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Bladder organoids: theory and practice of a 3D culture model

Tatiana Canelas Ferreira

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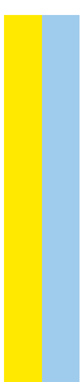

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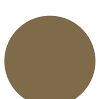

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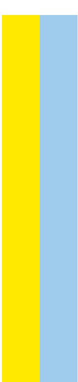

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Bladder organoids: theory and practice of a 3D culture model

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1. ABSTRACT

Bladder cancer (BC) is the 10th most common malignant disease, and the 13th most common cancer death cause worldwide. Although the ~75% of urothelial carcinoma are non-muscle-invasive bladder cancer (NMIBC) with a relatively favorable prognosis, between 50-70% of NMIBC will recur, and between 10-20% will progress to muscle-invasive bladder cancer (MIBC) with a poor prognosis. The demand for lifetime surveillance strategies and repeated treatment for recurrence disease makes BC have the highest cost per person of all cancer types. There is a need to establish new models that faithfully recapitulate BC. The novel 3D culture system, named organoids, allows the maintenance of the morphology and the molecular landscape of the tissue of origin, making it a powerful tool for translational research between the laboratory and the clinic.

Here we described the protocol used to establish human bladder organoids derived from tissue obtained from bladder cancer patients. From 47 samples, only 5 established organoid lines, which implies an efficiency rate of 11%. Then the protocol was compared with other bladder organoid protocols, and different approaches to improve organoid establishment are addressed, such as the application of hydrogels and growth factors.

In summary, the protocol needs to be modified to increase the efficiency rates and profits of the organoid culture system to improve the understanding of the biology of bladder cancer and find new treatments.

RESUMO

O cancro de bexiga (BC) é a 10ª doença maligna mais comum e a 13ª causa de morte por cancro no mundo. Embora ~ 75% do carcinoma urotelial seja cancro de bexiga não-músculo invasivo (NMIBC) com um prognóstico relativamente favorável, entre 50-70% do NMIBC irá reincidir e entre 10-20% irá progredir para cancro de bexiga músculo-invasivo (MIBC) apresentando um pior prognóstico. A necessidade de estratégias de vigilância ao longo da vida e tratamento repetido para doenças recorrentes faz com que a manutenção clínica dos doentes com BC apresente o maior custo por pessoa de todos os tipos de cancro. É necessário estabelecer novos modelos que mimetizem fielmente o BC. O novo sistema de cultura 3D, denominado organoides, permite manter a morfologia e a diversidade molecular do tecido de origem, tornando-se uma poderosa ferramenta de pesquisa translacional entre o laboratório e a clínica.

Neste trabalho descreve-se o protocolo usado para estabelecer organoides de bexiga humana derivados de tecido obtido de pacientes com cancro de bexiga. De 47 amostras, apenas foram estabelecidas 5 linhas organoides, o que se traduz numa taxa de eficiência de 11%. Seguidamente, o protocolo foi comparado com outros protocolos de organoides da bexiga e diferentes conceitos para melhorar o estabelecimento são abordadas, como a aplicação de hidrogéis e fatores de crescimento.

Em resumo, o protocolo precisa ser modificado para aumentar as taxas de eficiência e aproveitar assim o sistema de cultura organoide para melhorar a compreensão da biologia do cancro de bexiga e encontrar novos potenciais tratamentos.

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4. ABREVIATIONS

A1AT	α1-antitrypsin
ADAM10	Disintegrin and metalloproteinase domain-containing protein 10
ALI	Air-liquid interface
APC	Adenomatous polyposis coli
ASCs	Adult-tissue-resident stem cells
BC	Bladder cancer
BCG	Bacillus Calmette–Guérin
BMP	Bone morphogenetic protein
CagA	Cytotoxin-associated gene A
CBF1	Centromere-binding protein 1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CIS	Carcinoma in situ
CRC	Colorectar cáncer
CRISPR	Clustered regularly interspaced short palindromic repeats
CSL	CBF1 Suppressor of Hairless Lag-1
DBZ	Diazepine inhibitor of γ-secretase
ECM	Extracellular matrix
EGF	Epithelial growth factor
EHS	Engelbreth-Holm-Swarm
eIF2α	Eukaryotic Initiation Factor 2
ERBB2	Erythroblastic oncogene B 2
ERK	Extracellular signal-regulated kinases
ESCs	Embryonic progenitors stem cells
FGF	Fibroblast growth factor
FGFR3	Fibroblast growth factor receptor 3
GSTM1	Glutathione S-transferase-μ1
HES1	Hairy and enhancer of split-1
HTS	High-throughput screening
ISUP	International Society of Urological Pathology
LGR5^{+}	Leucine-rich repeat-containing G protein-coupled receptor 5
LOH	Loss-of-heterozygosity
MIBC	Muscle invasive bladder cancer

MMEC	Mouse mammary epithelial cells
NAT2	N-acetyltransferase 2
NECD	Notch extracellular domain
NICD	Notch intracellular domain
NMIBC	Non-muscle invasive bladder cancer
PDTX	Patient-derived tumor xenograft
PEG	Poly(ethylene glycol)
PEG-4MAL	Poly(ethylene glycol) with 4 maleimide groups at each terminus
PIK3CA	Phosphoinositide-3-kinase catalytic alpha polypeptide
PKD	Polycystic kidney disease
PLC	Primary liver cancer
PSCs	Pluripotent stem cells
PTEN	Phosphatase and tensin homolog
PUNLMP	Papillary urothelial neoplasm of low malignant potential
RAM	RBP-J kappa-associated module
RB	Retinoblastoma
RBPjk	J kappa-recombination signal-binding protein
RGD	Arginine-Glycine-Aspartate
RNA	Ribonucleic acid
TERT	Telomerase reverse transcriptase
TNM	Tumor-Node-Metastasis
TP53	Tumor protein 53
TTC7A	Tetratricopeptide repeat domain 7A
TURBT	Transurethral resection of bladder tumour
WHO	World Health Organization

5. INTRODUCTION

In the last decade, there has been an increase in the studies that adopted the organoids as a new cell culture system. Organoids are three-dimension structures derived from either pluripotent stem cells (PSCs), adult-tissue-resident stem cells (ASCs), or embryonic progenitors stem cells (ESCs) and consisting of organ-specific cell types that self-organize through cell sorting and spatially restricted lineage commitment like *in vivo* (1, 2). To fulfill the basic definition, a genuine organoid should satisfy several essential characteristic features of organs. First, the existence of more than one cell type, as in the organ itself; second, the self-organization of the cells should be similar to the organ it models; third, the exhibition of organ specific functions; and fourth, it should maintain the characteristic organization during the development of the organ (3), which implies that the way the organ establishes its characteristic organization during development has to be similar (4).

HISTORICAL CONTEXT

Although the term 'organoid' may seem new, this word was used for the first time in a tumor case study in 1946, in which the author used the term to describe histological features (5). At that time, the meaning was completely different. Indeed, the knowledge on the cell stemness, organogenesis, and the extracellular matrix obtained during the 20th and early 21st centuries was meaningful to the current definition of organoids.

As early as 1907, Wilson et *al.* showed that dissociated sponge cells had the potential to self-organize to regenerate a whole organism (6). This process of dissociation-reaggregation has also been observed in amphibian embryonic cells in 1944 by Holtfreter and colleagues (7). Later, in 1952, Moscona et *al.* (8) cultured early chick embryos cells from organ rudiments after enzymatic digestion and showed that the cells could reaggregate and reestablish the structural pattern of their tissue of origin. In these experiments, they used the watch glass culture method, which was established in 1929 by Fell and Robinson (9), and observed that cells aggregate faster in concave vessels, which implies that gravitation accelerates the process. A few years later, Weiss and Taylor (10), performed dissociation-reaggregation experiments with various organs of chick embryos and observed the same process.

In parallel, researchers were working on culturing cells in suspension and on the characterization of the extracellular matrix (ECM). In 1956, Robert Ehrmann and George Gey (11) grew cell lines in collagen extracted from rat-tail tendons, and they observed that in most cases, the cells grew better in dishes with dried collagen than on glass. A year after, Lasfargues and colleagues generated mammary organoids (duct fragments), lacking fibroblasts and adipocytes, from adult mouse mammary gland tissue previously dissociated

using collagenase from *Clostridium histolyticum* (12)*.* Two decades later, Richard Swarm and his group, studied the interaction between hyaluronic acid, collagen, and associated proteins of the extracellular matrix from chondrosarcomas. In the process, they isolated a laminin-rich gel with basement membrane characteristics, and they named it EHS sarcoma, using the initial of the three investigators (Engelbreth-Holm-Swarm) (13). This gel was what nowadays we know as Matrigel.

Starting in the eighties, thanks to the tools and knowledge provided by the works mentioned above, the new era in developing 3D cultures and organoids began. In 1984, Lee et *al.* (14), showed that mouse mammary epithelial cells (MMEC) exhibited basal and luminal polarity when cultivated in collagen gels. This polarity was verified by the synthesis of milk protein and secretion in the medium. A few years later, Bissell and her group (15, 16), demonstrated that MMEC grown in EHS gel could grow in 3D ducts and ductules. Moreover, when the cells were grown in EHS gel, the vast majority produced β-casein, fact that did not occurs when grown in the traditional culture method. These works supported the idea that ECM influences gene expression. In 1992, Petersen et a*l.* (17) published a work where human breast epithelial cells and primary human carcinoma breast cells were cultured in EHS matrix. They observed that all the normal cell lines were able to form spherical colonies very similar to the acini *in situ*. Conversely, none of the carcinoma cell lines were able to grow and form acini-like structures. These results suggested differences between both cell types due to a possible link between the ability to sense the basement membrane and a suppressor gene lost in malignant cells. These studies provided a rapid assay for discriminating normal and tumoral breast epithelial cells by cultivating them in EHS matrix.

In parallel to the development of the field of organoids and 3D cultures, stem cell biology also progressed during the eighties. In 1981, Evans and Kaufman (18) established cultures of pluripotent stem cells isolated from *in vitro* cultures of mouse blastocysts. At the end of the century, in 1998, the first human embryonic stem cell line was isolated and cultured from human blastocysts by Thompson et *al.* (19).

Despite the advances and the knowledge acquired about the extracellular matrix and stem cells, organoids, as we know them today, were not created until 2009 by Sato et *al.* (20). They published one of the main papers in the organoid field. On this study, the group showed that intestinal stem cells expressing Leucine-rich repeat-containing G protein-coupled receptor 5 ($LGR5^+$) could be established as 3D epithelial organoids. The purified $Lgr5^+$ stem cells were embedded in Matrigel as a substitute of the ECM and cultured with a specific culture medium containing epidermal growth factor (EGF), to promote cell proliferation; Noggin, a bone morphogenetic protein (BMP) inhibitor to allow for stem cell expansion; and R-spondin-1, a Wnt signaling pathway agonist to maintain stem cell

population. Through this culture method, the group obtained epithelial organoid cultures with definite crypt-villus compartmentalization as observed *in vivo.*

In 2012, Nakano et *al.* (21), generated self-organized optic cup structures from human ESC in 3D culture. These structures were organized in a multilayered neural retina containing cones and rods. The next year, Lancaster et *al*. (22) established a culture method to generate cerebral organoids from human PSC upon growth in Matrigel. These 3D structures were able to generate brain regions including, a cerebral cortex with populations of progenitors that organized and produced mature cortical neuron subtypes.

The works mentioned above are some of the starting points of organoids and 3D culture systems that we know today. Since then, numerous research groups have decided to use this tool for their projects, causing a surge of organoid culturing systems from a broad range of human and mouse organs (1-3, 23, 24).

APPLICATIONS

The increasing interest and use of this 3D culture system by a wide broad of groups is not surprising. Organoids offer the opportunity to mimic human and mouse tissue in a dish, from the beginning of its development to its adult stage or organogenesis (the formation and development of organs). They can be cultured long-term and are susceptible to the same molecular and cell-biological analyses developed for 2D cell lines. Hence, organoids appear as a new culture technique, a step between cell lines and *in vivo* studies, to study cellular development and gene functions. Moreover, organoid technology is a promising tool for translational research. Beneath, some examples of its translational applications will be exposed.

Study of cell and tissue development

Organoids enable researchers to study lineage specifications, embryogenesis, the principles of self-organization, and tissue homeostasis. They also allow us to look into the development of stem cells and their niches and highlight developmental similarities or differences between species by comparing human organoids with animal models.

For example, McCracken et *al.* (25), generated human gastric organoids and showed that the inhibition of BMP signaling is essential for specifying the human posterior foregut endoderm and the formation of the stomach.

In another study, through the use of stomach organoids derived from PSCs, the researchers found that Wnt/β-catenin is crucial to prompt the production of stomach acid-producing cells of both mouse and human (26).

Similarly, with the use of human lung organoids, Dye et *al.* (27), found that SHH (Sonic Hedgehog) signaling pathway specifies lung fate and that the human anterior foregut is specified by FGF (Fibroblast growth factor) signaling.

Disease modeling

When comparing the organoid cultures with traditional cell cultures, one of its main advantages is their capacity to mimic pathologies as in the organ itself. Besides, organoids can recapitulate specific human features when derived from human ASCs or iPSC (23). These characteristics facilitate their use as a model to study infectious disease and host-pathogen interaction, genetic diseases, and cancer.

Infectious diseases

An example of the utility of using organoids to study infectious disease and host-pathogen interaction are the studies related to the ZIKA virus. In brain organoids, it was possible to recapitulate the microencephaly produced in newborns when pregnant mothers are infected with ZIKA virus (28, 29). Furthermore, it was shown that infection triggers premature differentiation of neural progenitor cells and, in consequence, its depletion(30). The study also provided mechanistic insights on how the virus enters the cell and its pathogenic activity (31).
Another host-microbe interaction that benefited from the organoid technology was in studies related to *Helicobacter pylori (H. pylori)*, which is linked to gastritis and gastric cancer in humans. In these studies, they showed through the use of human gastric organoids that *H. pylori* efficiently colonize the luminal epithelia, leading to major physiological changes, such as an increase in proliferation caused by oncogenic *CagA* and increased β-catenin signaling, which could contribute to the loss of epithelial barrier function by the disruption of cell-cell junctions (32, 33).

Genetic diseases

In general, in the genetic field, organoids have been employed for two different approaches:

(1) Establishment of organoids from patient-derived biopsies. For instance, the generation of intestinal organoids harboring TTC7A deficiency derived from patients with multiple intestinal atresias. These organoids showed inversion of the apicobasal polarity in the epithelial cells that can be rescued by pharmacological inhibition of Rho-kinase signalling (34). In another study, liver organoids were generated from patients with α1-antitrypsin (A1AT) deficiency, an inherited disorder

predisposed to chronic liver disease and chronic obstructive pulmonary disease. As in the *in vivo* situation, these organoids showed an increase in apoptosis in the differentiated state and phosphorylation of eIF2α, a sign of endoplasmic reticulum stress (35).

(2) Introduction of specific genetic mutations in wild-type organoids. The introduction of mutations in organoids is useful to recapitulate features of diseases in different tissues. This strategy benefits from the arrival of a new technology that allows targeting easily any genomic locus recognizable by a specific RNA guide, the CRISPR/Cas9 system. For instance, in the work of Freedman et *al. (36)*, they investigated the potential of kidney organoids to model polycystic kidney disease (PKD). For that, loss-of-function mutations in *PKD1* or *PKD2* genes were introduced in hPSC. Then, they cultivated renal organoids derived from the previous hPSC. The researchers observed cyst formation alongside tubular organoids. These findings prove the utility of the renal organoids to mimic PKD through the CRISPR/Cas9 system and help understand the function of mutations in PKD genes in cystogenesis in polycystic kidney disease.

Therapeutics and drugs development

The concept that organoids can represent human pathologies due to their ability to maintain the genetic and phenotypic features of the tissue of origin makes them a valuable system for drug testing, screening application, and personalized medicine.

Drug screening

In the paper of Watanabe et *al. (37)*, there is a clear example of the applicability of the organoid technology on drug discovery. In this work, the researchers generated neural organoids, and they used it later for drug assays. First, they observed that Azithromycin, one of the first drugs identified as a potential ZIKA treatment, had little activity in their organoid platform. These results suggested a limitation on its protective effect on the fetal brain. Second, they identified two potential new drugs, Ivermectin and Duramycin, with significant efficacy in reducing ZIKA infection and the loss of neural tissue (37).
Another example is the work made by Czerniecki and colleagues, where they created an entirely automated high-throughput screening (HTS)-compatible platform of human kidney organoids. This platform improves cell differentiation, and phenotyping of organoids, therefore, it can help modulate toxicity and quantify disease phenotypes for safety and

efficacy prediction. To assess organ-specific toxicity, the researchers treated these organoids with cisplatin and they observed apoptosis and destruction to tubule organization. These results confirm the nephrotoxic side effects of cisplatin. The researchers also proved the potential of these organoids to model genetic disease by introducing mutations in polycystin-1 or polycystin-2 genes (loss of which causes polycystic kidney disease (PKD)) in kidney organoids. They observed the production of cysts from kidney tubules as in the disease. Finally, the authors conducted a small-scale screening to identify modifiers of PKD. They used eight factors that might modulate cell-microenvironment interactions. It was observed that blebbistatin, a specific inhibitor of non-muscle myosin II, induced a notable cyst formation increase (40% in PKD organoids). These unexpected findings suggest a possible involvement of myosin in cystogenesis promotion on PKD (38).

Personalized medicine

A proof-of-concept of personalized medicine using organoids was in testing drugs to treat cystic fibrosis (CF), a genetic disease caused by defects in the *CFTR* gene. In consequence, a faulty protein is generated, affecting ion channels of cells, leading to an accumulation of sticky and thick mucus in the lungs. Several papers showed the benefit of using rectum-derived organoids from patients with *CFTR* mutations to select efficacious treatments for patients and identify subjects who would benefit from CFTR-correcting treatment (39-41). As an example, the case for a CF patient from the Netherlands. The patient was in very bad shape, and his lung function was down to just 30%, however, because an unusual genetic defect caused his CF, he was not eligible for treatment with Kalydeco. This medication is highly effective but very expensive; for this reason, only patients with mutations that respond to this treatment and with proven efficacy are eligible. Due to the urgent need for treatment, the doctors grew organoids from a rectal biopsy from this patient. After treating organoids with Kalydeco, the ion channels opened, meaning that the drug was effective on this genetic defect. As a result, the patient received the treatment and he improved in only four hours. Finally, this patient was officially prescribed Kalydeco. This is one of the many examples of how the organoid system can be used for drug testing and personalized medicine and improve the health of the patients (41-44).

Regenerative medicine

The finite supply of tissue from healthy donors and the inherent complication of tissue rejection highlights the organoids system as a promising source of transplantable tissues and functional cell types for cell therapy in regenerative medicine. There are already reported experiments in animal models. The transplantation of colon organoids containing $Lgr5^{+}$stem cells into mice with acute colitis is an example. The authors observed a rapid integration of the donor cells into the mouse colon, leading to cover the area lacking epithelium (45). These results showed the potential use of organoids for regenerative medicine.

Biobanks

One characteristic of the organoids is that they can be derived not only from healthy tissue but also from diseased tissue. Taking this in mind, another applicability that has emerged consists of establishing living organoid biobanks. These are collections of organoid samples that have been characterized histologically and genetically. Besides, the diseased organoids are paired with normal organoids from a broad number of individuals. The biobanks are created for research purposes, as basic and clinical research and personalized medicine. Moreover, it pretends to encompass a wide range of population genetic variance to eventually make it easier to generate screening platforms (23, 46). With the exception for the biobank with intestinal organoids derived from CF patients (40), the majority are tumor organoid biobanks, including colon (47), glioblastoma (48), bladder (49), or breast (50) cancer, among others.

Cancer

Organoids have made their way into the field of cancer research, bridging the gap between 2D cancer cell lines culture and patient-derived tumor xenograft (PDTX) models. In contrast with 2D cell lines, organoids present genetic and phenotypical stability, conserving the tumor heterogeneity, and these can be expanded in long term. In addition, organoids offer the possibility to have normal tissue-derived control. On the other hand, when compared with PDTXs models, PDTXs are more expensive, time and resource-consuming, and may undergo mouse-specific tumor evolution (51, 52). These characteristics make the organoids useful for a broad application in cancer research; some of them will be illustrated below.
One of the main uses of organoids is the creation of living organoids biobanks (52). One of the possible applications of biobanks is drug screening; for example, in a paper from the

group of Meritxell Huch, they used six primary liver cancer (PLC) tumoroids (organoids derived from tumours) lines to assess their use to identify patient-specific sensitivities and as a platform to inform drug development. After observing substantial inhibition of tumoroid formation on three lines following inhibition of ERK1/2, they transplanted the tumoroids subcutaneously into NSG mice to further investigate. After injection of the drug (2-7 days), they observed a remarkable reduction of tumor growth. Then, the histological analysis revealed that tumor mass was necrotic and most of the cells were apoptotic. The results provided evidence of the potential therapeutic effect of ERK inhibition on PLC and the application of tumoroid for *in vitro* and *in vivo* drug testing (53).

Another example is the work made by Vlachogiannis et *al.* (54), where they use patient derived organoids of colorectal and esophageal metastatic disease as a predictor of treatment response in phase I/II clinical trials. The organoids were treated with the same therapeutics as the patients, and the results were compared with the response of the patients. They observed a 96% overlap in the mutational spectrum between the organoids and their biopsy from which they are derived. The researchers saw that the organoids with a genetic alteration, for example, *ERBB2* amplification, were the ones who responded better to specific therapeutics for this mutation, in this case, lapatinib. Besides, the differences between organoid derived from resistant and sensitive patients to regorafenib were identified. This study shows that organoids are useful to predict tumor-specific responses and their potential for personalized cancer treatment (54).

Another application of the organoids is genetic cancer modeling. For instance, Matano et *al*. (55), introduced multiple mutations into organoids derived from normal human intestinal epithelium using the CRISPR-Cas9 system. The mutated genes were the tumor suppressors *APC, SMAD4,* and *TP53* and the oncogenes *KRAS* and/or *PIK3CA*; these five genes are commonly mutated in colorectal cancer (CRC), and these are known as driver pathway mutations. They observed that the organoids carrying all five mutations could grow independently of niche factors, such as Wnt-3A, R-spondin-1, EGF, noggin, and A83-01 (TGF-β receptor inhibitor). In addition, they prove the capability of the organoids to form tumours after implantation under the kidney capsule in mice. Although the engineered organoids were able to form micrometastases, they were not able to form macrometastases colonies such as the organoids derived from chromosome-instable human adenomas did. On the other hand, macrometastases were also found when driver pathways mutations were combined with chromosomal instability. These results suggested that driver pathway mutations alone were not sufficient for malignant progression (55).

This work is an example of how genetic cancer modeling and organoids can be used for basic research to understand mutational processes underlying tumorigenesis.

BLADDER CANCER

Epidemiology

Bladder cancer (BC) is the 10th most common malignant disease and the 13th most common cancer death cause worldwide. The incidence increases with age, being more frequent between the age of 50 years to 70 years. In men, the incidence and mortality rates are 9.6 and 3.2 per 100,000, respectively, four times more than in women. This high incidence is also reflected in the disease ranks; in men, BC is the 6th most common cancer and 9th leading causes of death (56). If we look at the data in Spain, bladder cancer is the fourth most common cancer, the third in men and the seventh in women. These data place Spain as the second country with the highest incidence in BC of the world, below Greece.

The principal factor risk is tobacco smoking which accounts for 50% of all cases, and triples bladder cancer risk relative to never smoking (57). In addition to tobacco, chemical and environmental exposure to carcinogens such as paint, aniline-dyes, rubber, petroleum products, polycyclic aromatic hydrocarbons, chlorinated hydrocarbons, and arsenic drinking water are associated-risks too. Chronic infection with *Schistosoma spp.*, pelvic irradiation and chronic irritation could contribute to the risk of developing bladder cancer too(58-60). Albeit it does not occur in the majority of cases, there is some genetic predisposition to bladder cancer; polymorphisms in two genes involved in the metabolism of carcinogens, glutathione S-transferase-μ1 (*GSTM1*) and N-acetyltransferase 2 (*NAT2*). The slow acetylation of *NAT2* and the *GSTM1*-null genotype are related to increased risk to BC (59, 61).

Pathophysiology

The majority of bladder cancers (~90%) are originated from the urothelium, the epithelium that covers the inner surface of the bladder. The staging of the tumours is determined by using the Tumor-Node-Metastasis system (TNM), which describes the extent of invasion into the bladder wall (Tis-T4) (Figure1). Besides the TNM system, two classification systems exist to measure the grade of the tumor. The first one was created in 1973 by WHO, and classifies from grade 1 (the least degree of cellular anaplasia) to grade 3 (the most severe degree of cellular anaplasia) (62). On the other hand, the second system was created in 2004 by WHO and the International Society of Urological Pathology (ISUP), which classifies urothelial tumour into three categories: papillary urothelial neoplasm of low malignant potential (PUNLMP), low-grade carcinoma, and high-grade carcinoma (63).

Urothelial carcinomas are divided into two types, non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). NMIBC represents ~75% of the tumors, and has a relatively favorable prognosis. It can be sub-grouped into low-grade tumors (papillary tumors, stage Ta and T1) and high-grade tumors (carcinoma *in situ* (CIS)), which have a high risk of progression to muscle-invasive disease (58, 64). Importantly, 50-70% of these superficial tumours will recur, and around 10-20% will progress to invasive bladder cancer.

The remaining 25% of the tumors (stage T2 to T4) are classified as muscle-invasive and are associated to poor prognosis(58). Indeed, patients with MIBC have an approximate 5-year-survival of 50%, and if the tumor has metastasized, the patients have only a 5-year-survival of 15% (65).

NMIBC and MIBC differ histopathologically and in their molecular landscape. These differences are observed in the potential pathways of pathogenesis. Low-grade papillary tumors, can arise via simple hyperplasia and minimal dysplasia. These tumors frequently have loss-of-heterozygosity (LOH) of chromosome 9 (~50% of tumours), which affect the tumor suppressor gene *CDKN2A* (50-60%). There are also found point mutations on *TERT* (73-83%), activating mutations of *FGFR3* (60-70%), point mutations in *PIK3CA* (25%), and inactivating mutations in the cohesin complex tumour suppressor gene *STAG2* (32-36%) (Figure 2) (59, 61).

On the other hand, invasive carcinoma can arise via flat dysplasia and CIS. On these tumors are also frequent the LOH of chromosome 9, deletion and/or mutations in *TP53* (~50%) and alteration on genes encoding members of the p53–RB pathway (59, 64). The homozygous deletion and/or reduced expression of *PTEN* is also found in many cases (25-58%) (59). Low-grade papillary tumors can progress to the invasion of the muscle trough the loss of CDKN2A (50-60%) (Figure 2) (59, 66).

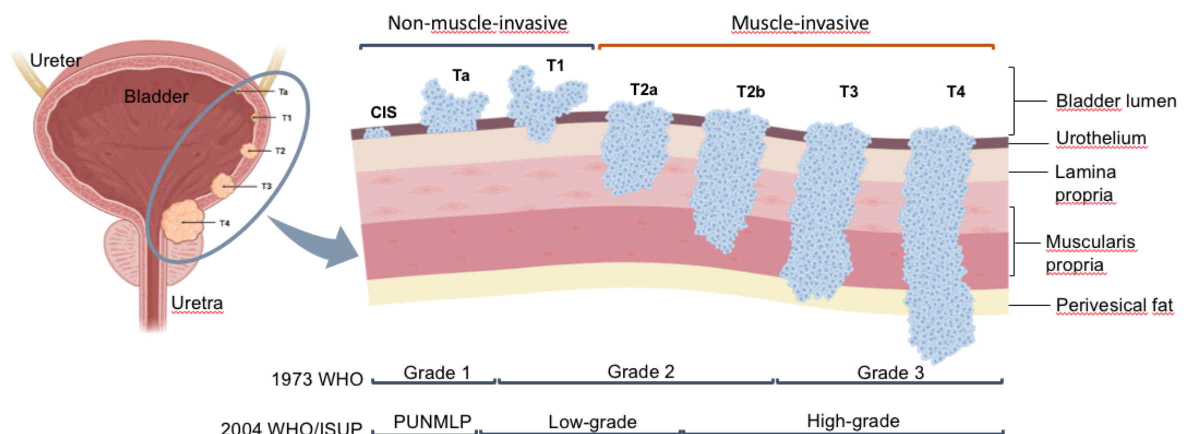

Figure 1. Types and stages of bladder cancer: Staging of bladder cancer according to the Tumour, Node, Metastasis (TNM) system is shown. Bladder cancer commonly originates from the urothelium. The Ta and T1 classification correspond to tumors confined to the mucosa or tumors that have invaded the lamina propria, respectively. CIS corresponds to the carcinoma in situ and is a flat, poorly differentiated tumor confined to the mucosa. T2a corresponds to tumors that invade the muscle superficially, and T2b invades the muscle deeply. When the invasion arrives into the perivesical fat, T3a corresponds to tumors with microscopic invasion and T3b with macroscopic. The T4 corresponds to metastatic disease, tumors that have invaded the prostate, uterus, vagina and/or bowel are T4a, whereas tumors that have invaded the pelvic or abdominal walls are T4b. The two histological classifications of bladder cancer are the created by the WHO on 1973 and the second one created by the WHO and ISUP in 2004 where the major difference is between low-grade and high-grade tumours (the greater propensity for invasion). Figure adapted from Sanli et *al.*[61]

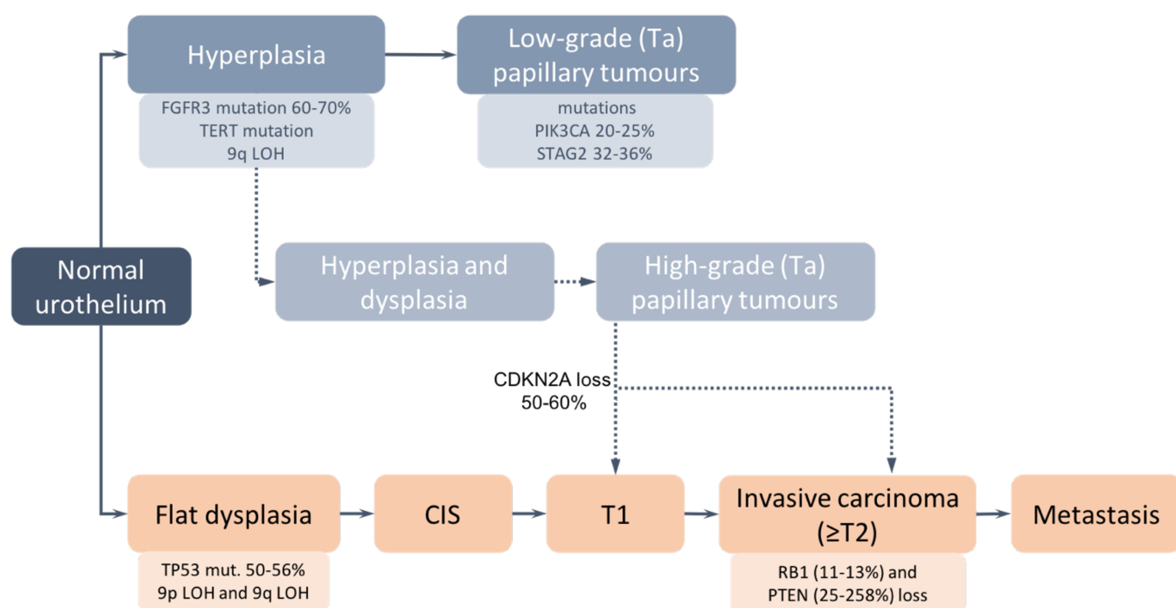

Figure 2. Possible pathways of urothelial carcinoma pathogenesis: The possible pathways are shown based on histopathological and molecular observations. The non-muscle invasive bladder cancer (NMIBC) arise via hyperplasia and flat dysplasia. These are characterized by loss-of-heterozygosity (LOH) of chromosome 9, mutations in *FGFR3, TERT, PIK3CA,* and *STATG2.* Muscle invasive bladder cancer (MIBC) may arise via flat dysplasia or carcinoma *in situ* (CIS) and usually presents mutations in *TP53* in addition to LOH of chromosome 9. There is a possible pathway where the high-grade papillary tumors can progress to MIBC through the loss of *CDKN2A.* Figure adapted from Knowles et *al.*[59]

Diagnosis and treatment

The most frequent symptom of bladder cancer is painless haematuria. The expert should also consider other symptoms such as urinary frequency, urgency, or irritation. These symptoms are more frequent in patients with carcinoma *in situ* (58, 65). Assessment of patients suspected to have bladder cancer is performed using voided urine cytology, cystoscopy, and radiographic imaging, CT, or RMI are the primary ones. Any abnormal finding such as erythematous patches, papillary or solid lesions needs a posterior histological evaluation. The histology sample is obtained from transurethral biopsy or resection of the entire area (58, 61, 65). After detecting an urothelial lesion, the following procedure is transurethral resection of bladder tumor (TURBT). This resection should include muscularis propria for an accurate diagnosis and clinical stage (58, 61). For NMIBC, the treatment will differ depending on the grade of the tumor (low, intermediate, or high). For low-grade tumors, the surgery may be followed by adjuvant intravesical therapy with a chemotherapy agent (for example, mitomycin or epirubicin), which prolongs the recurrence-free interval and early recurrences (67). This should be followed by periodic cystoscopy and voided urine cytology. The standard of care for high-grade tumors is the intravesical therapy with Bacillus Calmette–Guérin (BCG) (68) and lifetime surveillance. For MIBC and for NMIBC who failed intravesical therapy, the gold-standard therapy is the radical cystectomy. This procedure typically includes hysterectomy and partial resection of the vagina and urethra in women and prostatectomy in men. In addition to cystectomy, preoperative radiotherapy can be done, and neoadjuvant chemotherapy with a cisplatin-based combination (69). Finally, for metastatic diseases, the first-line treatment is chemotherapy with a cisplatin-based combination. For patients ineligible for cisplatin treatment, a carboplatin combination chemotherapy or single agents may be used (69).

Bladder cancer has an impact on the quality of life of patients since it affects sexual, urinary, and intestinal function. However, it also affects significantly the economy, having one of the highest lifetime per patient cost among all cancers. The elevation cost is due to the need for lifetime surveillance strategies and repeated treatment for recurrence disease (70, 71).

The need to have models that faithfully represent the biology of normal urothelium and bladder cancer make organoids a promising tool. These can genetically and phenotypically recapitulate the tissue of origin, thus allowing to elucidate the molecular roles of mutations in either NMIBC or MIBC. In addition, these can expand indefinitely and can be used to create bladder cancer biobanks. These characteristics make organoids useful for the discovery of new therapeutic targets and drug screenings to improve the treatments that already exist or to find new ones, and finally, decrease the risk of recurrence and progression, and improve the quality of life of bladder cancer patients.

6. AIMS

This dissertation aims to highlight the potential of organoids in bladder cancer research as models that can enhance the study of their biology and help find new treatments. For this, a fusion between the bibliographic review and the practical work performed will be carried out. To achieve the aims proposed the work has been developed in the following way:

1) A comprehensive introduction to the origin of organoids and their applications and bladder cancer.
2) Presentation of the protocol carried out to establish human bladder organoids and subsequent comparison with other bladder organoid protocols.
3) Propose new approaches such as biomaterials or niche factors to improve the bladder organoids protocol.
4) Highlight the use of organoids in co-cultures that better recapitulate the *in vivo* context.

7. MATERIAL AND METHODS

Human tissue

Human bladder tissue was obtained from patients undergoing TURBT at Hospital Universitario 12 de Octubre and Hospital Universitario La Paz from Madrid. Three patient-derived xenograft tumor tissue were obtained from Hospital Germans Trias i Pujol (Can Ruti) from Badalona.

Sample collection

During surgery, normal and tumor tissue samples were collected into a falcon containing Hepatocyte culture medium (Corning) supplemented with 5% charcoal-stripped fetal bovine serum (CS-FBS (Thermofisher Scientific), and 1X Glutamax (GIBCO)), placed on ice, and transported directly to the laboratory. Tissue samples were employed to establish primary organoid cultures and analysis of parental tumors.

Establishment and maintenance of urothelial organoids

Tissue dissociation

The normal and tumor tissues were washed in human organoid washing medium (Hepatocyte Medium with 5% CS-FBS and 1X Glutamax (Gibco)), and put in a petri dish to cut it in smaller pieces with a blade; a part of the tissue was put in a vial to frozen. Minced tissues were washed in 5mL of washing medium and centrifuge at 350g for 5min. After removal of the medium, tissues were then incubated in 1 mL of the organoid washing medium supplemented with 1:10 dilution of collagenase/hyaluronidase (StemCell Technologies) at 37 ºC for 15 min. In the meantime, a pre-heated 6-well plate was coated with 300µL of 100% Matrigel and it was put on the incubator for solidification. After the incubation time finish, minced tissues were resuspended in 5 mL of PBS, and centrifuged again. After removing the PBS, the tissues were resuspended in 1 mL of TrypLE Express (Gibco), followed by incubation at room temperature for 3 min and dissociated mechanically by pipetting. Then, 5 mL of Hank's balanced salt solution (HBSS; Life Technologies) supplemented with 5% CS-FBS, 10 mM of the ROCK inhibitor Y-27632 (Sigma-Aldrich) and 100 mg/ml Normocin was added to stop Trypsinization succeed by centrifugation at 350 g Dissociated tissues were resuspended with 5 mL of HBSS supplemented with 5% CS-FBS, 10 mM Y-27632 and 100 mg/ml Normocin, and filtered with a 100 mm cell strainer. Dissociated cell clusters were spun down and resuspended in 300µL of 100% Matrigel (Corning), and plated in the middle of one well of a pre-coated 6-well plate (Corning) with 100% Matrigel. After 30-minute incubation at 37 C and 5% CO_2 the drop was solidified. Next to solidification, was added to the well 2 mL of the organoid culture medium (hepatocyte media with 10 ng/ml EGF (Invitrogen), 5% CS-FBS, 10 mM Y-27632, and 1X Glutamax, supplemented with 100 mg/ml Normocin), and the medium was changed every 2-3 days.

When the cell pellet was too small, it was used a 24-well plate to minimize the distance between cells. The pre-coating and the drop with cells was done with 100μL of 100% Matrigel, and the volume of culture medium was 500μL.

Passaging

For maintenance, 500μg/ml dispase (Gibco) was added to the medium, succeeded by incubation for 30 min at 37ºC to digest the Matrigel. Afterward, organoids were collected from the plate to a 15 mL tube and washed with 5mL of PBS, and centrifuged at 350 g for 5 min. 1 mL TrypLE Express was added, and organoids were incubated at room temperature for 3 min, followed by mechanical dissociation to small cell clusters by pipetting. Organoids were passaged at a 1:2-3 dilution every 2–3 weeks. To generate stocks, organoids were frozen in 90% CS-FBS and 10% DMSO and stored in -80º freezer.

Table 1: List of organoid reagents

Reagents	Concentration	Source
Collagenase/Hyaluronidase	1:10	STEMCELL Technologies
Hank's Balanced Salt Solution	1x	Life Technologies
Hepatocyte medium	1x	Corning
HEPES	1x	Gibco
GlutaMAX	1x	Gibco
Matrigel, growth factor-reduced and phenol red-free	1x	Corning
Dispase II	40mg/mL	Gibco
Recombinant human EGF	10ng/mL	Invitrogen
Normocin	100mg/mL	
Y-27632	10µM	Sigma-Aldrich
Heat-inactivated,charcoal-stripped FBS	5%	Thermofisher Scientific
TryplE Express, no phenol-red	1x	Gibco

Software

GraphPad Prism 8 version 8.4.3 (471) was used to perform the statistical analysis.

8. RESULTS

The total number of samples received were 47, 44 from patients after surgery (22 from normal tissue, 22 from tumoral tissue), and 3 samples from PDTXs. Of all samples, 68% (n=17) came from men and 20% (n=5) from women (Figure 3a) being representative of bladder cancer incidence. The remaining 12% were unknown. The age of the patients was between 60-90 years for males, being the median age 78 years, and for women, the median age was 62 years (Figure 3b). The samples were divided according to the tumor grade established by the pathologist. Of the 25 samples, 14 were low-grade tumors (9= G1pTa and 5=G2pTa) and 11 were high-grade tumors (2= G3pTa, 4=G3pT1 and 3= G3pT2) (Figure 3c).

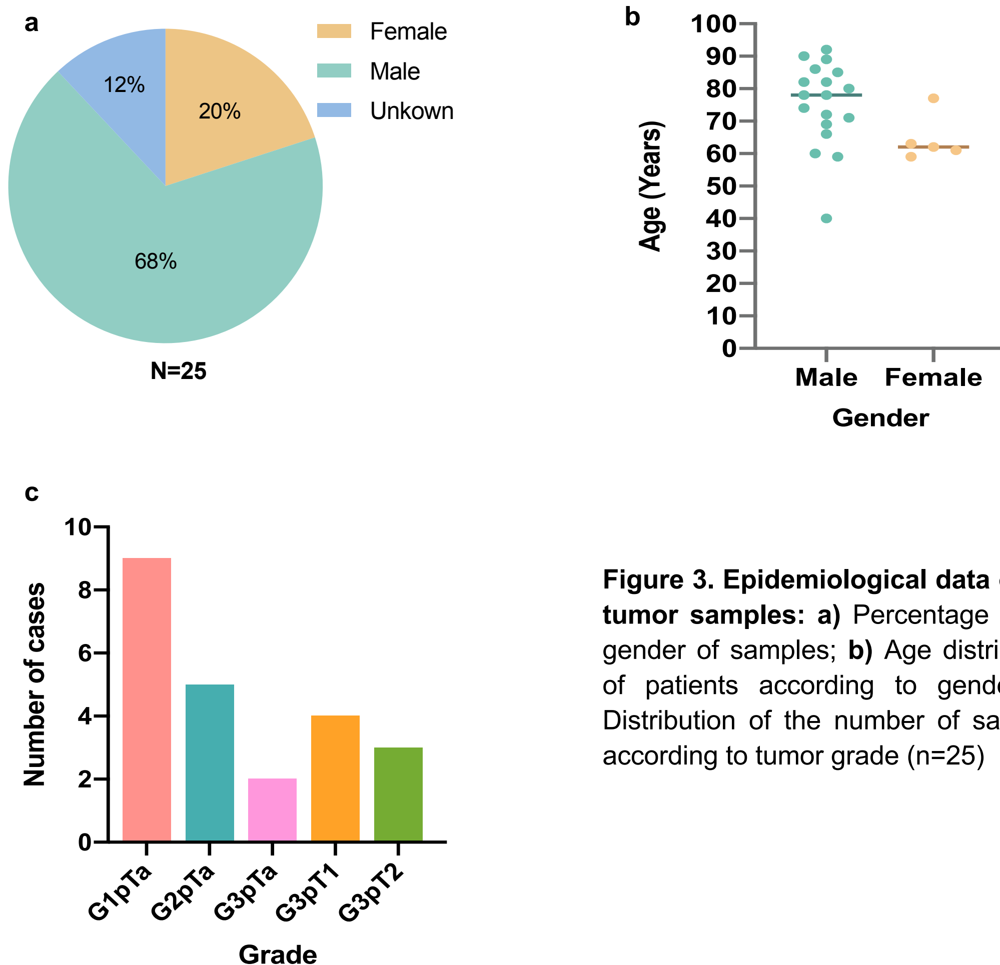

Figure 3. Epidemiological data of the tumor samples: a) Percentage of the gender of samples; **b)** Age distribution of patients according to gender; **c)** Distribution of the number of samples according to tumor grade (n=25)

From the 47 samples, 32% (n=15) formed organoid structures (11 from tumor tissue, 3 from PDTXs and 1 from normal tissue), but only five samples established organoid lines (3 from tumor tissue, 1 from a PDTX and 1 from normal tissue), which corresponds to 10.64%

(Figure 4a); we considered a line as established when it has been serially passaged at least six times. Among the samples that formed organoids structures, 33.33% (n=5) established an organoid line (Figure 4b). 46.67% (n=7) were only able to grow until the passage 1, 6.67% (n=1) grew until passage 3, and 13.33% (n=2) were able to grow until passage 4. Figure 5a shows the number of tumor samples that formed organoid structures according to the grade of the tumor. The grade that led to more organoid formation was G2pTa with 5 samples, then G1pTa and G3pT1 (2 samples from PDTX) with 4 samples each, and finally, G3pT2 with 1 sample from a PDTX. Lastly, 3 of the established organoids cultures came from G2pTa tumors and 1 from G3pT2 tumor from a PDTX (Figure 5b). These data suggest that samples from tumor tissue have a greater capacity to generate organoids than samples from normal tissue. However, this can also be due to the tumor samples being larger than normal samples, leading to more cellular content to form organoids. In addition, we can observe that the formation of organoid structures occurs in practically all tumor grades. Nevertheless, in our case, the established organoid cultures were mostly G2pTa. Although, our sample number was too small to draw conclusions about which degree is more favorable to establish organoids.

In figure 6 can be observed four of the five established organoid cultures (3 from tumor tissue, 1 from a PDTX and 1 from normal tissue). The organoid culture TPAZ24 was established from tumor tissue and reached passage 12. ID84T was established from tumor tissue, and it is the only with a paired organoid culture derived from normal tissue, ID84N; both cultures reached passage 11. ID96T was established from tumor tissue and reached passage 6. TRUTI11 was derived from tumor tissue from a PDX and reached passage 6. Aside from TRUTI11 that came from a G3pT2 grade tumor, the other organoid cultures were derived from G2pTa grade tumors. As observed in figure 6, TPAZ24, ID84T, and ID84N are proliferative organoids because of the lack of a lumen. The opposite is observed on TRUTI11 organoids, forming a lumen with the epithelial cells around it. Usually, established normal urothelial organoids present a proliferative phenotype and switch to a differentiated phenotype upon growth factor removal from culture medium (72). However, TRUTI11 spontaneously grew presenting a differentiated phenotype even in the presence of growth factors in the medium. This fact may be due to the greater aggressiveness of the tumor from which it comes or because it comes from a PDTX.

These results show that our efficiency rate on establishing organoids is low, being only 11%. Moreover, histological and molecular analyses have to be done to characterize the established organoids and verify whether they faithfully represent the tissue of origin.

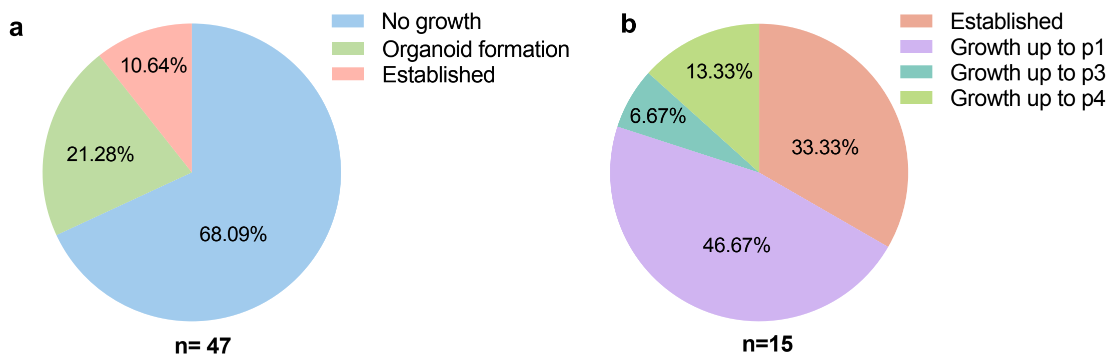

Figure 4. Outcomes of the establishment of organoids: a) Distribution of the samples according to the results obtained after the protocol of establishment of organoids; **b)** Distribution of the sample that formed organoids according to the outcome.

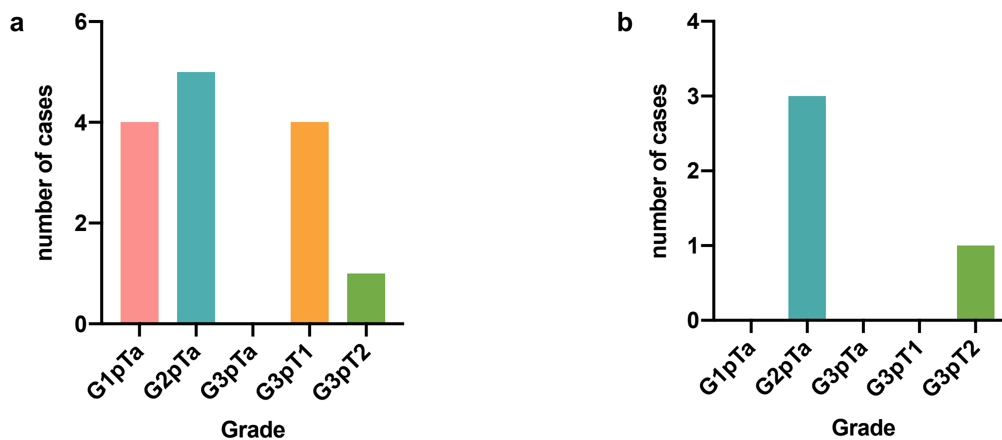

Figure 5. Distribution of the samples that formed organoids: a) Number of tumor samples that have generated organoids according to tumor grade (n=14). On G3pT1 2 samples came from PDTX and the G3pT2 sample came from a PDTX; **b)** Number of established organoid cultures according to tumor grade (n=4). The G3pT2 sample came from a PDTX.

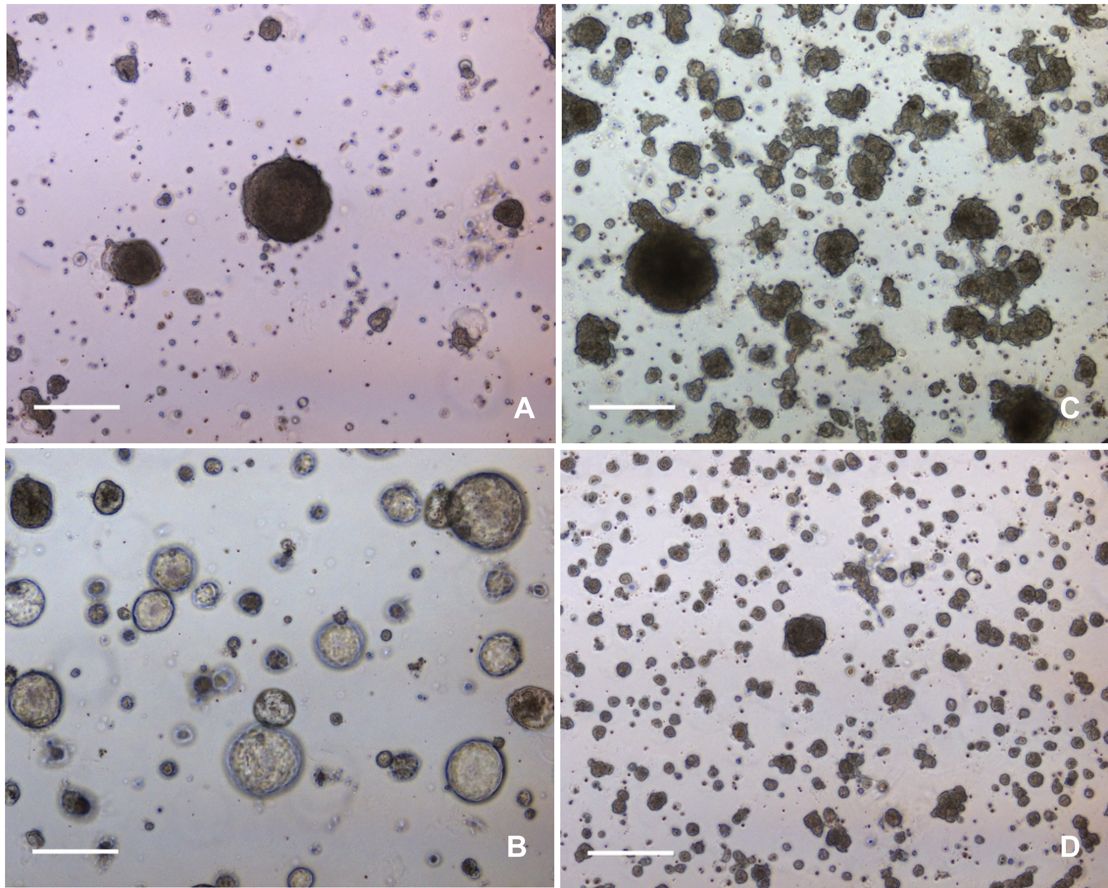

Figure 6. **Images of four established organoid cultures**: A) **TPAZ24** on passage10: Organoids derived from tumor tissue. B) **TRUTI11** on passage 6: Organoids derived from PDX. C) **ID84T** on passage 9: Organoids derived from tumor tissue. D) **ID84N** on passage 9: Organoids derived from normal tissue paired with ID84T. (Scale bar 500μm)

9. COMPARISON WITH OTHER PROTOCOLS

The protocol to establish bladder organoids was adapted from the work published by Shen et *al.* (49). Despite the similarity in the process, our efficiency rates are lower. They obtained an efficiency in establishing organoid lines around 70% and successfully passage them up to 26 passages. Through hematoxylin-eosin staining, the strong concordance between organoid lines and their parental tumors was verified. Moreover, 31% of the organoid lines are derived from female patients, concordant with the three-fold lower incidence of bladder cancer in women relative to men. Their organoid lines also recapitulate the mutational spectrum of human bladder cancer supporting the genomic representativeness of the models. These results show that the established organoid lines from Shen and colleagues are representative of bladder cancer by maintaining the phenotypically and molecular characteristics, and also being used to determine drug response (49).

One of the possible explanations is also one of the most significant drawbacks of the sample size. In general, the sample that reaches the laboratory from the surgery is usually tiny, between 2-5mm; instead, the group of Shen receives samples with a minimum of 7mm— thus limiting the amount of cellular content extracted after the enzymatical digestion. We have tried to overcome this drawback by growing the organoids in smaller wells so that the cells are closer to each other and thus stimulate their growth. Besides this, it is worth comparing the protocol used with other protocols of bladder organoids to compare which steps can be modified to increase efficiency.

When compared with the protocol used by Mullenders et *al*. (73), some differences are observed on the tissue dissociation process. After cutting the tissue into small pieces it is digested only with collagenase type IA in Advanced DMEM/F-12 with ROCK inhibitor (Y-27632, 10 μM) for 30 min at 37°C. After the incubation step, the tissue is filtered through a 70μm strainer and incubated again with the same method. For plate the cells, they used Cultrex as a matrix and a 24-well plate without pre-coating the wells. The culture medium is also different, they used Advanced DMEM/F-12, FGF10 (100 ng/mL), FGF7 (25 ng/mL), FGF2 (12.5 ng/mL), B27 (2%), A83-01 (5 μM), N-acetylcysteine (1.25 mM), and nicotinamide (10 mM).
From 133 tissue samples, they could establish 77 organoid lines, which implies a rate of around 50% efficiency. The organoid lines could be passaged for a long period of time (more than a year). More than 75% of the samples received came from radical cystectomy, which implies a superior amount of tissue to which we obtain for our procedures; thus, it could influence the success rate due to organoid-forming cells more significant presence. Furthermore, they verified by immunohistochemistry the urothelial origin of the organoids

and observed heterogeneity between samples (more luminal or basal types), thus representing the characteristic heterogeneity of bladder cancer (73).

Another paper to compare with is from Pauli et *al*. (74). In their protocol the enzymatic digestion after the mechanical dissociation was done with collagenase IV in combination with one third of 0.05% Trypsin-EDTA in a volume of at least 20 times the tissue volume. The incubation was made on a shaker at 200rpm at 37ºC of temperature. To inactive the digestion 10% FBS was added enriched DMEM and a posterior centrifugation at an average of 1,000 rpm for 5 minutes to pellet cells. The cell pellet was washed with 10% FBS-enriched DMEM and to eliminate residual FBS it was washed again with unsupplemented DMEM. The cells were resuspended to posterior plate them in a mix composed by 1:2 volume of growth factor–reduced Matrigel and a small volume of tissue-type specific primary culture medium (Advanced DMEM with 1x Glutamax, B27, 100 U/mL penicillin, 100 μg/mL streptomycin, Primocin 100 μg/mL, N-Acetylcysteine, EGF, FGF10, FGF-basic, Y-27632, A8301, SB202190, Nicotinamide, PGE2, Noggin and R-spondin). Finally, the cell suspension was plated in a 6-well plate forming about ten 50 to 80 μl drops. Once the drops are solidified, 4mL of culture medium was added.
The efficiency rate was around 33%, and the established organoid lines were concordant with the parental tumor.

The group of Usui et *al.* (75) designed a culture system that differs from the protocols mentioned above because the organoids do not derive directly from tissue biopsies, and the cells are from dog. The method used consist on establish dog bladder organoids from cancer stem cells present on the urine. First, the urine samples were centrifuge at 600g for 3min. Second, the pellets were washed with cold HEPES buffered saline and centrifuge again. Then the pellets were mixed with Matrigel on ice and plated on 24-well plates. After the solidification of the gel, the culture medium (Advanced DMEM with 50% Wnt, Noggin and R-Spondin conditioned medium; GlutaMax; B-27 supplement; 100μg/mL Primocin; 1mM N-Acetyl-l-cysteine; 10mM nicotinamide 50ng/mL mouse EGF; 500nM A83-01 3μM SB202190; and 10μM Y-27632) was added (76). From 17 urine samples, they established successfully 12 organoid lines, which implies a ~70% efficiency. The researchers proved the histological similitude between the organoids and their parental tumor. Urothelial markers were also expressed on the organoids, suggesting that the urine-sample-derived organoids from dogs with bladder cancer can recapitulate the bladder cancer characteristics present on the dogs (75). This protocol presents a novel and different straightforward approach to establishing organoid lines without a tissue biopsy and less time-consuming.

Table 2 summarizes the culture medium used in the protocols mentioned above. As observed on it, the culture mediums differ between them, being the culture medium from Shen et al. paper the more different. Even though the efficiency rate is high in their paper, the lack of growth factors present on the others culture medium could be one of the points to change to improve the establishment of bladder organoids. On the other hand, the other three culture media are quite similar, and in their works, they obtained reasonable efficiency rates, which indicates that it is worth trying each of them to see if the efficiency increases.

Table 2: Summary of the culture medium reagents in the papers

Shen	Mullenders	Pauli	Usui
Hepatocyte Medium with 5% CS-FBS	Advanced DMEM/F-12	Advanced DMEM with 1x Glutamax	Advanced DMEM with 50% Wnt
1x Glutamax	FGF10	EGF	1x Glutamax
EGF	FGF7	FGF10	EGF
Y-27632	FGF2	FGF-basic	B27
Normocin	B27	B27	A83-01
	A83-01	A83-01	SB202190
	N-acetylcysteine	SB202190	Y-27632
	Nicotinamide	Y-27632	Nicotinamide
		Nicotinamide	Noggin
		PGE2	R-spondin
		Noggin	N-acetylcysteine
		R-spondin	Primocin
		N-acetylcysteine	
		Penicillin, Streptomycin, Primocin	

10. NEW APPROACHES TO IMPROVE THE PROTOCOL

Basement membrane

One of the main features of the establishment of organoid cultures is its embedment into a 3D matrix. This matrix simulates the scaffolding support given by the native extracellular matrix of the tissue and it allows the free growth of the cells and the remodeling of the microenvironment. The majority of the organoid protocols use animal-derived matrices such as Matrigel, Geltrex, or Cultrex BME. These matrices are EHS matrix, a reconstituted decellularized basement membrane from mouse sarcoma. The EHS matrix is a heterogeneous blend of ECM proteins, growth factors, and proteoglycans that provide an elaborated environment for embedded cells. Another characteristic is the capability to be degraded and remodeled by the enzymes that the organoids express, and the adhesive domains of the matrix encourage the cell attachment (77, 78).

Despite the benefits of Matrigel and that it is the most used matrix for organoid culture, Matrigel also has some drawbacks. It suffers from batch-to-batch compositional and structural variability; indeed, only 53% of the mixture of proteins is consistently in each lot (77). Moreover, the cell cultures can be affected by the complex component mixture. For instance, EHS matrix carries growth-factor-binding proteins, which could drive heterogeneous cellular response and growth-factor sequestration (77, 79). Additionally, the biochemical and biophysical components cannot be adjusted to meet the diverse requirements of unique organoid niches. Finally, the mouse tumor origin makes the EHS matrices unsuitable for clinical applications posing risks of immunogen and pathogen transfer(80).

In order to overcome these limitations and to no be dependent on EHS matrices, engineered matrices derived from natural, synthetic, or hybrid molecules have been appearing on the organoid field as an alternative (81, 82). These matrices, called hydrogels, provide the possibility of tuning biochemical and biophysical parameters that impact on cell culture such as cell-adhesive ligand presentation, stiffness, stress, matrix geometry, and matrix degradation and remodeling (79, 83). The opportunity to control the characteristics make the hydrogel a more reproducible, completely chemically defined and suitable for clinal translation (79, 81, 82).

An example of a natural biopolymer hydrogel is the fibrin-laminin matrix, developed by Broguiere et *al*. (84). In this matrix, the fibrin is the base of the hydrogel, providing appropriate physical support giving stiffness and containing RGD domains required for integrins of cells bind to the matrix. Laminin-111 plays a role as a signaling molecule and on organoid growth and colony formation. The researchers established, grew, and expanded long-term intestinal organoids derived from murine Lgr5+ stem cells. The

organoids maintained their typical architecture, with crypt-like structures pointing outwards from the central lumen and contained Lgr5+ stem cells, Paneth, goblet, and enteroendocrine cells. They also tested the applicability of hydrogels on human organoid lines derived from the liver, small intestinal epithelium, pancreas, and pancreatic ductal adenocarcinoma. The authors designed a natural and effective alternative to Matrigel (84).

On the other hand, the hydrogel designed by Cruz-Acuña et a*l.* (85) is an example of a synthetic polymer matrix. In this work, the researchers developed a hydrogel based on a four-arm poly (ethylene glycol) (PEG) macromer with maleimide groups at each terminus (PEG-4MAL). They used human intestinal organoids derived from ESC to prove the viability of their matrix. The gels with a density from 3.5% to 4.0% showed the most significant viability and growth than gels with greater density, which decreased organoid culture viability. Moreover, for better survival and development of the organoids, RGD adhesive peptide was added to the gels. They also showed that the PEG-4MAL hydrogel allows the formation, growth, and differentiation of the organoids with similar rates as in Matrigel. Nevertheless, the organoids first require to be formed as spheroids in Matrigel. Besides, the researchers went a step further and used the hydrogel as an intestinal organoid delivery vehicle to heal colonic wounds. They generated intestinal organoids into PEG-4MAL hydrogel and mixed with the hydrogel precursor solution. Then the organoids were injected in lesions in the distal colon of immunocompromised mice. They observed a notable increase in wound closure contrasted with untreated wounds, wounds treated with hydrogel alone, or organoids without the hydrogel carrier. These results highlight the utility of the PEG-4MAL hydrogel as an injectable delivery vehicle that supports localized intestinal organoids engraftment in the colon and posterior wound closure. Lastly, to test whether the PEG-4MAL hydrogel was suitable for other human organoids, they embedded human lung organoids into the gel. After seven days, the hydrogel maintained high viability, and the organoids presented an organized epithelium, lumen formation, and airway basal cells. These results showed that PEG-4MAL hydrogel was suitable to generate human organoids derived from spheroids from different tissues (85).

Another example of a synthetic matrix is designed by Ranga et *al.*(86) to explore the role of the ECM in the neural tube patterning and focus on the relationship between apical-basal polarity and dorsoventral patterning. They worked with a PEG-based engineered hydrogel platform formed of a library of molecular building blocks. This synthetic matrix could be personalized by altering the physical properties such as the polymer content and the peptide domain linked to the PEG, to be more or less sensitive to metalloproteinases that degrade the matrix; and the biochemical properties adding bioactive elements involved in

morphogenesis such as collagen IV, entactin, laminin-111, and perlecan. Fibronectin, collagen I, and the growth factor bFGF were added. Then, they combined these elements with a factorial method based on the automatic liquid handling of hydrogel precursors. The researchers observed that the hydrogel with an intermediate stiffness (2-4kPa), with laminin and non-degradable, promotes apicobasal polarity and dorsoventral patterning, and in consequence, generation of neural tubes. Moreover, compared with EHS matrices, ESC cultured in PEG matrices formed more homogenous cultures of polarized neuroepithelial colonies. This high-throughput platform, particularly the manipulation of matrix properties, allowed the authors to observe that patterning events in neurogenesis such as apicobasal polarity and posterior dorso-ventral symmetry are mediated by the shape of the organoids (86).

Another interesting culture method is the air-liquid interface (ALI), a double-dish culture system. The inner plate contains a permeable membrane or nitrocellulose at the bottom and collagen type-I layer on top. The cells will then be introduced into the collagen solution and poured into the inner dish above the bottom layer. The inner dish will be placed in an outer dish. After the solidification of the collagen, the culture media will be added into the outer dish without covering the cells of the inner dish (87, 88). This culture system was used by Li et *al. (89)* to establish intestine, stomach and pancreas organoid from neonatal or adult mouse. They observed a multilineage differentiation and sustained growth for more than sixteen days. In addition, they growth the organoids without addition of exogenous growth factors. DiMarco et *al.* (90) also used this technique to establish intestinal organoids. They observed that use an ALI culture rather than a conventional submerged culture enhances oxygen transport and drives to enhanced organoid maturation, as measured by spontaneous contractility. They also showed that organoid contractility depends on material density and geometry in collagen gel, with a tight optimal range of approximately 20% porosity and 30 Pa stiffness (90).

The hydrogels are presented as an alternative to overcome the drawbacks of EHS matrices, such as the batch-to-batch variability or the unknown chemical composition. The hydrogels can be derived from natural or synthetic polymers; it is possible to add cell-binding domains and bioactive molecules to improve the establishment of organoids, growth, and differentiation. The complete knowledge of the gel composition enables reproducibility and the possibility to adapt the biophysical and biochemical properties to improve the organoid viability. Moreover, the hydrogels permit relating the interaction between the ECM components and composition with microenvironment and cell fate.

Niche factors

The culture medium to establish, grow, and maintain organoids lines is characterized by contain bioactive molecules that promote stem cell expansion and maintenance and cell proliferation. These molecules typically are present in the cell microenvironment; for example, Wnt, R-spondin-1, Noggin, and EGF are used in the culture medium for mouse bladder organoids described by Santos et al. (72). The addition of these molecules is an interesting approach to be explored to improve bladder organoids establishment.

Notch inhibitor

The Notch signaling pathway is an extremely conserved interface of receptors, ligands, and effector proteins that interfere with the crosstalk between neighboring cells and control basic cellular processes such as proliferation, differentiation, and apoptosis (91-93). Notch is a single-pass transmembrane receptor protein with an extracellular domain (NECD) containing EGF repeats, the transmembrane domain, and an intercellular domain (NICD) containing the RAM23 domain to interact with nuclear transcription factors. The Notch pathway is activated when the Delta/Serrate/Jagged family of ligands bind to the EGF domain leading to the cleavage of the NECD by the ADAM10/Kuzbanian metalloprotease and the cleavage of the NICD by the γ-secretase enzymes. The second cleavage leads to the release of the NICD and posterior translocation to the nucleus. At the nucleus, the RAM domain of the NICD binds to the DNA-binding transcription factor CSL (CBF1/RBPjk) and the Mastermind co-activator, which triggers the activation of Notch target genes (94-96). In the paper by Santos et *al.* (72), it was observed how mouse bladder organoids were more proliferative rather than differentiated when the Notch pathway was inhibited with the γ-secretase inhibitor DBZ. This change on the organoids morphology, no lumen formation in differentiating organoids, was accompanied with a decreased expression of the Notch target HES1 at the mRNA and protein levels. Furthermore, mRNA expression of basal markers suffered a moderate increase, an upregulation of *TP63*, and a notable decrease of the expression of luminal markers. These results support the urothelial differentiation role of the Notch signaling. Because the Notch and Wnt pathway are tightly associated, these results may be due to crosstalk between them (95, 97). On the one hand, the decrease of Notch signaling can lead to an increase of unphosphorylated (active) β-catenin and posterior translocation to the nucleus, bind to transcription factors and consequently activate Wnt target genes (94, 98). On the other hand, the decrease of Notch signaling can trigger an increased activation of the Wnt pathway (95, 97). These processes will increase the implication of the Wnt pathway on maintaining and stimulate stem cells (99). The

addition of DBZ to the culture medium could be a stimulus for the organoid forming cells by inhibiting their differentiation and boosting the proliferation of stem cells.

CHIR99021

The Wnt/β-catenin pathway is a conserved signaling pathway that controls the cytosolic and nuclear levels of β-catenin. In the lack of Wnt ligands, β-catenin molecules are present in the cytosol bounded to a destruction complex constituted by the Axin and adenomatous polyposis coli (APC) proteins, the glycogen synthase kinase-3β (GSK3β), and the Casein kinase 1 (CK1). This complex phosphorylates β-catenin, allowing its subsequent proteasomal degradation and maintaining the Wnt transcription factors repressed. Moreover, the pathway is initiated when Wnt binds to the receptor complex, constituted by Frizzled and LRP5/6 proteins. This binding activates the cytoplasmatic protein Dishevelled, which inhibits the degradation complex allowing the stabilization of the β-catenin. The active (unphosphorylated) β-catenin translocates to the nucleus, and it binds to the TCF/lymphoid enhancer factor (LEF) transcription factors family and activates the Wnt target genes (94, 95). CHIR is a Wnt/β-catenin pathway activator by inhibiting GSK3 and subsequent phosphorylation and degradation of β-catenin, which accumulates in the nucleus and activate Wnt-responsive genes (99). The canonical Wnt/β-catenin pathway has significant roles in regulating cell proliferation, survival, migration and polarity, cell fate specification, and self-renewal in stem cells.(99, 100). In a paper by Yoshida et *al.*(101) CHIR99021 was used to assess the effects of Wnt/β-catenin pathway activation in bladder organoids. They observed an activation of the pathway by translocation of β-catenin into nuclei and upregulation of AXIN2 expression; in consequence, an increase of proliferation in primary bladder cancer cells in organoid culture. Besides, they observed a reduction in cytokeratin 20 (CK20) over CHIR-induced proliferation in bladder cancer organoids. CK20 is a marker for terminal differentiation and is expressed in more luminal cells. These data suggest that CHIR would spur the proliferation of basal-like cancer cells within an organoid, leading to less expression of CK20 or the possibility that luminal-like cancer cells transform into basal-like (expressing less CK20) as an effect of CHIR. In a paper by Yin et *al.* (102), they establish mice intestinal organoid cultures from small-intestinal crypts. The authors verified that the culture medium containing EGF, Noggin, and R-spondin was not enough to sustain the capacity of Lgr5+ stem cells to self-renew when losing their contact with Paneth cells. After the addition of CHIR, there was an increase of Lgr5+ stem cell numbers, and the size of the organoids was larger than the organoids without CHIR; these results indicate a promoted proliferation of crypt cells. The authors then combined CHIR with Valproic acid (a

histone deacetylase (HDAC) inhibitor) related to Notch activation. Combining these two molecules results in a more significant increase in the stem cell numbers and a raised self-renewal of stem cells.

The capacity of CHIR99021 to activate Wnt/β-catenin pathway and consequently stimulate stem cell renewal and proliferation makes this molecule a candidate to improve the establishment of bladder organoids.

Heregulin

Heregulin (HRG) or also known as Neuregulin (NRG), is a ligand for human epidermal growth factor receptors 3 (HER3) and 4 (103, 104). The ligand-binding leads to receptor dimerization and activation of tyrosine kinases and c-terminal tail phosphorylation of tyrosines. This leads to the recruitment of adaptor proteins that trigger activation of downstream PI3K/AKT, MAPK, and JAK/STAT pathways, impacting on several biological factors such as cell proliferation, growth, and differentiation (103, 105). In a paper by Okuyama et *al.* (106) used HRG to promote human urothelial cancer organoid growth. They observed growth improvement in 4 of the 7 patient-derived organoids that used Heregulin, which implies a promoted growth in more than 50% of the organoids. Because the richest source of HRG is the mesenchymal cells (107), such as fibroblasts, add HRG to the culture medium could be a proliferation stimulus that supplies the lack of stromal cells. In a paper by Jardé et *al.* (108), in an attempt to improve long-term maintenance of both proliferation and functional differentiation of mammary organoids, they supplemented the culture medium with Neuregulin-1(Nrg1) and Noggin. After 15 days, the organoids treated with Nrg1 compared with the organoids treated with EGF and Noggin presented a threefold increase in cell viability and looked larger, an indicator of promotion in mammary development. After 30 days in culture, the organoids were able to form complex lobular mammary structures. Due to the important role of the Wnt pathway on mammary gland development, the researchers decided to add to culture medium R-spondin (Rspo) to potentiate Wnt signaling. The mammary organoids cultured with Rspo and Ngr1 could recapitulate the murine mammary duct differentiation since they contained distinct basal and luminal compartments and progenitor cells that allows the expansion of organoids for multiple passages over two and a half months. In this work the Neuregulin or Heregulin, improved the maintenance, expansion and differentiation of mammary organoids. Another paper of Jardé et *al.* (109) showed the role of NRG1 to sustain efficient regeneration, proliferation, and differentiation on small intestinal organoids. They observed that the organoids were longer and contained buds composed of several proliferating cells when

cultured with NRG1 and without EGF. They also observed an increase in the organoid growth by 4-fold compared to organoids cultured with EGF. NRG1 was more potent than EGF in inducing phosphorylation of both HER2 and HER3 receptors. This correlates with the increased stimulation by NRG1 of PI3K-AKT and MAPK signaling in comparison with EGF.

Heregulin or Neuregulin-1 seems to have a role in maintaining the proliferation and differentiation of organoid cells by activating the PI3K / AKT, MAPK, and JAK / STAT pathways, which makes this molecule a potential candidate to stimulate the establishment of organoids in the bladder.

11. DISCUSSION AND FUTURE PERSPECTIVES

The organoid culture models have arrived to bridge the gap between the 2D cell culture and the animal model or PDX. Organoids can be applied in basic research to study the basic biology of the tissue and the diseases, organogenesis, and interaction cell-microenvironment. It can also be used in clinical research in high-throughput drug screenings, personalized medicine, and regenerative medicine. Due to its benefits, it is not surprising that in bladder cancer research also profits from organoid culture, as illustrated by the two main papers by Mullenders et *al.* (73) and Shen et *al.* (49) in the bladder cancer organoid field. They successfully established normal and tumoral bladder organoids, maintained for an extended period, and generated biobanks. They also verify that the organoid lines were representative of the bladder cancer population and showed how organoids can be used on drug testing to evaluate drug response and toxicity.

As shown in the results, although our protocol is adapted from the article by Shen and colleagues. Our efficiency rate was only 11%, far from the 70% efficiency they achieved. One of the main differences between both studies is the size of the sample. While Shen and colleagues received samples with a minimum of 7 mm, we usually received samples between 2 and 5 mm. The size of the sample can affect the amount of cellular content placed on the plate and, consequently, the growth of the organoid structure. When our protocol was compared with other protocols, enzymatic digestion and culture medium were the most significant differences. This leads us to consider trying other protocols to evaluate whether we can improve the percentage of established organoid lines. Furthermore, other approaches, such as basement membranes and growth factors, can also be addressed.
As mentioned above, one of the possibilities that may help to promote the establishment of organoids is to change the commonly used EHS matrices as a base membrane for engineered membranes, the hydrogels. The hydrogels can be derived either from natural biopolymers or from synthetic polymers. The biophysical and biochemical properties can be modified and personalized according to the cell type and cell needs. Moreover, the engineered hydrogels allow the study of the interaction between the cell and the extracellular matrix components by adding and subtracting bioactive elements present on the natural matrices and observing the cell/organoid response to the changes.
Another option to enhance it is the addition of molecules on the culture medium that promotes the expansion of stem cells, proliferation, and differentiation. For example, the inhibitor of the Notch pathway (DBZ); the activation of the canonical Wnt/β-catenin pathway with Wnt agonist CHIR99021; or the activation of the downstream pathway of HER3 with Heregulin.

In the present work, I have exposed the relevance of organoid cultures as well as I have compared the protocol used with similar protocols and addressed new approaches to improve the establishment of bladder organoids.

Co-cultures: new approaches to organoid cultures

Once a significant efficiency rate is achieved, the organoids are stable to be passed for long periods and faithfully represent the tissue of origin; it is necessary to move towards more complex constructions that combine several cell types in order to enhance the potential of this technique to better recapitulate the in vivo context; this is the case of co-cultures. One of the applicability of the co-culture is to model the diversity and physical architecture of the tumor microenvironment as in the paper by Neal et *al.* (110). They cultured patient-derived organoids (PDO) from tumor samples in an air-liquid interface. Because of the lack of enzymatic digestion of the samples, the ALI organoids preserve integrated stromal cancer-associated fibroblasts (CAFs). Besides, the ALI PDOs contained immune stroma with tumor-infiltrating lymphocytes (TILs), B-cells, T-cells, macrophages, natural killer, and natural killer T cells. The authors verified that TILs exhibit activation, expansion, and cytotoxicity responses to PD-1/PD-L1 checkpoint blockade. This organoid methodology allows the study of the tumor microenvironment because of the conservation of the stromal elements, and could be used to study *in vitro* immunotherapies/immune checkpoints (110). Other articles such as Takebe et *al.* (111) have shown the usage of the coculture in regenerative medicine by creating human induced pluripotent stem cell (iPSC) derived liver buds (iPSC-LB). The iPSC-LB were obtained by culturing human iPSC hepatic endoderm cells (iPSC-HEs) with human umbilical vein endothelial cells (HUVEC) and human mesenchymal stem cells (MSC). The iPSC-HE self-organized into a 3D structure and the HUVECs formed an endothelial network between cells. After the transplant of the iPSC-LBs into mice, the authors observed the generation of a complete functional and vascularized liver by connecting to host vessels. Besides, iPSC-LBs were competent to perform specific liver functions, such as protein production and drug metabolism specific to human cells. Finally, they transplanted liver buds in the mesentery and verified their ability to rescue the model of lethal drug-induced liver failure, thus holding a grand promise for regenerative medicine.

Organoid co-culture method can also be used to improve the establishment of organoid cultures as shown in the article by Zhao et *al.* (112), where they generated, in parallel, organoids and CAFs from patients with oral squamous cell carcinoma (COCE). After the generation of both cell lines, the organoids and CAFs were co-cultured together. The experts observe an increase in colony size and organoid formation efficiency compared to

organoid culture alone, indicating the role of CAFs in promoting self-renewal of OSCC cells. They also verified the influence of CAFs on cancer stem cells (CSC) by increasing the efficiency of organoid formation of these cells after co-culturing with CAF. In addition, the investigators also observed an increase in the organoid-forming capacity of CSC cells when treated with conditioned medium harvested from CAF, indicating a role for secreted CAF molecules in CSCs.

Tumoroids and cancer treatment

Bladder cancer is a very heterogeneous disease that means that current treatments to treat it are not enough to cure it and reduce the high frequency of recurrence (59, 61). According to that, appears the need to better understand the mechanism of carcinogenesis and develop more efficient drugs and treatments. Here is where organoids play a leading role in searching for new, more personalized, and effective treatments.

An interesting work that reflects the applicability of the organoids in immunotherapy is the paper by Scognamiglio et *al.* (113). They used patient-derived organoids to predict response to the programmed cell death-1 receptor (PD-1) and its ligand-1 (PD-L1). The selection of patients that would benefit from treatment with the PD-1/PD-L1 checkpoint inhibitors is difficult due to the heterogeneity of the tumors and the variable biomarker detection. The researchers generated organoids from surgical chordoma specimens from positive PD-L1 patients. The cultures contained positive and negative PD-L1 organoids. Then, organoids responded to the treatment with Nivolumab, a human antibody that blocks the PD-1 receptor. These results implie that some sarcomas with low or no immunohistochemically detectable PD-L1 expression respond to therapy, and in consequence, these patients could benefit from immunotherapy with Nivolumab.

Otherwise, organoids could also play a key role in predicting the response to chemotherapy, as in the paper by Ooft et *al.* (114). The authors established patient-derived colorectal cancer (CRC) organoids metastatic lesions to identify non-responders to standard-of-care chemotherapy in CRC. They observed that PDOs derived from lesions classified as progressive disease (PD) are less sensitive to treatment with Irinotecan than PDOs derived from lesions classified as stable disease (SD). Similar results were observed when PDOs were treated with 5-Fluorouracil (5-FU)–irinotecan combination therapy (FI). Moreover, they observed that the in vitro sensitivity to FI was associated with the clinical response because the most sensitive PDOs to FI had a significantly higher progression-free survival. These data suggest the predictive value of PDOs for Irinotecan monotherapy and FI combination

therapy. However, the results shown in this paper could be used to prevent cancer patients from undergoing ineffective irinotecan-based chemotherapy.
In addition to the proven application of organoids in predicting response to chemotherapy, some works such as Yao et *al.* (115), have shown the impact of organoids in predicting chemoradiation response. The authors used PDOs to infer chemoradiation responses of locally advanced rectal cancer (LARC). The standard treatment for LARC patients is neoadjuvant chemoradiation (NACR). They established organoids from LARC patients that were treatment-naïve at the time of PDO generation. The response of the PDOs to irradiation, 5-Fluorouracil, and Irinotecan was compared with the clinical outcome of the patients. The results showed that when the PDOs were sensitive to at least one of the three treatment components, the patients achieved a good clinical response, and when the PDOs were resistant to irradiation and drugs, the patients had a poor clinical response. These data suggest using organoids as a biomarker to help radiation oncologists determine which patient is sensitive to NACR therapy either to the three treatments together or separately. Moreover, the organoids can be used to select another targeted agent in patients with radio- and chemorefractory LARC to avoid the overtreatment and reduce the side effects.

Although organoid culture is a promising technique to better understand tissue biology and tumorigenesis and thus improve cancer prognosis and treatments, we might not forget that like all models, organoids also have some disadvantages. Compared to 2D cell cultures, organoid cultures take longer to establish and expand, which could lead to a long waiting time between patient-derived organoids being established stably and being used for drug screening. On the other hand, although organoids seem to be a good model to predict the response to some treatments, these will always have to be verified in an *in vivo* context. This is because organoids are usually only made up of epithelial cells, thus lacking the interaction with the microenvironment. Despite the advances made to establish organoid co-cultures to better mimic the *in vivo* context, this is not enough for replacing animal models. For this reason, drugs tested on organoids must also be tested on animals to have the action of the drug in a complex model in which epithelial cells, stroma, immune system, and microenvironment are interconnected. Despite the needs mentioned above, organoid cultures can be used to make a more discriminatory selection of drugs, thus reducing the proposals to be tested.

12. CONCLUSION

Bladder cancer can benefit from organoids to make more reliable models that better mimic the morphological and molecular heterogeneity of this cancer. Organoids can be co-cultured with other cells, such as stromal cells or immune cells, to create a more complex model that better recapitulates the context *in vivo*. Besides, organoids offer the possibility of searching for new treatments that improve the prognosis and therapeutic options that can help reduce the high recurrence of bladder cancer and, in consequence, reduce the costs that this disease carries through constant monitoring and treatment. To achieve this objective, our protocol must be improved taking into account the techniques and methods used in similar studies as well as the change of the base membrane or the introduction of growth factors into the culture medium that boost the proliferation and expansion of the organoids. In summary, the organoid culture is a novel research model that holds promising results on bladder cancer understanding and treatment.

13. LIMITATIONS

Due to the pandemic caused by COVID-19, the dissertation project was altered in June. Consequently, the project became more focused on a bibliographic review than a laboratory project. For this reason, the results presented are those that could be carried out until the beginning of March 2020.

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