relevant 3D cell culture technologies to study the biological, mechanical and chemical interplay between cells and their natural ECM [9]. The continuous improvement of biomaterial and scaffold design, modification and fabrication and the cellular analytical methodologies of 3D cell culture matrices have fostered tissue engineering for clinical applications [108]. Interdisciplinary approaches aligning tissue engineering with cell biology and medical research contributed to tumour-engineered microenvironments to decipher cancer development, progression and responses to current treatment regimes and to screen novel anti-metastatic therapeutics [109-111].

4.1. Cell-derived matrices

A tissue-like 3D architecture and composition can be produced by cell-secreted matrices. Cell-derived matrices are grown to a thin, microscopy-compatible thickness, and hence permit high-resolution imaging in a 3D context [112]. These experimental ECM models, grown by different cell types (*e.g.* mesenchymal cells, fibroblasts), harbour a complexity and diversity in their molecular and structural organization to represent the wide range of *in vivo* matrices [113, 114]. As such, a bone marrow-derived ECM was used as an engineered bone marrow tissue model of metastasis of prostate cancer cells, thereby regulating factors, such as androgen-independent growth and survival, cell phenotype, adhesion and chemoresistance to docetaxel, that promote cancer progression [115].

Our group established a mineralised human primary osteoblast-derived bone matrix to investigate molecular processes implicated in prostatic bone metastasis of LNCaP and PC-3 cells. This cell-derived matrix has shown high reliability and displays biomimetic properties similar to those of native bone. Within this bone-like microenvironment, prostate cancer cells displayed morphological plasticity and altered hormonal and proteolytic responses that were not seen in traditional cell monolayer cultures. Hence, this decellularised matrix demonstrates a robust model system to mimic key features of prostate cancer bone metastases and tumour-bone interactions [116]. This engineered bone-like matrix can also be used to delineate the molecular mechanisms of colonization of other cancer cell types that metastasise to the skeleton, such as breast cancer cells [117].

4.2. Semi-synthetic and synthetic matrices

A library of biomaterial-based cell culture models is available to address questions of cell-cell/cell-matrix/cell-surface receptor interactions, signalling pathways, mechanisms of cell motility and migration, mechano-biology and tensional homeostasis in a physiologically

relevant, tissue-like context. Cells grown in a 3D context respond differently to growth, polarity, apoptotic and proteolytic stimuli [118, 119].
The gold standards for 3D cell culture systems are Matrigel, a reconstructed matrix produced by Engelbreth-Holm-Swarm mouse sarcoma cells, and other naturally-derived, protein-based hydrogels, like collagen-based hydrogels [13, 120, 121]. For example, Matrigel has been used for ovarian cancer cell-encapsulating droplet patterning to control cell density, growth kinetics, cell-to-cell distance and viability [122], for metabolic profiling of ovarian cancer cell responses to chemotherapeutics [123] as well as for prostate cancer biomarker expression profiling [124], for prostate cancer-stroma 3D co-cultures [125] and to mimic the prostate glandular structure [126]. This basement membrane extract has also been used to carry ovarian cancer cells within a bioreactor system that allows constant diffusion of oxygen to monitor their morphology and growth under hypoxic conditions [127]. Collagen-based hydrogels representing a spatial-mechanical 3D culture model [128] and provide a collagen-rich sub-mesothelial matrix for ovarian cancer cells [129, 130] and an invasive matrix for prostate cancer cells [131].
To overcome their drawbacks (*e.g.* high batch-to-batch heterogeneity, undefined matrix composition, restricted modification), engineered semi-synthetic and synthetic biomaterials have been applied as 3D culture platforms, creating a link between cell monolayer cultures and animal experiments [132-134]. These bioengineered matrices offer a combined advantage of having native ECM components and tunable material properties resulting in less complexity, high reproducibility and comparability between different studies [135]. Such tailored matrices can be used to mimic, for example, the 3D architecture of circulating tumour cells in a microfluidic microenvironment, where prostate cancer cells form clonogenic 3D spheroids within a synthetic biomimetic hydrogel [136].
A variety of different hydrogel-based microenvironments to study ovarian and prostate cancer (Table 1) will be discussed in the following sections.

4.2.1. Hyaluronan-based hydrogels

Hyaluronan (also known as hyaluronic acid or hyaluronate) is an integral ECM component connective and epithelial tissues and the local tumour microenvironment contributing to cell proliferation and migration as well as inflammatory responses during wound repair. Hyaluronan-based hydrogels are also suitable as 3D cancer models as they recreate the tumour microenvironment (*e.g.* bone marrow) and allow malignant invasion and the evaluation of chemotherapeutics [137-140]. This biopolymer has been used as synthetic ECM to grow subcutaneous and orthotopic tumours using injectable hyaluronan-based hydrogels (*e.g.* into the ovarian capsule) *in vivo* leading to increased vascularisation, thus representing a cancer cell delivery vehicle for animal studies [141, 142]. Hyaluronan-based hydrogel invasion assays are well suited for the analysis of hyaluronan-interacting factors (*e.g.* CD44, CD168, hyaluronidase) and invadipodia formed by embedded prostate cancer cells [143]. Hyaluronan presents an increased selectivity when used in bio-conjugate drug delivery systems as its receptors (*e.g.* CD44, RHAMM) are over-expressed in ovarian cancers and mediate intracellular drug release. As such, the conjugation of paclitaxel with hyaluronan has shown greater efficacy and less host toxicity upon intraperitoneal treatment of ovarian cancer xenografts compared to paclitaxel alone [144].

4.2.2. Alginate-based hydrogels

Alginate represents a biocompatible and physico-chemically stable biopolymer that allows in contrast to naturally-derived ECM systems (*e.g.* Matrigel, collagen) the study of cancer cell signalling, for example, upon integrin engagement due to incorporation of integrin cell binding motifs [145]. Alginate-based hydrogels have been used as 3D organoid cultures to provide a scaffold on which cells from ovarian and oviductal tissues can grow and to study the origin of ovarian cancer and carcinogenic transformation [146].

4.2.3. Gelatine methacrylamide (GelMA)-based hydrogels

The semi-synthetic origin of GelMA-based hydrogels combines the features of naturally-derived matrices, integrin cell binding motifs and protease cleavage sites, with high stability and reproducibly tailorable characteristics, thereby allowing binding to cell adhesion receptors and degradation by cell-secreted proteases [147]. GelMA-based hydrogels are composed of gelatine, mostly denatured collagen type-I, and offer a 3D cell culture system for cancer cells as demonstrated by our group [18] and others [148]. Our group designed a quality control and validation protocol to measure the physical and diffusion properties of GelMA-based hydrogels performing undefined compression tests and fluorescence recovery after photobleaching (FRAP) and demonstrated their application as spheroid carriers within an ovarian cancer animal model, thereby promoting local tumour growth and metastatic spread as observed in patients with advanced disease [18].

4.2.4. Polyethylene glycol (PEG)-based hydrogels

Our group designed a synthetic PEG-based hydrogel model that possesses integrin cell binding and protease cleavage motifs to decipher cell-ECM interactions of cancer cells. OV-MZ-6 and SKOV-3 ovarian cancer cells formed multicellular spheroids in this 3D culture model that were representative of metastatic outgrowth within the peritoneal cavity as seen in patients with advanced disease [15]. Our group also used this bioengineered ovarian cancer spheroid model to assess protease- and integrin-associated functions. Within this 3D system, KLK4, KLK5, KLK6 and KLK7 (KLK4-7)-cotransfected ovarian cancer cells formed larger spheroids and proliferated more compared to vector controls, particularly upon integrin engagement, which was reduced upon integrin inhibition [16]. Moreover, the same biomimetic hydrogel model was employed to mimic the prostatic gland microenvironment by encapsulation of LNCaP prostate cancer cells. Prostate cancer cells showed morphological plasticity and altered their androgenic response compared to traditional cell monolayer cultures, implying the presence of tumour-ECM interactions [20].

5. Engineering the tumour’s cellular niche

Complex interactions between the tumour and their surrounding host cells, or niche, are crucial for carcinogenesis, whereby tumour cells (the 'seed') home to specific organs (the 'soil'); hence, metastases only occur when the 'seed'/tumour cells and 'soil'/stroma are compatible [149-152]. The understanding of the role of the microenvironment in tumour biology has recently started to shift since proof has emerged that there is a functional link between tumour cells and their surrounding cells and tissues, regulating malignant processes

[153, 154]. This dynamic reciprocal tissue network includes intercellular (*e.g.* cell-cell contacts, ECM, chemokines, cytokines, growth factors, proteases) and intracellular (*e.g.* cell surface receptors, kinases, genomic instability) elements [155, 156]. Findings from cell culture systems are not as powerful in validating a drug target as animal models are, but they do indicate the potential effects of therapeutics. However, experimental confirmation can be problematic if the function of a protein in a pathway requires other proteins or cofactors that are not present in cell culture approaches using one cell type, such as co-factors or substrates produced by different cell types [157]. Hence, a variety of different engineered co-culture approaches for ovarian (Table 2) and prostate (Table 3) cancer with stromal-derived cells have been used as both tumour entities are a mixture of epithelial, stromal, immune and endothelial cells [158, 159].
Host and stromal factors, such as ECM components, stromal-derived and inflammatory cells and macrophages, in the tumour microenvironment are equally important to early and late events in carcinogenesis [74, 160]. Mesothelial cells secrete growth factors (*e.g.* VEGF, FGF2, TGF-β), chemokines (*e.g.* CXCL4, CXCL6, CXCL8) and cytokines (*e.g.* IL-6) that support the homing of cancer cells to the peritoneal lining. Proteases (*e.g.* matrix metalloproteinase (MMP) 2, MMP9, MMP13, MT1-MMP), present in the tumour fluid (ascites), enhance the release of soluble factors from both cancer and mesothelial cells, further promoting interactions between both cell types that facilitate the implantation of cancer cells onto the peritoneal stroma [161-163].
Depending on extracellular stimuli and the interaction with ECM ligands, integrins enhance cell survival through several mechanisms, including the activation of signalling pathways upon crosstalk with other cell surface receptors (*e.g.* VEGFR-2), to promote angiogenesis [164, 165]. Tumour angiogenesis leads to the formation of blood vessels in a tumour, which in turn support cancer cell survival, local tumour growth and the development of distant metastasis. Various angiogenesis inhibitors have been approved for anti-cancer treatment as single agents or in combination with chemotherapy [166-168].
Tumours are organs, and they constantly evolve as they encounter different microenvironments [153]. Combinatorial approaches of therapeutics targeting specific factors will be required not only to inhibit tumour cell function but also to restore the stromal niche. The cytokine network (*e.g.* CXCR4, CXCL12, TNF-α, IL-6), particularly in the advanced disease, is activated and mediates peritoneal tumour spread [169]. As such, IL-6 has tumour-promoting functions on both tumour and stromal cells. There is preclinical evidence that this cytokine enhances ovarian cancer cell survival and mediates treatment resistance due to its angiogenic properties. In patients with advanced ovarian cancer, high IL-6 levels correlate with poor prognosis [170]. Given its inflammatory role as part of the tumour-stroma niche [171], IL-6 antagonising agents are suitable for combinatorial therapy regimes [172].

5.1. 3D ovarian cancer co-cultures

Abnormal extracellular factors can enforce cancerous growth; in fact, inflammatory signals associated with wound healing promote the development of epithelial tumours [155]. Tumours activate parts of the normal wound healing cascades, such as the formation of a fibrin clot and the release of growth factors and proteases [173-175]. Hence, our group employs a bioengineered microenvironment that is based on a cross-linking reaction that

occurs during the fibrin clot formation in wound healing in order to provide the tumour's cellular niche [15]. An integrated 3D co-culture model of ovarian cancer spheroids with mesothelial cells was developed to reflect the tumour-stroma niche of the advanced disease. In patients, ovarian cancer cells form multicellular spheroids that accumulate in the tumour fluid (ascites), grow anchorage-independently and adhere to the stromal layer. To replicate this interaction, spheroids were grown within PEG-based hydrogels that comprise ECM features due to incorporation of protease cleavage sites and integrin cell binding motifs. These were layered onto electrospun-fabricated meshes that allow adhesion of mesothelial cells, representing the abdominal lining [176]. Initial high-throughput gene expression analyses and signalling profiling show that cancer spheroid growth is enhanced, and that genes regulating cell cycle and growth are increased in 3D co-cultures, thus highlighting the importance of the tumour-stroma network in disease progression (our unpublished data).

Another co-culture approach includes the seeding of ovarian cancer spheroids onto mesothelial cell monolayers to demonstrate the clearance of mesothelial cells from beneath the cancer cells, which is indicative of what is seen in patients with ovarian tumours. This spheroid-mesothelium crosstalk occurs through integrin- and talin-dependent activation of myosin and contractile forces that are generated by spreading cancer cells [177].

As ovarian tumours predominantly metastasise to intra-abdominal organs like the omentum, ovarian cancer cells have been co-cultured with adipocytes. Indeed, adipocytes mediate tumour growth and facilitate intra-abdominal spread due to the enrichment of fatty acids, indicating their contribution to the tumour-stroma network [178]. A complex organotypic 3D co-culture model harbouring multiple cell types and a cell-secreted ECM has been developed to mimic the early steps of ovarian cancer metastasis within the peritoneal microenvironment [179]. Through the analyses of ovarian cancer cells cultured on top of a mesothelial layer, which is replicated by primary mesothelial cells seeded on top of primary peritoneal fibroblasts that have been mixed with collagen type-I, factors mediating cancer cell adhesion and invasion (*e.g.* fibronectin, vitronectin, different collagens and laminins) have been identified [180]. A different 3D co-culture approach utilised mesenchymal stem cell-rich amniotic membrane scaffolds that were co-cultured with ovarian cancer cells, mimicking early cancer cell migration and infiltration into the sub-mesothelial layer accompanied by increased IL-6 levels [181].

5.2. 3D prostate cancer co-cultures

The rationale for 3D co-culture models for prostate cancer progression and metastasis to the bone using different non-engineered approaches like rotary wall vessel modules has been reviewed elsewhere [182]. As disseminated prostate cancer cells have a high affinity to skeletal bone, co-cultures of cancer cells with osteoblasts, endothelial and immune cells allow signal transduction (*e.g.* TGF-β/BMP, Wnt signalling) and interaction with tumour-secreted proteins (*e.g.* endothelin-1, ephrins, IL-6, RANKL) and growth factors (*e.g.* FGF-2, VEGF, PDGF) occurring during bone remodelling [183]. RANKL can promote prostate cancer metastatic colonisation through autocrine and paracrine RANK-mediated signalling. Interestingly, the RANKL-RANK network is associated with an invasive phenotype present upon disease progression or therapeutic intervention [184].

A 3D tumour growth assay using co-cultures of PC-3M prostate cancer cells and human mesenchymal stem cells was developed, using a commercially-available basal membrane extract, to mimic the prostatic niche and to quantitatively screen responses to anti-cancer compounds. This high-throughput approach is based on automated microscopy and image analyses, applicable to other prostate cancer cells (*e.g.* LNCaP, DU-145) and suited for large-scale drug discovery [185]. Another 3D cancer-bone metastasis approach utilised free-floating murine calvarial bone organs that were co-cultured with prostate cancer cells in a roller tube system. Bone formation or resorption was correlated with the metastatic behaviour of the prostate cancer cells, demonstrating that the metastatic PC-3 cells induced osteoblastic activity and new bone formation, whereas the less aggressive LNCaP cells showed no bone resorption and only limited bone formation, accompanied by changes in the gene expression pattern (*e.g.* cysteine proteases, IL-6) [186].

Our group designed a tissue-engineered 3D co-culture model of human primary osteoblasts with prostate cancer cells employing a cell sheet-based technique. Osteoblast-derived sheets are wrapped around a polycaprolcatone-tricalcium phosphate scaffold to fabricate a tissue-engineered bone construct which is seeded with LNCaP or PC-3 prostate cancer cells to mimic osteoblastic and osteolytic responses occurring during bone metastasis. Proteolytic and hormonal interactions between both cell types underscored the physiological relevance of this engineered bone-like microenvironment [19]. This bone-like microenvironment promoted osteomimicry and altered levels of androgen-responsive genes in LNCaP cells under androgen-deprived conditions [22].

Another 3D co-culture approach utilises BPH-1 non-tumourigenic prostate epithelial cells seeded on top of human primary prostate stromal cells (*e.g.* normal prostate and cancer-associated fibroblasts) which have been pre-cultured onto coverslips for optimal ECM production. This cellularised co-culture leads to phenotypical plasticity and directed migration of the non-tumourigenic prostate epithelial cells induced by cancer-associated fibroblasts, suggesting a more invasive phenotype. This engineered ECM-like microenvironment allows the quantitative assessment of the tumourigenic potential of cancer-associated fibroblasts [12].

A 3D double layered microsphere model was designed to elucidate paracrine interactions occurring within the tumour-stroma network. Alginate hydrogel microspheres were generated by co-culturing prostate cancer cells (*e.g.* C4-2) with normal prostate stromal cells (*e.g.* WPMY-1) that were viable for over a month independent of cell type layer combination as measured by levels of a soluble E-cadherin fragment, indicative of the secretory function [187]. A 3D co-culture approach, which recapitulated the metastatic tumour microenvironment by employing 3D collagen-encapsulated lung fibroblasts (*e.g.* MRC-5) or pre-osteoblast-like cells (*e.g.* MC3T3-E1) layered with prostate cancer cells (*e.g.* PC-3), demonstrated that α3β1 integrin is crucial for tumour-stroma interactions to suppress cancer cell growth [188]. A 3D model of tumour-driven angiogenesis was developed to study the effects of angiogenic factors produced by prostate cancer spheroids (*e.g.* LNCaP), grown within a basement membrane extract, on arterial explants from human umbilical cords. These co-cultures increased angiogenic outgrowth and were less responsive to the anti-angiogenic drug Sunitinib [189]. To model the physical interaction of prostate cancer cells (*e.g.* LNCaP) with immune cells *in vitro*, 3D porous chitosan-alginate scaffolds were seeded with cancer

cells and co-cultured with human peripheral blood lymphocytes. The lymphocyte population (*e.g.* T cells, B cells, natural killer cells) homed to the cancer spheroids, thus representing a physiological and high throughput tool to assess the efficacy of immunotherapeutics [190].

5.3. Genetic implications of engineered co-cultures

Genetic polymorphisms, of which single nucleotide polymorphisms (SNPs) are the main type of these sequence variations, have been implicated in ovarian and prostate cancer, for example in the human *KLK* genes [191, 192]. The *KLK* locus harbours the largest cluster (15) of homologous protease genes [193]. SNPs in the *KLK* genes (*e.g. KLK3*, *KLK15*) have been linked to a poor ovarian cancer survival [194, 195], while other *KLK*-related SNPs (*e.g. KLK4*, *KLK14*, *KLK15*) have been associated with risk and aggressiveness prostate cancer [196-198]. Some of the identified SNPs in the *KLK* locus (*e.g. KLK2*, *KLK3*, *KLK15*) are predicted to alter miRNA binding sites, short non-protein-coding RNA molecules that deregulate gene expression by sequence-specific binding to mRNA, and thus, potentially interfere with miRNA function [191]. Such miRNAs represent an important control mechanism of KLK expression at the post-transcriptional level as a single miRNA can target multiple KLKs [199]. Genome-wide miRNA profiling has linked their deregulation to ovarian cancer pathogenesis, and their signatures can potentially be used as diagnostic and prognostic tools for this disease [200]. Interestingly, miRNAs can modulate the sensitivity to PARP inhibitors and can potentially serve as biomarkers to identify a subset of ovarian cancer patients that benefit from a therapy with PARP inhibitors [201].

In our integrated 3D ovarian cancer co-culture model (our unpublished data), some of the miRNA that are reported to be altered in cancer, including ovarian [202-204] and prostate cancer [205-207], and affect tumour development, progression and angiogenesis [208]. The gain or loss of a cell type-specific miRNA network can act as an oncogene and/or tumour suppressor [192]. Interestingly, alterations in miRNAs expressed by the stroma (mesothelial) cells have been shown to play a role in the tumour microenvironment underlining their tumour growth-promoting and/or growth-suppressing functions [209]. The ovarian tumour microenvironment can re-program normal fibroblasts into cancer-associated fibroblasts due to changes in the miRNA expression in the quiescent fibroblasts, thereby promoting the up-regulation of chemokines (*e.g.* CCL5) that drive invasion and metastasis [210]. Strikingly, a novel targeted approach combining an EphA2, a receptor tyrosine kinase that is linked to poor outcome in ovarian and prostate cancer, tumour-suppressing miRNA and RNA interference (*e.g.* small interfering RNA) represents an innovative technique for a dual gene silencing therapy for cancer with high efficacy [211].

6. Cancer-associated protease function in 3D microenvironments

Susceptibility to enzymatic degradation allows for cell-mediated remodelling and migration within cell-laden biomaterials that are crucial for normal developmental and homeostatic processes as well as pathological processes occurring during wound repair and disease progression. Proteolytic systems, such as MMPs, cysteine and serine proteases, have been linked to ovarian [212] and prostate [213] cancer progression as they are frequently up-regulated in malignant tissues and modulate ECM components, process secreted molecules and cell surface receptors, alter signalling functions and cellular behaviour, thereby

facilitating carcinogenesis [157, 214, 215]. However, several MMPs and KLKs harbour tumour-protective or dual cancer-modulating properties in different stages of disease progression by numerous distinct pathways [214, 216-219]. To decipher KLK-mediated proteolysis of putative substrates in ovarian and prostate cancer, our group is using mass spectrometry-based proteomic approaches in a biologically-mimetic context, and is currently validating the functional consequences of the KLK degradome in various engineered 3D models [220]. To detect cell-dependent proteolytic activity locally within 3D matrices, various fluorescent substrates, which cause a dramatic increase in fluorescent intensity upon proteolytic degradation, have been used [221].

Although cell-mediated proteolytic activity is detectable in different 3D microenvironments, there is also evidence of a non-proteolytic cell migration through 3D matrices [112]. Migration through 3D matrices (*e.g.* collagen matrices) occurs in a multi-step mode with alternating stationary and migratory phases [222]. Cancer cells maintain their migratory ability after broad-spectrum inhibition of cell-dependent proteolysis (*e.g.* MMPs, cysteine and serine proteases) exhibiting a non-proteolytic amoeboid migration type. The transition of a mesenchymal to an amoeboid movement is accompanied by morphodynamic (*e.g.* constriction ring, diffuse actin distribution) and molecular changes (*e.g.* integrin and MT1-MMP clustering) [223]. When pericellular proteolysis is blocked by a cocktail of inhibitors, some cancer cells exhibit a rounded phenotype and require Rho-ROCK signalling, ultimately increasing the invasive behaviour of these cells [224]. However, the extent of proteolysis depends on the specific cell type, combination of protease inhibitors, type and composition of the 3D matrix and architecture [112, 222]. The migratory behaviour depends on the matrix stiffness; while low matrix stiffness fosters a non-proteolytic 3D migration mode, a higher stiffness allows proteolytic migration in artificial ECMs [225].

A variety of different approaches to measure cancer-associated proteolytic activity in 3D and live organisms (Table 4) will be discussed in the following sections.

6.1. Proteolytic factors in the tumour's cellular niche

The tumour-promoting functions of proteases are embedded within a multidirectional interactive network to modulate angiogenesis, cancer cell invasion, ECM composition and signalling cascades in the tumour microenvironment. Hence, it needs to be taken into account that proteases are not only expressed by cancer cells but also stromal-derived cells which increases the diversity of the proteolytic machinery because proteases usually not expressed by cancer cells are now present [226]. Most importantly, tumour-stroma interactions can modulate the expression levels of proteases [227]. Proteases are not only produced by invasive cancer cells but also by non-malignant cells of the tumour microenvironment (*e.g.* MMPs, cysteine cathepsins, serine proteases) facilitating cancer cell invasion, intravasation and dissemination to distant organs. Individual proteases cleave cell-adhesion molecules (*e.g.* E-cadherin) leading to the disruption of cell-cell contacts, degrade ECM and basement matrix proteins allowing invasive cancer cells to migrate into the surrounding tissues and process pro-domains subsequently activating growth factors, cytokines and signalling cascades [175]. For example, the expression of MT1-MMP within the tumour microenvironment has been linked to its proteolytic activity and has led to the design of a tumour-targeted prodrug as a therapeutic strategy for solid tumours like prostate cancer [228]. Altered tumour

microenvironment dynamics influence the behaviour of both cancer and stromal, endothelial and immune cells, thereby actively contributing to cancer cell proliferation and survival [229].

The degradation of quenched-fluorescent (DQ) collagen type-I/IV by various proteases can be imaged and quantified by confocal microscopy in 3D cultures of prostate cancer cells [230] and 3D organotypic cultures using multiple cell types [231]. Recently, quantitative image analyses has entered the fourth dimension, namely time, and cancer cell-associated proteolysis can be measured in 3D co-cultures of cancer spheroids with stromal-derived fibroblasts for up to 23 days via degradation of quenched-fluorescent (DQ) collagen type-I/IV [232].

6.2. MMPs

Biodegradability of synthetic hydrogels is engineered by incorporation of peptides containing specific sequences sensitive to cleavage by MMPs [233]. Fluorogenic substrates, such as fluorophores-labelled native ECM proteins, have been used to visualise protease activity, whereby cleavage of the fluorescent protein by cell-secreted MMPs separates the fluorophores and releases a fluorescence signal [221, 231]. To overcome the high background signals of these fluorescently-labelled proteins, fluorogenic peptide substrates have been incorporated into synthetic hydrogels [234, 235], whereby close proximity of two fluorophores on the non-cleaved peptide results in quenching, and cleavage by MMPs separates the fluorophores generating a fluorescence signal that corresponds to MMP activity and collagen type-I matrix degradation by migrating prostate cancer cells [227]. Hence, fluorogenic MMP-sensitive peptide substrates have been used to quantitatively monitor the spatial distribution of cell-mediated MMP activity by real-time measurements in 3D cell cultures [236]. Fluorescein (DQ)-conjugated collagen becomes fluorescent when fluorophores are separated due to pericellular proteolysis occurring during cancer cell invasion of a 3D nylon mesh-supported gelatine matrix [237].

With the help of sophisticated cancer cell-ECM interfaces a variety of different proteolytic structures have been identified to contribute to pericellular ECM degradation and remodelling during proteolytic cancer cell invasion through 3D fibrillar fibrin or collagen matrices [238].

6.3. Cysteine proteases

Molecular imaging technologies comprise, for example, fluorescently-based probes that become activated by cancer-associated proteases (*e.g.* cathepsins) in conjunction with 3D optical imaging to detect very small tumours and response to therapy *in vivo* [239]. Magnetic resonance imaging coupled with 3D fluorescence molecular tomography was used for *in vivo* fluorescent imaging of ovarian tumours and their response to therapy. Upon cathepsin activity a fluorescent signal was detectable and positively correlated with tumour size. In the same study, fluorescent molecular imaging probes, relevant to ovarian cancer, were also used to detect MMP activity and αvβ3 integrin binding but resulted in a lower signal compared to that for the cathepsin activity [240]. Thus, probe activation and retention in tumours are directly indicative of the activity of protease expressed by cancer cells.

Activity-based probes are highly selective in detecting catalytically active forms of proteases within living cells or organisms using modern imaging technologies, thereby also visualising

the localisation and distribution of active proteases [241]. Fluorescently-quenched activity-based probes have been used to monitor the activity of multiple cathepsins in 3D cultures *in vitro* and tumour microenvironments *in vivo* [242, 243]. Computational modelling predicted the interactive proteolysis of cysteine cathepsins in the local microenvironment, allowing the separation of individual cathepsin activity. Fluorogenic substrate assays revealed that cathepsin S degrades cathepsin K, and consequently results in reduced ECM remodelling, including collagen type-I degradation [244]. An interesting approach utilising a split luciferin ligation reaction has determined the activity of caspase 3 and caspase 7 in live ovarian tumour-bearing animals by bioluminescence imaging upon drug-induced apoptosis [245].

6.4. Serine proteases

The enzymatic activity of the KLK serine proteases can be evaluated by the design of small molecule inhibitors that selectively target the active form of the protease to prevent the tumourigenic behaviour [246]. In a 3D suspension culture that represents the ascites microenvironment, the selective active site KLK4 sunflower trypsin inhibitor reduced the multicellular aggregation of ovarian cancer cells and increased their sensitivity to paclitaxel, thereby providing a potential combination strategy for anti-metastatic therapies [247].

7. Drug screening in 3D microenvironments

For preclinical screening of drug efficacy and the assessment of drug responsiveness the cell line and culture models used need to be carefully chosen to reflect maximal molecular similarity to tumour tissue of a distinct subtype. Cell line models that are genetically characterised can guide the selection of cancer patients, depending on genomic heterogeneity, suitable for clinical trials specifically designed for a particular histological subtype, to yield higher response rates [248, 249]. As the minority of new drugs show anti-cancer activity in clinical trials, there is an urgent need for pre-clinical drug screening models that better mimic the complex *in vivo* architecture of primary tumours. High-throughput technologies provide the opportunity for genomic-based drug discovery studies, thereby allowing systems-level analyses of cancer cell behaviour in a physiological 3D microenvironment [250, 251].

The chemo-response of epithelial ovarian cancer, the most common subtype of ovarian cancer, was evaluated using a 3D microenvironment allowing multicellular aggregation of the cancer cells. It was reported by us [13, 15] and others [252] that cells grown in 3D were more chemo-resistant compared to traditional monolayer (2D) cultures, thus implying a critical impact on drug testing approaches, drug design strategies and mechanism underlying drug-resistance. In a comprehensive study, 31 epithelial ovarian cancer cell lines were characterised for their chemosensitivity towards cisplatin (0.1 mg/ml) and paclitaxel (100 nM) when grown in 2D and 3D cultures. The authors showed that 3D-cultured cells had a significant increase in survival to both chemotherapeutics, including a 30% increase upon cisplatin treatment, compared to 2D cultures. Additionally, the 3D microenvironment reflected the characteristics of primary ovarian cancer cells *in vivo* and their histological classification, such as reduced proliferation rates and enhanced E-cadherin, cytokeratin, PAX8 and CA125 expression. Hence, this high-throughput approach is suitable for drug discovery and preclinical drug development [253].

Our group developed a hydrogel microwell array platform to analyse the survival of ovarian cancer cell aggregates in response to paclitaxel (100 nM) using a high-throughput mode. This technology allows the precise control of the aggregate size layered on top of hydrogel microwell arrays and to assess the efficacy of drug treatment dependent on aggregate size. Within this hydrogel-based model, the expression of β1 integrin was increased upon paclitaxel treatment, underscoring the critical function of this integrin in paclitaxel-related resistance. The administration of paclitaxel reduced aggregate area but not numbers. KLK4-7-expressing ovarian cancer cells showed a higher ability to aggregate and to survive independent of paclitaxel in all microwell sizes tested compared to vector controls [17, 254]. These findings imply that biomedical engineering technologies will advance the current cancer cell modelling and ultimately lead to novel anti-metastatic therapeutic interventions that translate into improved clinical strategies and prolonged patient survival [111].
A high-throughput 3D invasion assay has been used to screen the response of invasive cancer cells, including prostate cancer cells, to various anti-invasive and cytotoxic drugs. Cancer cells were encapsulated within collagen I-based hydrogels to allow spheroid formation and drug treatment to develop a standardisation and automated quantification protocol. Co-staining with nuclear (*e.g.* Hoechst33342) and apoptotic (*e.g.* propidium iodide) markers helped to simultaneously evaluate the anti-invasive and/or cytotoxic effect of a specific compound [255]. This study shows that high-throughput 3D platforms represent a precise, reproducible, quantitative and phenotypic screening tool for large scale anti-cancer drug discovery.

8. Engineered cancer animal models

The determinants and key mechanisms of cancer progression have not yet been fully delineated, mainly due to the lack of reproducible animal models that represent the biological, genetic and clinical features seen in patients. While transgenic and syngenic animal models can answer specific questions about metastatic processes, they fail to predict drug efficacy against a panel of human tumours with a given histology. Furthermore, the time it takes to develop tumours in these models is variable, and usually experiments are limited to long-term studies [256, 257]. For example, in ovarian cancer, intraperitoneal injections of single cell suspension of cancer cells derived from a primary tumour tissue do not replicate the early metastatic processes. Orthotopic ovarian xenografts mimic more closely the dissemination of cancer cells from the primary tumour, and therefore, reflect the metastatic phenotype [158]. In prostate cancer, transgenic and syngenic models do not allow the replication of the advanced disease as they usually lack a robust skeletal metastatic phenotype [258].
Therefore, most insights about the progression of ovarian and prostate cancers are still derived from xenograft models [21, 159, 259, 260]. Xenograft injections of human cancer cells into murine tissues have been routinely used as *in vivo* models to study various aspects of human tumour pathogenesis and responsiveness towards therapeutics. However, there are fundamental differences between both species in terms of anatomy, lifespan, cancer susceptibility and cytogenetic alterations. Humanising the host organism with human stromal-derived cells or factors prior to engrafting of human cancer cells substantially

improves the value of xenograft models to fully mimic the relationship between the human tumour cells and the complex human microenvironment [261].
Within this context, tissue engineering approaches and material sciences, which originally had been focused on repair and reconstitution of damaged tissue structure and function [3, 262], can offer 'cancer research the third dimension' [8]. It is apparent that these powerful and modular tools are not only used to resemble the physiology of healthy tissues but also to replicate the physical and biochemical microenvironment of human primary and secondary tumours [21]. To replicate the complex metastatic processes, intratumoural and paracrine signalling events of cancer progression, the pre-metastatic sites, including its various cell types *(e.g.* mesothelial cells, osteoblasts, fibroblasts), need to be considered and engineered. The metastatic microenvironment of ovarian cancer is restricted to the organs (*e.g.* omentum, liver, intestine, kidney, spleen) of the peritoneal cavity, while prostate cancer metastasises primarily to the skeletal bone causing osteoblastic, osteolytic or mixed lesions [175].

8.1. Ovarian Cancer – Engineered mesothelium

The only non-human animal model that spontaneously develops ovarian cancer with a high incidence is the laying hen, with evidence of abdominal metastasis and accumulation of tumour fluid (ascites) that is similar to that found in patients with late stage disease. However, the abundance of the histological subtypes and hormones differs between birds and humans [263]. Transgenic engineering is very limited as it can only be performed on chicken embryos using, for example, gelatine-based nanoparticles [264]. In order to decipher the genetic and molecular alterations occurring during ovarian cancer progression, *D. melanogaster* has been used as a simple model organism as border cells share some features (*e.g.* migratory behaviour) with ovarian cancer cells [265]. The majority of ovarian cancer animal models are based on xenografting of human ovarian cancer cells into immunologically compromised mice [266]. But these xenograft models have drawbacks, including the incomplete replication of tumour-stroma interactions and the inability to recapitulate the early stages of tumour development [267]. Genetically modified animal models of ovarian cancer overcome some disadvantages of these xenograft models, as tumours arise in the appropriate location and mimic the initiation and clinical route of metastasis seen in patients [71, 268, 269].
Our group established a xenograft model of ovarian cancer growth using cancer cells that have been modified to allow bioluminescence imaging to non-invasively monitor tumour growth in living animals over time (Figure 2). Ovarian cancer spheroids were grown for two weeks within biomimetic PEG-based hydrogels *in vitro* prior to implantation into NOD/SCID mice. During surgical procedure, the reproductive tract is exposed, revealing the ovaries between the oviduct and the surrounding fat pad. A spheroid-containing hydrogel is placed in direct contact to the ovary (one implant on the left ovary, one implant on the right ovary) and the adjacent fat tissue which ensures a constant and reproductive growth rate of the cancer spheroids. The structural integrity of the bursa is maintained, and a tumour mass is formed on the ovaries (Figure 3). The implants are eventually surrounded by peritoneal fluid that enables the ovarian cancer cells to escape their engineered microenvironment and attach preferentially to the mesothelium of the peritoneal cavity, resulting in the typical pattern of metastatic lesions seen in the clinical sequence of metastases, in loco-regional lymph nodes,

the omentum, liver and sigmoid colon (Figure 4). Our spheroid-based animal model is more physiological and reflects more closely the disease progression seen in patients as tumour growth is observed over 4-8 weeks using 100 times less cells compared to commonly used animal models which administer 10 million cells as single-cell suspension and grow tumours over 8-12 weeks. Using this bioengineered intraperitoneal animal model, combined KLK4-7 expression significantly promoted early-phase tumour growth (after 4 weeks), thus validating the *in vitro* protease-mediated enhancement of spheroid growth *in vivo*. After 8 weeks, no significant differences in tumour weight and volume in both KLK4-7-expressing and KLK4-7-deficient groups were detected. However, in this late-phase, the number of metastases was distinctly enhanced upon the presence of KLK4-7 over-expression in the tumour cells [16]. This spheroid-based animal model has already provided new insights into ovarian cancer pathogenesis [16] and has proven useful for preclinical testing of novel therapeutics that target specifically ovarian cancer cells (*e.g.* integrin antagonists [18]), stromal-derived molecular factors (*e.g.* IL-6), and signal transduction (*e.g.* mediated by tyrosine kinases) inhibitors [76].

Currently, this 3D co-culture model of ovarian cancer spheroids with mesothelial cells is validated *in vivo* using orthotopic implants to enable organotypical interactions between human tumour cells and human stroma. Other *in vivo* human tumour-promoting niche models include human embryonic stem cell-derived microenvironments to study the tumourigenic heterogeneity among cancer cell subpopulations from ascites-derived ovarian cancer cells in order to distinguish cancer cells amenable to anti-cancer therapy screening [270].

8.2. Prostate Cancer - Engineered Bone

The xenotransplantation of human prostate cancer cells or tumour tissue into immuno-compromised murine hosts provides the possibility to simulate early and late stages of the human disease. In conventional xenograft models of metastatic prostate cancer, the skeleton as the predominant homing site of human cancer cells is of murine origin [271, 272]. As a consequence, human prostate cancer cells grow within and interact with the murine bone microenvironment. Hence, these models are not suited for the analyses of species-specific interactions of human prostate cancer cell osteotropism. To overcome these limitations, a humanised approach using subcutaneously xenotransplanted human bone fragments as homing sites for inoculated human cancer cells was designed [273-278]. However, these attempts to successfully transplant functional human bone into a murine host have failed [21]. The transplanted bone does not sufficiently mimic the morphological and functional characteristics of vital human bone as it is poorly vascularised and necrotic. Furthermore, the implanted bone tissue cannot recapitulate a functional vascular and endosteal niche for haematopoietic stem cells which results in the progressive replacement of the haematopoietic marrow by fat cells and fibrotic tissue [279-281]. To date, it is known that metastatic cancer cells usurp the homing pathways of haematopoietic stem cells to establish footholds in their microenvironment [282-284]. Therefore, the establishment of a pre-metastatic haematopoietic niche seems to be indispensable for the development of prostate cancer bone metastasis.

Tissue-engineered humanised bone can overcome the limitations of conventional xenograft models of prostate cancer bone metastases by creating bone constructs with vital human bone cells and a functional bone marrow compartment. This approach simulates the conditions

seen in the clinic more precisely as prostate cancer bone metastases are mainly found in the axial skeleton characterised by a high content of proliferative red bone marrow [285]. Several heterotopic bone models have been developed that use tissue engineering techniques to mimic the physiological conditions of a functionally intact organ bone. Hence, it is possible to enhance heterotopic bone formation, increase vascularisation and ultimately create a tailored niche for haematopoietic stem or prostate cancer cells. Most models apply cell-seeded scaffolds based on ceramic materials [286-291], synthetic polymers [292] or scaffolds that contain naturally occurring components, such as collagen meshes [288, 293], gelatine sponges [294, 295] and cellulose matrices [296]. Others used de-mineralized bone matrices [297] or gelatinous protein mixtures, such as Matrigel [298], to induce the formation of new bone with haematopoietic marrow.

Schuster and colleagues [299] were the first to use this approach to study the growth of human prostate cancer cells within a humanised tissue-engineered bone construct. Hydroxyapatite-coated collagen sponges seeded with human osteoblasts were subcutaneously implanted into male SCID mice. After *de novo* bone formation, PC-3 prostate cancer cells were injected into these bone constructs. Histological analyses confirmed tumour masses in all transplanted scaffolds. Unfortunately, the viability and composition of the newly formed bone was not further analysed [299]. Moreau and colleagues [300] cultured human bone marrow-derived stromal cells on silk fibroin sponges that were coupled with BMP-2. After bone formation, luciferase-labelled SUM1315 human breast cancer cells were orthotopically implanted into the mammary fat pad of female NOD/SCID mice. Metastatic spread was only detected to the implanted human bone constructs but not to the murine skeleton. In a later study from this group, metastatic spread was also evident in the murine skeleton [301, 302]. Therefore, the species-specificity of this approach has still to be proven.

So far, only these two groups [299, 300] applied tissue-engineered principles to analyse the mechanisms of human cancer cell osteotropism within a murine host. However, in these models, the bioengineered bone constructs were not sufficiently characterised in terms of their bone biology. Histological analyses did not show the morphological characteristics of a functional organ bone as the bone marrow spaces were mainly filled with connective tissue and not with the typical cellular components of physiological bone marrow. Furthermore, the bone matrix was interspersed with the carrier material which interferes with the development of a coherent physiological tissue network [300, 302]. Therefore, metastatic lesions occurred within a non-physiological, immature bone microenvironment.

At present, our interdisciplinary group is creating a novel tissue-engineered xenograft model of prostate cancer bone metastasis to recapitulate more subtle, species-specific aspects of the mutual interaction between human prostate cancer cells and a humanised physiological bone microenvironment (Figure 5). Our results show that homing of the human prostate cancer cells indeed occurs at a humanised organ bone with a morphologically intact marrow compartment [24]. A large amount of human osteocytes and human-derived matrix proteins are incorporated in these constructs. In contrast to human bone, these humanised tissue-engineered bone constructs are reproducible, consistent and not limited in supply [23]. The amount and viability of the cells seeded onto the scaffold material can be monitored before implantation into the murine host. This approach reproducibly tailors the characteristics of the humanised constructs according to the experimental purpose and ensures the quality of

the cell-seeded construct prior to implantation [24]. In the future, human haematopoietic elements can be introduced into the construct to further increase the value of the model to allow interactions between human immune cells and the tumour within the murine host.

9. Mechanical and metastatic properties of cancer cells

The rapidly increasing research into the dynamic biological and functional characteristics of metastatic cells and their heterotypic niche has uncovered a number of new anti-metastatic targets to move the field of personalised cancer therapies forward [303]. Consequently, there is a constant progress to decipher interactions between biomechanical effects and intracellular and intercellular signalling in order to understand cancer growth, its interaction with the surrounding stroma, cell invasion and metastasis [304, 305]. The invasive behaviour of ovarian cancer cells and spheroids is inversely correlated with their stiffness leading to cytoskeletal remodelling and altered adhesion to ECM [306, 307]. A decrease in cell stiffness seems to be greater with higher malignant and metastatic potential [307]. However, the detailed mechano-biology of ovarian cancer spheroids and tumour tissue has not yet been determined. With the exception of Xu and colleagues [306], there is limited information regarding the biomechanics of ovarian cancer cells. There are few methods available for characterising cell mechanics, and of these, atomic force microscopy offers unique advantages over other popular methods to determine cell mechanics in an extremely localised area [308]. The cells (*e.g.* from pleural fluids), harvested for atomic force microscopy nano-mechanical testing are most commonly cultured in monolayers, but not in spheroids [309].

Our unpublished results suggest that cancer spheroids derived from spheroid-based xenografts were softer than normal ovarian tissue suggesting that stiffness may be a useful indicator to evaluate the aggressive potential of this disease. Interestingly, paclitaxel treatment of cancer spheroids and tumour tissues increased the elastic modulus, implying that altered biomechanical properties are an indication of therapy responsiveness and anti-metastatic approaches (Figure 6). The analyses of the nano-mechanical profiles and nano-structural organisation of the cytoskeleton of ovarian cancer cells have revealed a potential chemoresistance-associated mechanism, implying an inter-linkage between actin remodelling and drug-induced increase in cell stiffness [310].

These mechano-biological data built sub-cellular parameters that can be integrated into computational simulation approaches to advance the current knowledge of ovarian cancer progression, in particular multicellular cancer spheroids, and treatment [311]. Computational modelling is a proven tool to assist and improve *in vivo* treatment methods and to demonstrate the efficacy of various treatment combinations [312]. Our confined compression tests showed that normal ovarian tissue was softer than tumour tissue, which has also been reported for other epithelial cancers and control tissues [313]. This altered micro-rheology can be advantageous during cell invasion at metastatic sites and extra-vasation of the vasculature, thereby promoting cancer progression. Indeed, tumour rigidity is a response to the increased interstitial tissue pressure and solid stress upon a perturbed vasculature and tumour growth [314]. It was shown that interstitial flow-induced forces increased the metastatic cell motility, involving integrins, MMPs and CD44, and mechano-transduction through hyaluronan and heparan sulphate in a 3D collagen-based hydrogel suspension model [315].

Computational modelling has provided insights into the changes of the mechanical architecture and cell migration of prostate cancer cells that occur over time in a 3D microenvironment [316]. Araujo and colleagues developed an integrated computational model to predict the cellular interactions upon treatment of bone metastasis occurring during prostate cancer progression to the bone [317]. The interdependence of cell motility on matrix stiffness and protease activity was experimentally quantified, indicating that the invasive and proteolytic (*e.g.* MMP2, MMP9) capabilities of cells depend on the rigidity of the microenvironment [131]. Mathematical simulation combined with experimental 3D systems help to understand the cellular behaviour at a systems-level during cancer progression, suggesting that invasive cancer cells more actively remodel the ECM and its dynamics to facilitate metastasis [318].

The tissue microenvironment and the ECM architecture control the force acting on cancer cells, and reciprocally, these cells respond via their mechano-receptors (*e.g.* integrins) to the exogenous forces (*e.g.* matrix stiffness) by cell-generated forces creating a mechanical interdependence [128]. The stiffening of the extracellular microenvironment can predict the presence of malignant cells. The rigidity of a tumour is accompanied with an increased stiffness of the surrounding stroma and mechano-regulatory events (*e.g.* Rho-dependent cancer cell growth, contractility, integrin clustering) that facilitate an oncogenic phenotype [314, 319]. Mechanical tension from the extracellular microenvironment also affects the physical properties of the nucleus, thereby regulating gene expression, nuclear rheology and cell fate [320]. LaminA was identified as key factor of the tension-mediated transcriptional regulation resulting in the tissue-specific activation of mechano-sensitive signalling pathways [321]. The emerging challenges in understanding and targeting the complex cell-biological and mechano-chemical interactions occurring during tumour metastasis require integrated and multi-disciplinary approaches [8, 121, 153, 311, 322].

10. Conclusion and future directions

Although several breakthroughs in anti-cancer drug development have been accomplished in the last decades, it is estimated that, especially in developing countries, the cancer burden will significantly increase in the near future. This is mostly due to the fact that the understanding of cancer cell biology and the contribution of the tumour microenvironment to disease progression and implicated signalling pathways is still rudimentary. *Sensu stricto*, most conventional cell culture approaches fail to model the tumour's niche as they usually focus on single-scale events in cancer development or progression. However, as highlighted in this review, carcinogenesis and metastasis are multi-scale processes including alterations at the genetic, molecular, cellular, tissue and organ level which influence each other in a reciprocal way. Thinking outside the box and applying tissue engineering principles with a multi-disciplinary *modus operandi* can lead to a better understanding of the individual steps promoting carcinogenesis and finally enables the study of cancer in multi-scale and systems-level platforms according to the principle of 'the whole being greater than the sum of its parts'.

The contributions of the tumour microenvironment to cancer progression and signal transduction are multi-facetted. Engineered tumour models have already provided biologically and physiologically relevant insights into the interdependence of carcinogenesis

and the surrounding milieu, thereby affecting responses to therapeutics. Tissue engineering principles can further be used to develop personalised tumour models that allow for growing patient-derived primary cells or tumour tissue within its native microenvironment to develop patient-specific, individualised therapeutic strategies. However, tumour engineering will only have a lasting impact on the cancer research field if it allows the translation of novel anti-metastatic drug candidates from bench to bedside, and thus, improves the overall survival of cancer patients.

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Figure legends

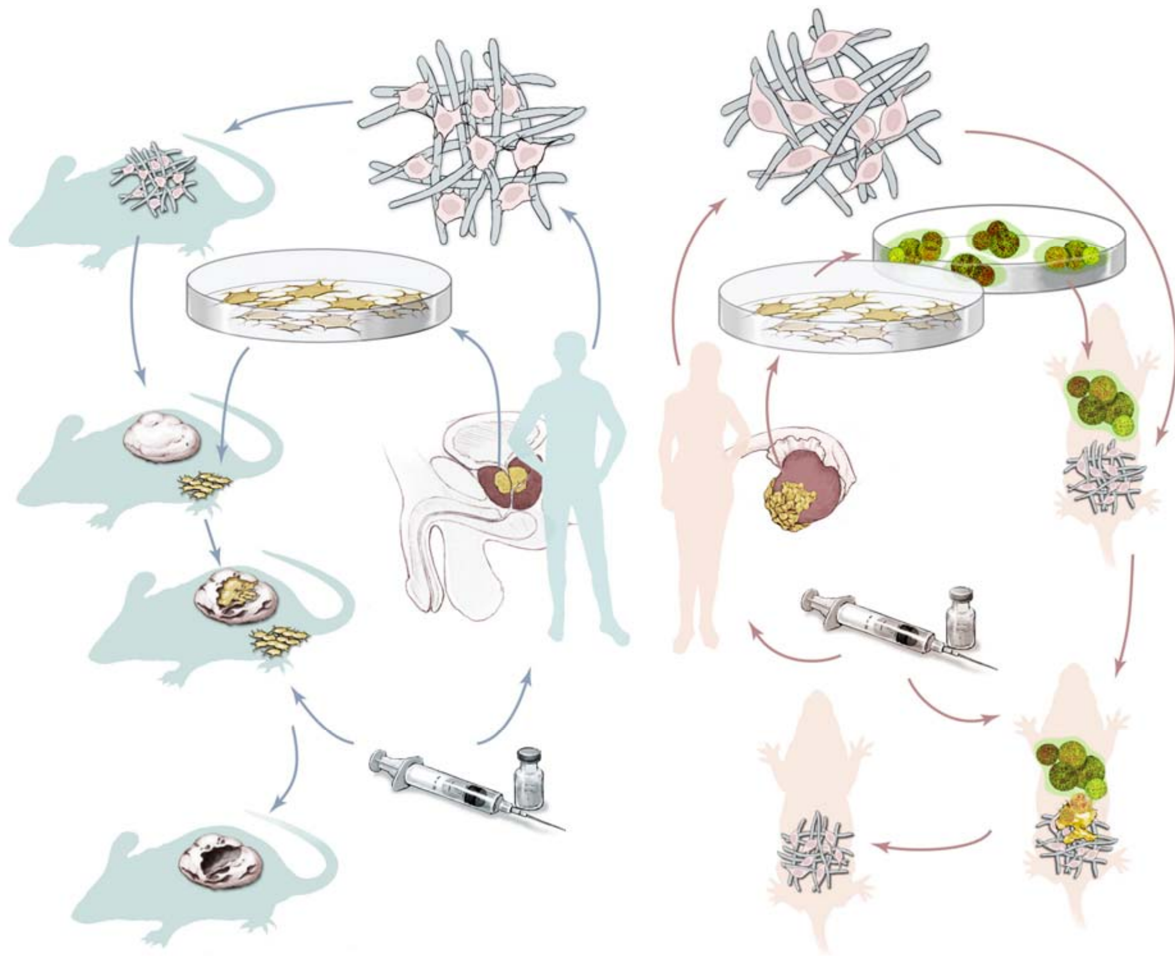


Figure 1. Tissue engineering provides a platform to study species-specific homing mechanisms of human cancer cells. This approach allows testing of drug efficacy against a panel of human tumours within an immunocompromised murine host and developing of individualised, patient-specific therapeutic strategies. Left panel: Mesenchymal cells are obtained from prostate cancer patients, cultured under osteogenic conditions and seeded onto composite scaffolds. After implantation, a tissue-engineered humanised organ bone develops within the host. Human prostate cancer cells are cultured and inoculated into the animal via intracardiac injection or orthotopic implantation into the prostate capsule. Finally, the effects of novel therapeutic strategies on the development of bone metastases can be investigated. Right panel: Mesothelial cells are obtained from non-cancerous individuals, cultured on an electro-spun scaffold and implanted into the animal's abdominal cavity to mimic the human mesothelium as the native soil for metastasizing ovarian cancer. Tumour cells are cultured and embedded into a 3D matrix to develop multicellular spheroids. After implantation of these constructs into the abdominal cavity, homing of human ovarian cancer cells to the humanised mesothelium can be analysed. Finally, the efficacy of novel anti-cancer drugs can be tested to inhibit tumour growth and metastasis.

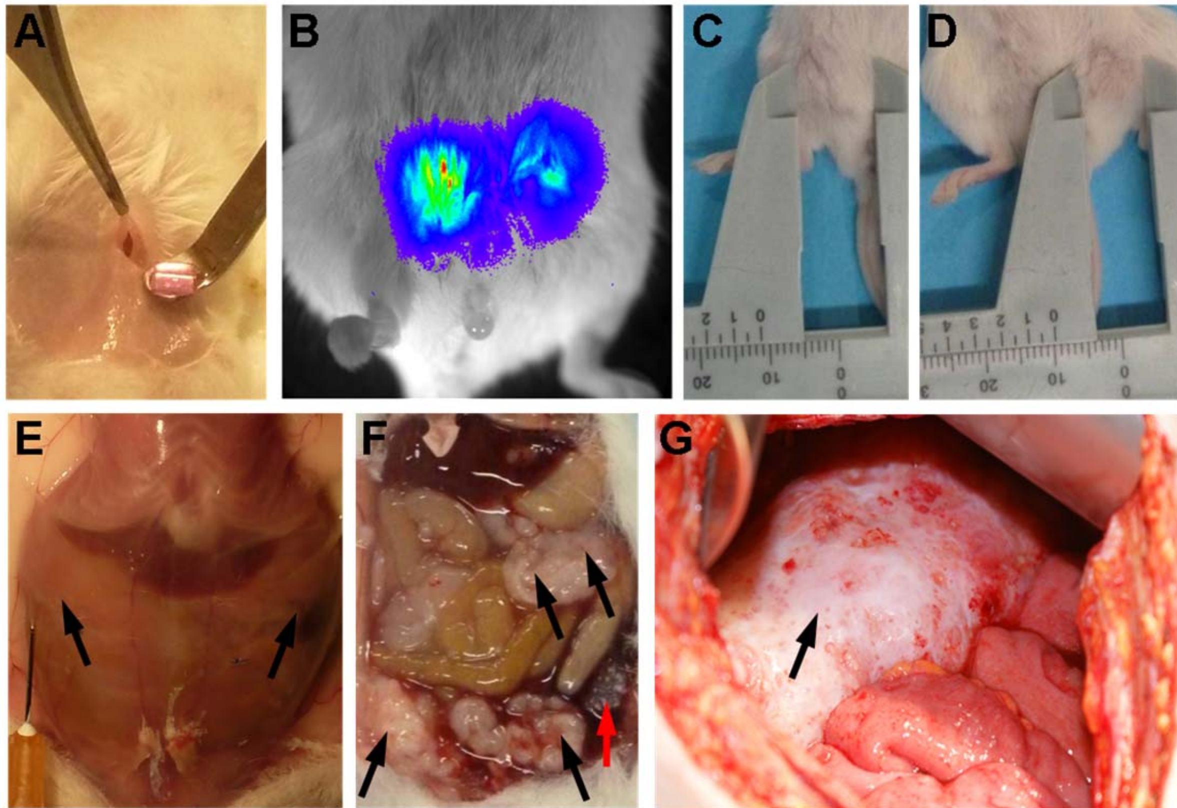


Figure 2. Xenograft model of intraperitoneal ovarian cancer growth using a bioengineered 3D construct. A. Ovarian cancer OV-MZ-6 cell spheroids were grown for 2 weeks within a bioengineered 3D model prior to implantation into 6 weeks old female NOD/SCID mice. An abdominal incision parallel to the longitudinal body axis was made to expose the peritoneal cavity and to place the spheroid-containing hydrogel implant adjacent to both ovarian fat pads. **B.** The correct placement of the spheroid-containing implants was confirmed via bioluminescence imaging (7.5 mg/ml D-luciferin; Caliper Life Sciences) directly after surgery using a live organism imaging system (IVIS® Spectrum 200, Perkin Elmer; Living Image® software v.4.3.1). **C**, **D.** Intraperitoneal tumours developed over the duration of the studies and were visible (about 11mm in diameter) at the time of termination after 8 weeks. **E.** The accumulation of tumour fluid (ascites) within the peritoneal cavity was evident (arrows). **F.** Cancer spheroid implants (red arrow) caused tumour spread throughout the abdominal cavity (black arrows). The metastatic distribution pattern observed in the intraperitoneal animal model was comparable to the clinical route of metastasis with the largest secondary tumour masses seen in the omentum **G.** Intra-operative situs of a 48 year old patient diagnosed with ovarian cancer and peritoneal carcinomatosis showing the typical omental cake, an extremely thickened omentum caused by massive tumoural infiltration (black arrow; picture was kindly provided by Prof Dr Joerg Pelz, Department of Surgery, University Clinic Wuerzburg, Germany).

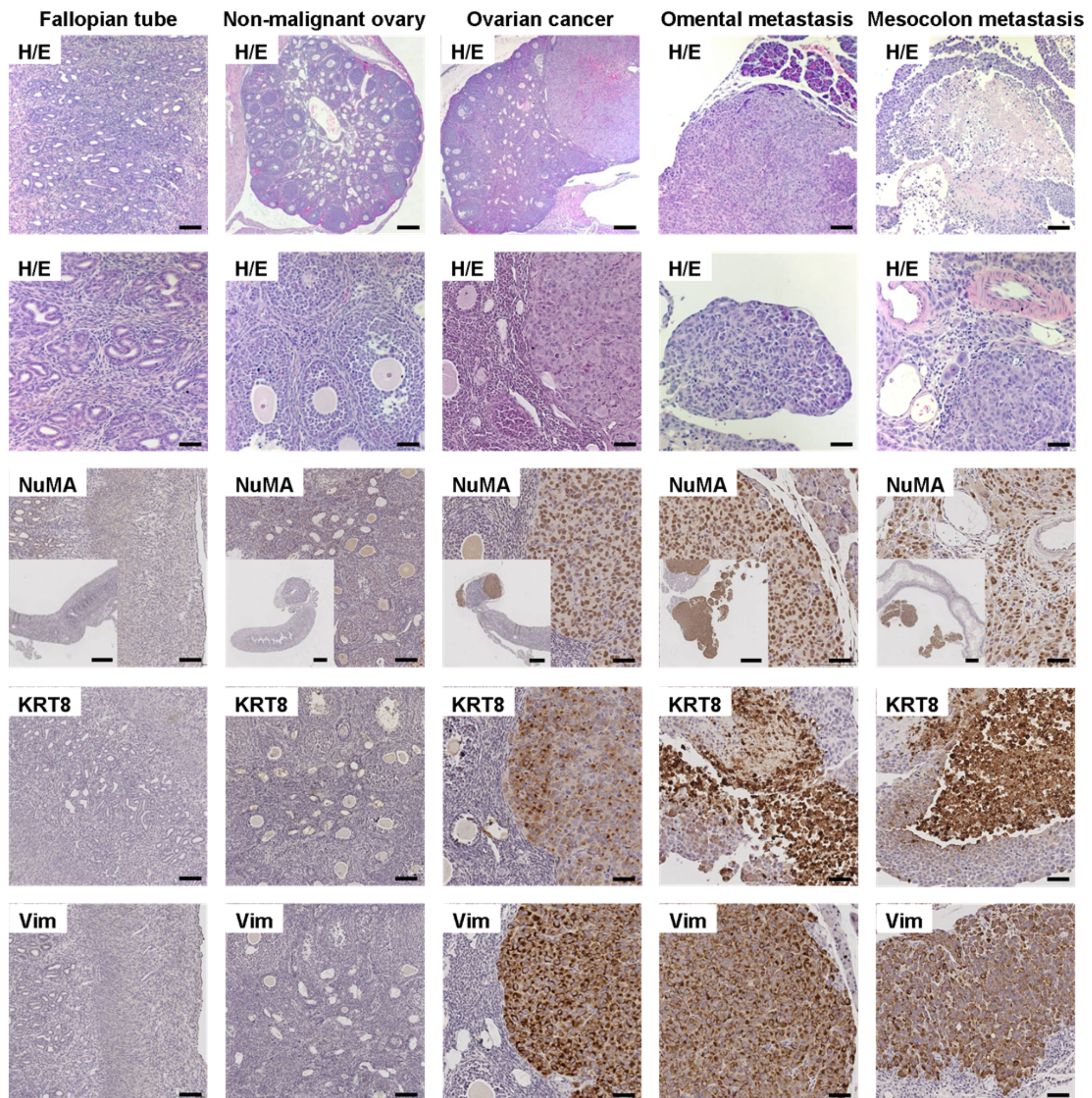


Figure 3. Immunohistochemical characterisation of ovarian cancer development and progression in the bioengineered 3D model. To evaluate the growth of the human cancer cells observed in the intraperitoneal ovarian cancer animal model, paraformaldehyde (PFA)-fixed, paraffin-embedded serial sections (5µm) of spheroid-based xenografts and the metastatic lesions were immunohistochemically processed using an EnVision™ Flex, high pH detection system (Dako). Antigen retrieval was performed by 95ºC treatment using the provided target retrieval solution (high pH) followed by blocking in 2% BSA/PBS. Human-specific antibodies against the nuclear mitotic apparatus protein 1 (NuMA; 1:100; Epitomics), cytokeratin8 (KRT8; #M20; 4 µg/ml; Abcam), a epithelial cancer cell marker, and vimentin (Vim; #V9; 1 µg/ml; Abcam), a mesenchymal cancer cell marker, were incubated for one hour at room temperature, and their binding visualised using 3,3'-diaminobenzidine as chromogen (Dako). Sections were counterstained with Mayer's hematoxylin and imaged with a widefield microscope (LaborLux, Leitz; DXM1200C digital camera, Nikon; ACT-1C software v.1.01) with a 10x and 20x air objectives. While no staining of the fallopian tube

and the healthy ovary was detected, human-derived malignant growth in the neoplastic ovary and lesions in the omentum and mesocolon were evident as indicated by positive staining for NuMA, KRT8 and Vim. Scale bars, 1 mm (overview insert in NuMA staining), 200 µm (top H/E panel), 50 µm (second H/E panel, NuMA, KRT8, Vim staining).

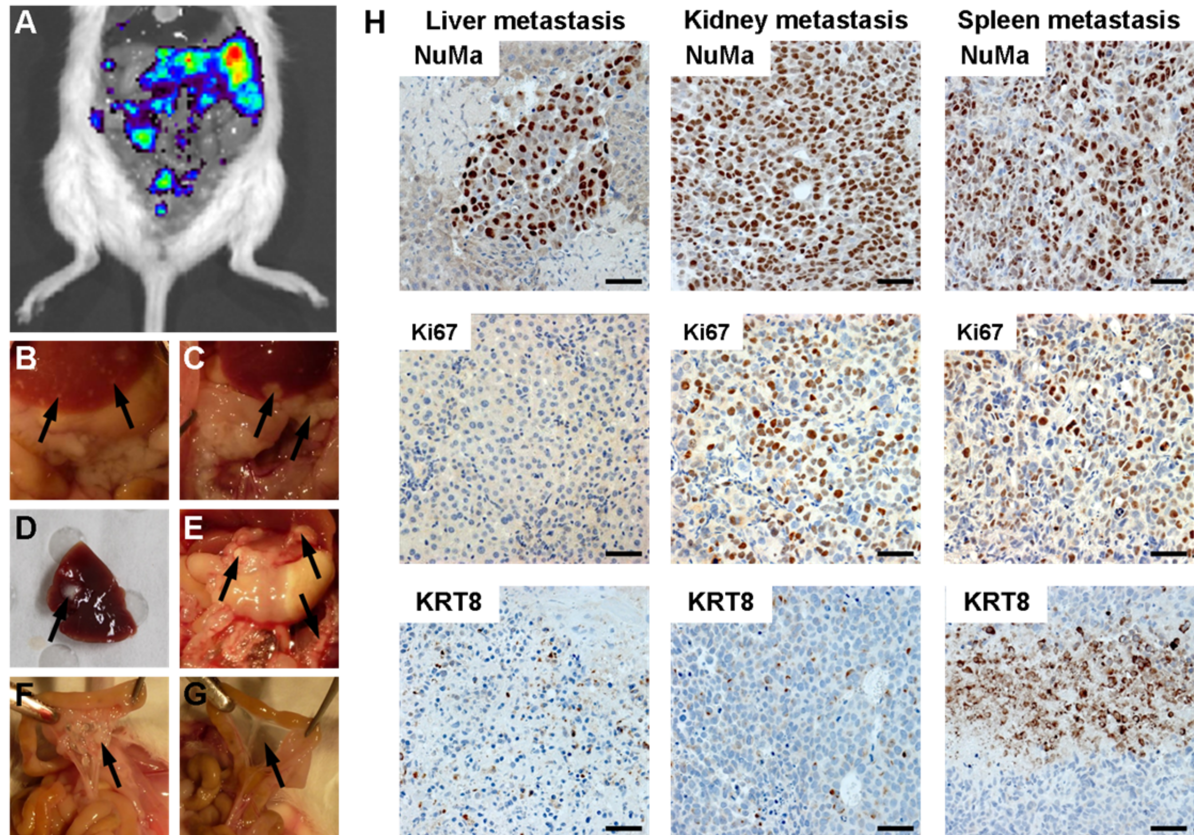


Figure 4. Ovarian cancer metastasis in the bioengineered 3D model. A. Within the intraperitoneal ovarian cancer animal model, widespread abdominal lesions were detected by bioluminescent imaging and evident in the liver (**B**, **C**, **D**; arrows), omentum (**C**, **E**; arrows) and mesothelium of the intestine and colon (**F**; arrows). **F**, **G.** The omental metastatic spread was remarkably reduced by treatment with paclitaxel (4 weeks after tumour growth, animals were treated with 10 mg/kg paclitaxel twice per week over 4 weeks; arrows). **H.** Immunohistochemical analyses of PFA-fixed, paraffin-embedded metastatic tissues of the liver, kidney and spleen indicates a positive staining for the human-specific antibodies directed against NuMA, Ki67 (#MIB-1; 1:75; Dako), a cell proliferation marker, and KRT8. Scale bars, 50 µm.

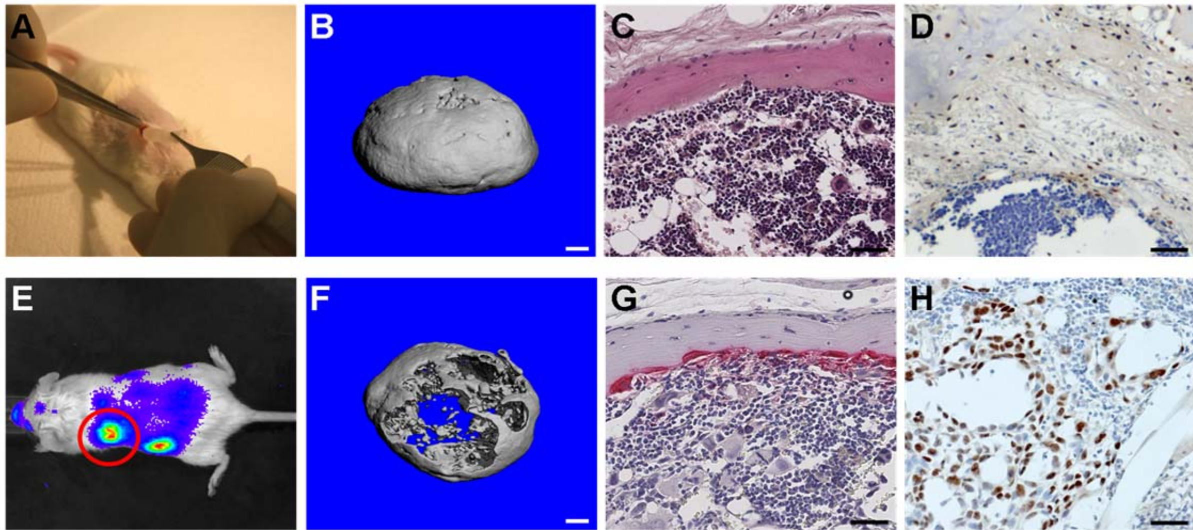
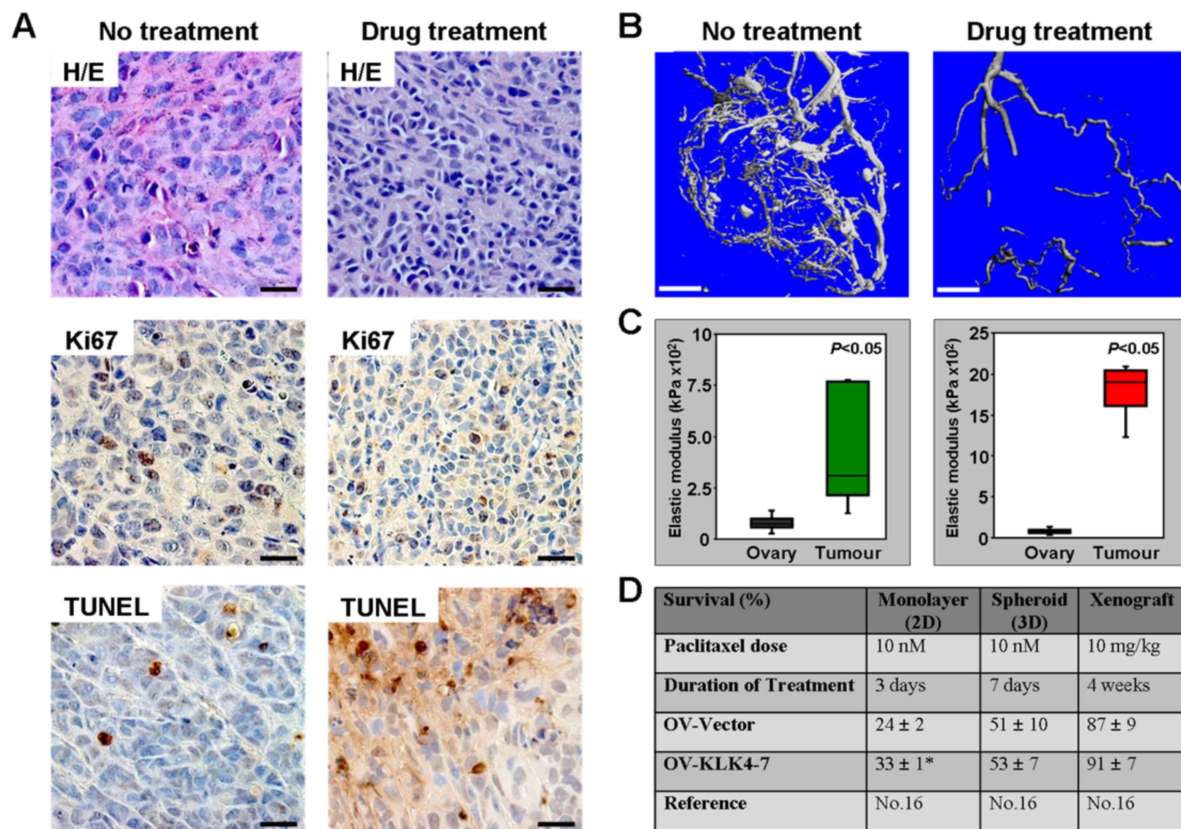


Figure 5. Establishment of a humanised xenograft model of prostate cancer bone metastasis. A. Melt-electrospun tubular polycaprolactone scaffolds seeded with human osteoblasts and loaded with recombinant human BMP-7 were subcutaneously implanted into the flanks of male NOD/SCID mice. **B.** Micro-computed tomography 3D reconstruction demonstrates the development of a bone organ 14 weeks after implantation of the tissue-engineered construct. Scale bar, 1 mm. **C.** Histological analysis (H/E) shows newly-formed bone matrix and a physiological bone marrow compartment with well-expanded sinusoids and haematopoietic cells of various lineages and differentiation stages. Scale bar, 50 µm. **D**. Immunostaining for human-specific NuMA proved that human-derived cells (brown staining) contributed to the formation of the bone microenvironment (murine cells stained blue with Mayer's hematoxylin). Scale bar, 50 µm. **E.** Bioluminescence imaging after intracardiac injection of luciferase-labelled human PC-3 prostate cancer cells revealed a positive signal at the implanted bone construct (red circle) indicating homing of the cancer cells to the humanised bone. **F.** Micro-computed tomography 3D reconstruction shows an osteolytic growth pattern of the human prostate cancer metastases within the ossicles. Scale bar, 1 mm **G.** Staining for tartrate-resistant acid phosphatase (TRAP) confirmed these results by visualising a large number of osteoclasts (red staining) adjacent to tumour cells that degrade the bone matrix. Scale bar, 50 µm. **H.** Immunostaining for human-specific NuMA confirmed the presence of human prostate cancer cells (brown staining) scattered within the bone marrow. Scale bar, 50 µm.



Survival (%)	Monolayer (2D)	Spheroid (3D)	Xenograft
Paclitaxel dose	10 nM	10 nM	10 mg/kg
Duration of Treatment	3 days	7 days	4 weeks
OV-Vector	24 ± 2	51 ± 10	87 ± 9
OV-KLK4-7	33 ± 1*	53 ± 7	91 ± 7
Reference	No.16	No.16	No.16

Figure 6. Effects of paclitaxel treatment seen in an intraperitoneal animal model using a bioengineered 3D construct. A. After 4 weeks of tumour growth within the intraperitoneal animal model, mice were treated with paclitaxel (10 mg/kg; DMSO as no treatment control) twice per week over 4 weeks. After 8 weeks, mice were sacrificed, and tumour tissues processed for subsequent analyses. The effect of treatment is indicated by H/E staining (top panel). Immunohistochemical analyses of PFA-fixed, paraffin-embedded ovarian cancer xenografts without and with paclitaxel administration indicate a reduced staining for the proliferation marker Ki67 and increased TUNEL staining (DeadEnd Colorimetric TUNEL System; Promega) upon treatment. Scale bars, 20 µm. **B.** To visualise the tumour vasculature, MICROFIL® (MV-122; Flow Tech Inc.) perfusions were carried out and were imaged by micro-computed tomography. The tumour vasculature was widely branched without treatment and dramatically reduced and less branched upon paclitaxel exposure. Scale bars, 1 mm. **C.** To determine the biomechanic properties of healthy ovarian and tumour tissues, non-fixed tissues underwent a confined compression test to measure the elastic modulus. The cancerous tissue was significantly stiffer than the non-malignant ovarian tissue. A further increase in tumour stiffness was detected upon paclitaxel treatment. **D.** This table summarises our reported treatment protocols and findings of ovarian cancer cells grown *in vitro* as monolayers (2D) and spheroids (3D) and *in vivo* in a spheroid-based animal model upon combined expression of the kallikrein-related peptidases (KLK) 4, KLK5, KLK6 and KLK7 (KLK4-7). Cell monolayers expressing KLK4-7 were more resistant to paclitaxel treatment (media containing 10nM paclitaxel for 3 days) than vector controls. Spheroids grown over 7 days and then treated with paclitaxel-containing media (10 nM) for another 7 days showed an enhanced cell survival, for both KLK4-7-expressing and KLK4-7-deficient cells. To validate

these *in vitro* effects upon paclitaxel treatment *in vivo*, the spheroid-based animal model, as described above, was used. Therefore, mice were treated with paclitaxel (10 mg/kg) intraperitoneally, twice per week, starting in week 4 over 4 weeks. Both KLK4-7-expressing and KLK4-7-deficient tumour-bearing mice showed a response rate of 87-91% (* - P<0.05).

Table 1: Hydrogel-based microenvironments for ovarian and prostate cancer. Examples for engineered biomaterial platforms for *in vitro* and *in vivo* applications are summarised.

Biomaterials	Advantages	Disadvantages	References	Authors, Year
Hyaluronan-based hydrogels	injectable at room temperature for *in vivo* cell delivery	many physical forms; chemical cross-linking or radical polymerimerisation	[140]; [142]	Burdick and Prestwich 2011; Liu et al. 2007;
Alginate-based hydrogels	controllable stiffness and pore size; controllable ligand density; study of cell-cell/ECM-crosstalk	non-fibrillar structure; cross-linked in the presence of divalent cations (Ca^{2+})	[145]; [146]	Fischbach et al. 2009; King et al. 2011
GelMA-based hydrogels	controllable stiffness and pore size; natural ligand distribution; study of cell-cell/ECM-crosstalk	non-fibrillar structure; cross-linked in the presence of a photoinitiator and UV light	[18]; [147]; [148]	Kaemmerer et al. 2014; Levett et al. 2014; Nichol et al. 2010
PEG-based hydrogels	controllable stiffness and pore size; controllable ligand density; study of cell-cell/ECM-crosstalk	non-fibrillar structure; physical or chemical cross-linking	[15]; [16]; [20]	Loessner et al. 2010; Loessner et al. 2013; Sieh et al. 2012

Table 2. Engineered models for ovarian cancer. Examples for engineered culture techniques of ovarian cancer cells for *in vitro* and *in vivo* applications are summarised.

Microenvironment	Cell line(s)	*In vitro*	*In vivo*	Reference(s)	Authors, Year
Cell encapsulation within PEG-based hydrogels	SKOV-3; OV-MZ-6; OV-Vector; OV-KLK4-7	X		[15]; [16]	Loessner et al. 2010; Loessner et al. 2013
NIH3T3 fibroblast-derived 3D matrix	PA-1; PA-1/E6	X		[114]	Serebriiskii et al. 2008
Cell-encapsulating droplet patterning	OVCAR-5	X		[122]	Xu et al. 2011
On top cell culture of rat tail collagen I-based	DOV13	X		[129]	Barbolina et al. 2007

hydrogels					
Cell encapsulation within rat tail collagen I-based hydrogels	DOV13; OVCA433	X		[130]	Moss et al. 2009
Organoid cultures within alginate-based hydrogels	ovarian and oviductal organ pieces	X		[146]	King et al. 2011
Organotypic 3D co-cultures of cancer cells with primary mesothelial cells and fibroblasts	IOSE; Hey A8; SKOV3ip.1	X		[180]	Kenny et al. 2007
Mesenchymal stem cell-rich amniotic membrane scaffold co-cultures with cancer cells	OVCAR-3; SKOV-3	X		[181]	Touboul et al. 2013
Xenograft model of intraperitoneal implantation of spheroid-containing PEG-based hydrogels	OV-Vector; OV-KLK4-7		X	[16]	Loessner et al. 2013
Orthotopic model using hyaluronan-based hydrogels injected intracapsularly in ovaries of athymic nude mice	SKOV-3; OVCAR-3		X	[142]	Liu et al. 2007

Table 3. Engineered models for prostate cancer. Examples for engineered culture techniques of prostate cancer cells for *in vitro* and *in vivo* applications are summarised.

Microenvironment	Cell line(s)	*In vitro*	*In vivo*	Reference(s)	Authors, Year
3D co-cultures using normal prostate and cancer-associated fibroblasts for ECM production with BPH-1 cells	NPF; CAF; BPH-1	X		[12]	Clark et al. 2013
3D co-cultures of cancer cells with osteoblasts	LNCaP; PC-3	X		[19]	Sieh et al. 2010
Cell encapsulation within PEG-based hydrogels	LNCaP	X		[20]	Sieh et al. 2012
3D silk fibroin scaffolds	PC-3	X		[51]	Kwon et

coupled with BMP2					al. 2010
bone marrow-derived ECM using whole bone marrow aspirates	LNCaP; PC-3; MDA-PCa-2b	X		[115]	Lescarbeau et al. 2012
Mineralised human primary osteoblast-derived bone matrix	LNCaP; PC-3	X		[116]	Reichert et al. 2010
Cell encapsulation within rat tail collagen I-based hydrogels	LNCaP; DU-145	X		[131]	Harjanto et al. 2011
Cell encapsulation within hyaluronan-based hydrogels	LNCaP	X		[137]	Xu et al. 2012
Cell encapsulation within hyaluronan-based hydrogels	LNCaP; C4-2; C4-2B; C4	X		[138]; [143]	Gurski et al. 2009; Gurski et al. 2012
Cell encapsulation within hyaluronan-based hydrogels	PC-3	X		[139]	David et al. 2004
3D cancer-bone metastasis model using murine calvarial bone organs co-cultured with prostate cancer cells in a roller tube system	LNCaP; PC-3	X		[186]	Curtin et al. 2012
Double layered, alginate hydrogel microspheres to co-culture cancer cells with stromal-derived cells	C4-2; C4-2 PKD1; WPMY-1	X		[187]	Fang et al. 2013
3D rat tail collagen I-based co-cultures of cancer cells with lung fibroblasts or pre-osteoblast-like cells	C4-2B; GS689.Li; MRC-5; MC3T3-E1	X		[188]	Varzavand et al. 2013
3D model of tumour-driven angiogenesis using human arterial rings co-cultured with cancer spheroids	LNCaP	X		[189]	Seano et al. 2013
3D porous chitosan-alginate scaffolds seeded with cancer cells and co-cultured with human peripheral blood lymphocytes	LNCaP; C4-2; C4-2B	X		[190]	Florczyk et al. 2012
Hydroxyapatite coated collagen I-based scaffolds seeded with human osteo-lasts and subcutaneously implanted into NOD/SCID	PC-3		X	[299]	Schuster et al. 2006

mice					

Table 4. Measurement of cancer-associated protease activity in 3D. Examples for different techniques to measure the activity of cancer-associated proteases, including MMPs, cysteine and serine proteases, are summarised.

Model	Method	Cancer type(s)	Protease(s)	Reference	Authors, Year
Cell encapsulation within PEG-based hydrogels	Bioactivity assay	Ovarian cancer	MMP9	[15]	Loessner et al. 2010
On top cell culture using rat tail collagen I-based hydrogels	Gelatin zymography	Ovarian cancer	MT1-MMP; MMP2	[129]	Barbolina et al. 2007
Organotypic 3D co-cultures; DQ-collagen	Gelatin zymography; spectro-fluorometry	Ovarian cancer	MMP2; MMP9	[179]	Kenny et al. 2008
Tumour-bearing TgMISIIR-Tag mice injected with fluorescent molecular imaging probes	Magnetic resonance imaging coupled with 3D fluorescence molecular tomography	Ovarian cancer	Cathepsins; MMPs	[240]	Hensley et al. 2012
Tumour-bearing (NU(Ico)-Foxn1^{nu} mice injected with caspase 3/7-specific peptide substrates	Bioluminescence imaging	Ovarian cancer	Caspase 3/7	[245]	Godinat et al. 2013
3D suspension mimicry culture; active site KLK4 inhibitor	WST-1 Proliferation assay	Ovarian cancer	KLK4	[247]	Dong et al. 2013
3D co-cultures of osteoblasts with cancer cells	Gelatin zymography	Prostate cancer	MMP2; MMP9	[19]	Sieh et al. 2010
Mineralised human primary osteoblast-derived bone matrix	Bioactivity assay	Prostate cancer	MMP2; MMP9	[116]	Reichert et al. 2010
Cell encapsulation within rat tail collagen I-based hydrogels	Gelatin zymography	Prostate cancer	MMP2; MMP9	[131]	Harjanto et al. 2011
DQ-collagen IV mixed with matrigel or DQ-collagen I	Confocal microscopy	Prostate cancer	Cysteine proteases, MMPs, trypsin	[230]	Podgorski et al. 2005

mixed with Vitrogen-100 bovine collagen I on glass coverslips					
Cells co-polymerised with rat tail collagen containing FITC-labelled collagen monomers	Spectro-fluorometry (FITC release assay)	Breast cancer	MMPs; cysteine and serine proteases	[223]	Wolf et al. 2003
3D organotypic cultures of cancer cells and fibroblasts on DQ-collagen I/IV	Confocal microscopy	Breast cancer; Colon cancer	MMPs; cysteine and serine proteases	[231]	Sameni et al. 2009
3D organotypic cultures of cancer cells and fibroblasts on DQ-collagen I/IV	Confocal microscopy; analysed in real-time	Breast cancer	Collagenases	[232]	Sameni et al. 2012
Tumour-bearing Balb/c mice injected with fluorescently quenched activity-based probes	Optical live and *ex vivo* fluorescence imaging	Breast cancer	Cathepsins	[242]	Verdoes et al. 2013
3D reconstituted basement membrane overlay cell culture, cell-permeable activity-based probes	Fluorescence microscopy	Breast cancer	Cathepsins	[243]	Mullins et al. 2012

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