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Prostate Cancer Organoids in the Fight Against Prostate Cancer

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Abstract

Prostate Cancer is one of the leading diseases that increase morbidity and mortality rates in men worldwide. Despite high intertumoral and inter-patient heterogeneity, prostate cancer is, in most cases, androgen-sensitive at its early stages and thus can be treated using androgen deprivation therapy and/or surgical castration. However, in certain cases, it can become resistant to androgens, primarily due to hijacking of several cellular pathways, such as the AR pathway and/or the JAK/STAT pathway, as well as due to other genetic and epigenetic factors. As a result, the tumor acquires a more aggressive, metastatic, castration-resistant phenotype. This form of prostate cancer remains uncurable. Cell lines and animal models have been used extensively for the discovery of diagnostic biomarkers as well as for the development of novel therapeutic approaches. However, in the last few years, prostate cancer organoids are being developed to recapitulate the tumor microenvironment and its 3-dimensional nature, more precisely. This bibliographical review focuses on presenting the contribution of prostate cancer organoids to the field. More specifically, we will discuss the use of Prostate Cancer Organoids in the a) identification of diagnostic biomarkers, b) drug repurposing and c) development of novel therapeutic approaches. This review shows that the use of organoids, even though still at its infancy, paves the way to precision medicine and facilitates drug testing in a patient-tailored manner, to achieve better treatments.

Maria Th. Panteli

Dedication

To my family and friends.

Maria Th. Panteli

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I am sincerely grateful to my thesis supervisor, Dr. Anna Charalambous, for her invaluable advice and guidance, while pursuing my thesis.

Maria Th. Panteli

Composition of the Examination Committee

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Seminar Announcement

University of Cyprus
Department of Biological Sciences

BIO 680 Scientific Methodology in Molecular Biology

Student Presentation

Wednesday, 13 December 2023 at 11:00
Building ΘΕΕ02, Room B172, Panepistimioupoli Campus

This seminar is open to the public

Maria Panteli

Thesis Supervisor: Special Teaching Staff, Dr. Annita Charalambous

"Prostate Cancer Organoids in the Fight Against Prostate Cancer "

Prostate Cancer is one of the leading diseases that increase morbidity and mortality rates in men worldwide. Despite high intertumoral and inter-patient heterogeneity, prostate cancer is, in most cases, androgen-sensitive at its early stages and thus can be treated using androgen deprivation therapy and/or surgical castration. However, in certain cases, it can become resistant to androgens, primarily due to hijacking of several cellular pathways, such as the AR pathway and/or the JAK/STAT pathway, as well as due to other genetic and epigenetic factors. As a result, the tumor acquires a more aggressive, metastatic, castration-resistant phenotype. This form of prostate cancer remains uncurable. Cell lines and animal models have been used extensively for the discovery of diagnostic biomarkers as well as for the development of novel therapeutic approaches. However, in the last few years, prostate cancer organoids are being developed to recapitulate the tumor microenvironment and its 3-dimensional nature, more precisely. This bibliographical review focuses on presenting the contribution of prostate cancer organoids to the field. More specifically, we will discuss the use of Prostate Cancer Organoids in the a) identification of diagnostic biomarkers, b) drug repurposing and c) development of novel therapeutic approaches. This review shows that the use of organoids, even though still at its infancy, paves the way to precision medicine and facilitates drug testing in a patient-tailored manner, to achieve better treatments.

Maria Th. Panteli

Table of Contents

Maria Th. Pantelli

Maria Th. Panteli

List of Figures

Maria Th. Panteli

Introduction

1. The Molecular and Cellular Identity of Prostate Cancer

1.1. The Anatomy of the Prostate Gland

The Prostate Gland (PG) is a conical-shaped gland, that consists of three different zones: the peripheral, the transition, and the central zone. It belongs to the male urogenital system and each zone surrounds different compartments of it. Firstly, the urethra is surrounded by the transition zone, while the ejaculatory glands are surrounded by the central zone [(Figure 1), (Ittmann, 2018)].

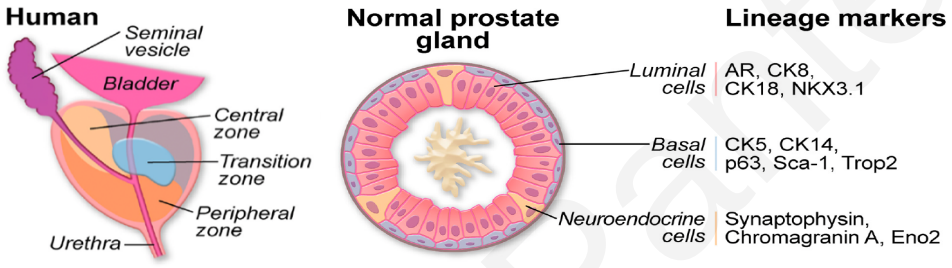


Figure 1: The anatomy of the human prostate gland and the lineage markers of its differentiated cell types. In the leftmost panel, the association between the three main zones of the PG and the compartment of the urogenital system is shown. Some of the major lineage markers of each cell type involved, are mentioned. Modified from Wang et al., 2018.

Because of its pivotal role, it is one of the major glands, which is directly associated with male fertility. Its main function is to supplement the semen with fluids rich in nutrients, metabolites, and enzymes, so that its fluidity, motility, and quality can be maintained, for optimal fertilization. One of the most important ingredients of the PG's secretions are the Kallikreins (such as Prostate-Specific Antigen), which are serine proteases, used for semen liquefaction. Zn^{2+}, and citrate, are also ingredients of major importance since they are mainly used for the homeostasis of the gland and the control of ejaculation (Verze et al., 2016).

The PG is composed of three major cell types, all having distinct roles in PG's function: Basal, neuroendocrine, and luminal cells (Figure 1). These terminally differentiated cell types can be distinguished by the expression of specific lineage markers. Different proteins of the Cytokeratin family are expressed in both luminal and basal cells, while Chromagranin A is expressed in neuroendocrine cells (Wang et al., 2018). PG's function is highly dependent on

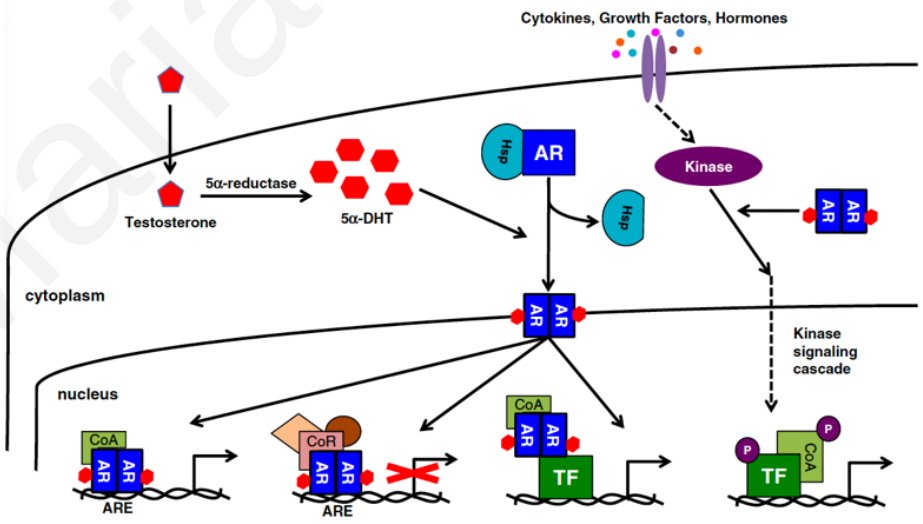
Maria Th. Pantelli

androgen signaling (Shafi et al., 2023); thus, Androgen Receptors (AR) are expressed by and found on the basal and luminal cells (Wang et al., 2018).

The anatomy of the PG is known to be altered in several pathological conditions, including Prostate Cancer (PCa), thus it can be monitored for diagnostic purposes.

1.2. Molecular Pathways Involved in Prostate Cancer Development

As it is widely known, PCa is a disease that heavily relies in the presence of Androgens (e.g. testosterone) and thus one of the major signaling pathways that are crucial for tumorigenesis is the Androgen Receptor Pathway. What happens in the canonical pathway is that, because of its steroid nature, the Androgen Receptor (AR) is localized in the cytoplasm of the cells. In the absence of androgens, the AR is attached to chaperon proteins (such as heat shock proteins), that inhibit its function as a nuclear transcription factor. In the presence of testosterone and after its conversion to dihydrotestosterone (DHT), the chaperon proteins are released from the AR, and the nuclear localization signal (NLS) is released making it possible for the DHT-AR complex to translocate to the nucleus, and act as a transcription factor (TF). The complex can either promote transcriptional activation or inhibition, depending on which co-regulators will be recruited. This signaling pathway can also be activated by the interaction of the DHT-AR complex with specific kinases (e.g., SRC or AKT), and through the ongoing kinase activity cascade, other TFs can be recruited, so that gene transcription can be promoted, without the immediate binding of the DHT-AR complex on the DNA [(Shafi et al., 2013), (Figure 2)].



Maria Th. Pantelli

Figure 2: The canonical and non-canonical AR pathway. The DHT-AR complex in the canonical pathway leads to either transcriptional activation or inhibition, after its translocation to the nucleus. The complex can also interact with kinases and promote transcriptional activity without it being within the nucleus. DHT: Dihydrotestosterone; AR: Androgen Receptor; Hsp: Heat shock protein; CoA: Co-Activator; CoR: Co-Repressor; TF: Transcription Factor. Modified from Shafi et al., 2013.

In a healthy PG, this pathway serves as a means for the proper development and function of the organ since it is involved in the maintenance of proliferation and differentiation of all PG's cell types. In epithelial cells, the AR pathway is also responsible to produce PG-specific proteins (Wang et al., 2018; Shafi et al., 2013). On the other hand, in the event of the development of a malignancy, the AR pathway is dysregulated, so that it favors the events of tumorigenesis. This pathway is in constant crosstalk with other signaling pathways that seem to be vital for the progression of the disease.

An example of these pathways is the phosphatidylinositol-3-kinsase/Akt/ Mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway, whose activity is maintained in most cancer cases, as a consequence of its crucial function in helping cancer cells survive from stress and producing crucial proteins for the tumor to be sustained (Porta et al., 2014). This pathway is initiated by the autophosphorylation of tyrosine residues of Growth Factor Receptors (GFRs) and the recruitment of PI3K on these residues. The cascade goes on with further phosphorylation of downstream kinases, such as Pyruvate Dehydrogenase Kinase 1 (PDK1), which then phosphorylates Akt. Phosphorylation of Akt inhibits Tuberous Sclerosis Complex 2 (TSC2), which results in the activation of mTORC1 (Shorning et al., 2020). mTORC1 (mTOR in association with Raptor and other regulatory proteins) is known to be associated with metabolic functions related to cell growth, such as translation and protein synthesis (Xia et al., 2015). Moreover, mTORC2 (mTOR in association with Rictor and other regulatory proteins) is also activated directly by the phosphorylation of PI3K, and this complex seems to be associated with cell survival (Oh et al., 2012).

Another example of a crosstalk of signaling pathways, affecting the trajectory of PCa, is the one between AR and Wnt signaling pathways. Wnt is a protein that acts as a ligand, and binds to its transmembrane receptor, called Frizzled. In the absence of the ligand, Casein Kinase and GSK3 phosphorylate β-catenin to mark it for ubiquitination and proteolysis. In the presence of the ligand, another protein gets activated, called Disheveled, and inhibits the function of GSK3 and CK1, making it possible for β-catenin to avoid proteolysis and finally translocate to the

Maria Th. Panteli

nucleus, where it can promote transcriptional activation (Komiya and Habas, 2008). One of the target genes of this pathway is the AR, which along with Wnt/β-catenin can be transported to the nucleus and transcribe more AR-related genes (Shorning et al., 2020).

All the aforementioned signaling pathways can not be studied individually, as they all function collaboratively to orchestrate the onset and progression of tumorigenesis (Figure 3). In addition, many molecular key players involved are crucial as they serve as biomarkers or therapeutic targets. By elucidating as much of the crosstalk mechanism, the exact function of these molecules and their contribution to the progression of PCa, will be uncovered. On the other hand, by studying only the molecular identity of PCa is not enough, because as it progresses, many intracellular and genetic changes are made, after which the PCa cells do not rely on AR signaling anymore (especially after treatment), but on PI3K-Akt-mTOR signaling pathway and this makes PCa a life-threatening disease (Militaru et al., 2023). This case is known to be the Aggressive Variant Prostate Cancer (AVPCa) with more aggressive clinical impact (Montironi et al., 2020).

1.3. Genetics and Prostate Cancer

As PCa is a multifactorial disease, it is expected that genetic factors would also affect its trajectory and be partially responsible for its emergence. To begin with, higher probabilities of developing PCa are observed in men, who had a close relative suffering from PCa. Evidence show that genetic predisposition has a vital role in PCa's emergence. Some genes are predisposed with higher probabilities than others. Some of them are associated with other cancers, such as Breast Cancer genes, BRCA1 and BRCA2 genes, but they seem to be associated with the increase of possibility of developing PCa (Messina et al., 2020). The proteins encoded by those genes are involved in the DNA repair mechanism of the cell (by homologous recombination-HR). Other genes are the mutS Homolog (MSH) family genes, whose products are involved in the mismatch repair (MMR) mechanism of the cell (Kornberg et al., 2018). Both mechanisms and genes are vital for the proper duplication of the cells, so every mutation that happens in these genes, might lead to the malfunction of these processes, and lead to tumorigenesis (Vietri et al., 2021). These genes might also serve as biomarkers for controlling and even treating PCa.

More mutations were also discovered either in housekeeping genes, or in genes that serve as transcription factors in PCa's pathways or as oncogenes and tumor suppressors. For example, in the AR pathway, there are some regulators, that heavily depend on androgens. One example is the transcription factors of the ETS family, which were observed to be overexpressed in cells

Maria Th. Partelli

undergoing tumorigenesis (Sizemore et al., 2017). Overexpression and gain of function were also observed in genes acting as co-activators in the AR pathway, such as Nuclear Receptor Co-Activator (NCOA1/2), while some co-repressors were silenced or under expressed, such as Nuclear Receptor Co-Repressor (NCOR1/2) (Taplin et al., 2008). As for the PI3K pathway, the most well-studied gene, that its problematic expression seems to increase the probability of tumorigenesis, is the gene that encodes for the Phosphatase and Tensin homolog, called the PTEN gene. In various PCa tumors, the under expression or the loss-of-function of this gene seems to be paired with uncontrolled proliferation and increased cell survival, because it does not interact with the mTOR complexes in such cases (Pourmand et al., 2007). All the aforementioned gene expression alterations, seem to be also paired with the progression of PCa (Wang et al., 2018), as such events are more profound in Castration-Resistant Prostate Cancer (CRPC) and metastatic Castration-Resistant Prostate Cancer (mCRPC), which are developed after Androgen Deprivation Therapy (ADT), which aims to reduce the levels of androgens (discussed more in section Current Treatments).

Gene alterations also affect the AR gene. This gene consists of 8 exons, that give rise to the standard domains observed in a steroid receptor, such as the ligand binding domain, the DNA binding domain, the hinge region, and the N-terminal domain. In some cases, because of mutations and alternative splicing, a much shorter variant of the AR will be produced that lacks the ligand binding domain, making it functional without needing to be in complex with androgens, to act as a TF (Shafi et al., 2013). The truncated variant is called AR-V7 and is more common in cases of CRPC (Antonarakis et al., 2014). Even in cases where no alternative splicing occurs the AR gene is still susceptible to overexpression, and that eventually leads to the increased translation of the receptor (Visakorpi et al., 1995).

All these genes act in collaboration with signaling pathways and are indicators of how heterogeneous and complex the studying of the disease can be.

1.4. Epigenetics and Prostate Cancer

Epigenetic alterations seem to also be responsible for development of PCa. Such changes do not impact the DNA sequence of the cells, but they do impact the whole transcriptional landscape and phenotype of cells. The epigenome alters gene expression as it is associated with the accessibility of the chromatin to the transcriptional machinery of the cell (Pardo et al., 2022). The first epigenetic change is DNA methylation which is catalyzed by DNA methyltransferases (DNMTs), which are responsible to covalently attach a methyl group on the fifth carbon atom (5C) of a cytosine. This alteration can be more profound in regions where

Maria Th. Partelli

guanine and cytosine nucleotides are found. The genome can be either hypo- or hyper-methylated and this affects its transcription further on. Hypermethylation is linked with gene silencing, whereas hypomethylation with an increased gene expression, because they both affect the interaction of the transcription machinery with the methylated region of the DNA and the recruitment of either transcription inhibitors or enhancers, respectively (Moore et al., 2013). In PCa, many gene regulatory regions, such as promoters of tumor suppressor genes are found to be hypermethylated. One example is the PTEN's regulatory region, that when hypermethylated is not transcribed and uncontrolled proliferation and inhibition of apoptosis starts occurring. Such events are mostly observed from as early as the initiation of tumorigenesis (Graca et al., 2016). The exact opposite observation is made for tumor promoting genes, whose promoters seem to be hypomethylated and fully accessible to the transcriptional machinery, such as Heparanase (HPSE), which is associated with extensive angiogenesis, and cell migration. This is even observed in genes that are responsible to encode subunits of the DNMTs (Pardo et al., 2022).

Histone modifications are also contributing to the course of the disease. Histones are the proteins responsible for chromatin packaging within the cells. The histones are prone to modifications, such as the addition of different chemical groups (e.g., methyl groups, acetyl groups etc.) on specific amino acids on the tails of histones, that can subsequently alter the way that chromatin is wrapped around them. It can be either more compacted (less accessible to the transcriptional apparatus) or acquire a more open structure (more accessible to the transcriptional apparatus). These modifications require specific enzymes to catalyze the addition of the chemical group. Some histone modifications are known to benefit transcriptional activation, while some others favor transcriptional repression. In PCa it was shown that enzymes that catalyze some of these alterations are highly expressed in specific subtypes of PCa (e.g., Histone Deacetylases, Histone Methyltransferases in CRPC), while in different cases specific modifications are highly observed [(e.g., H3K4me1/2/3 and H3K18Ac in CRPC), (Bianco-Miotto et al., 2010)]. Such alterations are useful as they can serve as biomarkers for the diagnosis of the disease.

Maria Th. Pariel

1.5. Lineage Plasticity and Tumor Heterogeneity in Prostate Cancer

As it was previously mentioned, the PG consists of three discrete cell types: the luminal; the basal and the neuroendocrine cells. It is yet not known what the cell of origin in PCa is, but there are data which support that the prostate intraepithelial neoplasia (PIN), which precedes the formation of the tumor, occurs in either luminal cells (Choi et al., 2012) or basal cells (Lawson et al., 2010). After development of the tumor, all the cells are dependent on AR signaling. Hence, the standard of treatment is ADT, which works well in the first stages of treatment. The reason why CRPC, or mCRPC occurs after therapy, is that the cells acquire a different cellular identity that makes them AR-independent. This shift gives rise to the Neuroendocrine Prostate Cancer (NEPC), in which cells are characterized as neuroendocrine cells, because of the expression of specific markers of this cell type (Ge et al., 2020). This differentiation was attributed to alterations in the epigenome of the cell, and not in random clonal selection (Beltran et al., 2016). These changes involve changes in the methylation state of the regulatory regions of the genes, in histone modifications and in altered gene expression of epigenetic modifiers that aim on reprogramming the cells, to acquire neuroendocrine characteristics, such as the loss of the AR, or Retinoblastoma Protein (Rb: responsible for S-phase induction and increased proliferation) (Davies et al., 2020).

Even though PCa is a strictly characterized disease, genomic and phenotypic heterogeneity of tumors, as well as inter-patient heterogeneity make it difficult to study the progression of it, understand the onset of the disease and find therapeutic techniques that will benefit equally all PCa patients (Haffner et al., 2021). To begin with, when PCa progresses multiple cancer foci arise, that when sequenced appear to have differences in their genome and epigenome, making it difficult to address the molecular identity of the tumor (Boutros et al., 2015; Gundem et al., 2015). As for the extracellular matrix of PCa tumors, its composition changes in order to evade the surveillance of the immune system (Gabrilovich et al., 2009), help with Epithelial-To-Mesenchymal Transition (EMT) and maintain tumoral homeostasis (Hannah and Weinberg, 2011). All these eventually result in inter-patient heterogeneity, which explains the variety of responses in the therapeutic techniques that these patients undergo, or the progress of the disease among each one.

Maria Th. Pariel

2. Clinical Aspects of Prostate Cancer

2.1. Diagnosis of Prostate Cancer

Morbidity and mortality rates of PCa have been constantly increasing. Some of the main reasons are the lack of obvious symptoms that might indicate PCa's onset and the limited means and resources for diagnostic purposes. The standard procedure is the detection of the PSA antigen in blood circulation. Most of the times it can precisely detect the chance of developing cancer or the onset of it, but there are also some caveats in using this blood test. Cases were documented in which increased PSA levels were not associated with PCa, while on the other hand in PCa patients PSA levels did not indicate the presence of the tumor in early stages (Adhyam and Gupta, 2012). Another diagnostic procedure is Digital Rectal Examination (DRE), in which the PG's size or any abnormalities are manually examined by the physician. Magnetic Resonance Imaging (MRI) is another technique that images all abnormalities of the PG and precedes biopsy analysis, in which tissue samples from the organ are analyzed in the lab, either by genome sequencing to observe critical biomarkers or by histochemistry. In combination, these techniques can successfully diagnose PCa, and address the stage of the tumor.

2.2. Current Treatments and Newly Emerging Therapies

For the treatment of PCa there are various treatment methods, that all aim in prolonging the patients' life without affecting dramatically the quality of their lives. As soon as the cancer the cancer is diagnosed, as well as when the tumor progress, constant surveillance is mandatory. Apart from surveillance, usually in early cancer stages, the most common treatments used are prostatectomy and radiotherapy. These aim on limiting the expansion of the tumor and prevent metastasis (Lima et al., 2019; Sekhoacha et al., 2022).

When the disease progress and further therapeutic measures are needed to be taken, Androgen Deprivation Therapy (ADT) is the standard process, in which patients are either surgically or hormonally castrated, to reduce the production of testosterone and other androgens. For the inhibition of growth of cancer cells, as well as for their killing, anticancer drugs are used in chemotherapeutic methods. Combinatorial use of the aforementioned methods is what prolongs the life of the patients (Crawford et al., 2023; Sekhoacha et al., 2022). For example, it was shown that when ADT was used in combination with abiraterone acetate, which inhibits the function of the protein CYP17A1 (a 17–20 lyase and 17-α hydroxylase of the cytochrome P450 family), hormonal castration was improved, and androgens' production

Maria Th. Pantelli

was dramatically diminished. This lowered the chances of mCRPC (Nevedomskaya et al., 2018).

mCRPC still remains one of the most challenging events in the progress of PCa, as it remains uncurable. Researchers aim on uncovering novel, molecular therapeutic targets by first observing how the signaling pathways are being hijacked by the cancer cells to acquire their metastatic features. However, to speed up the process of the development of drugs and surpass the time limits that is needed to go through clinical trials, drug repurposing is also under study, by several laboratories. Bioinformatics, genomics, and proteomics can also contribute to these studies, to speed up the process or propose possible models or outcomes regarding these drugs (Bahmad et al., 2022).

3. Cancer Organoids as a Mean of Surpassing Cancer Cell Lines' Limitations

3.1. Generation and Advantages of Cancer Organoids in Research

Studying diseases like cancer, which are caused by several (and sometimes unknown) factors, can be challenging, because the extracellular and intercellular conditions can not be easily reproduced in cell lines, or animal models. Thus, the scientific community created three-dimensional cancer organoids, with protocols that will be described further on, to be able to represent and study tumor heterogeneity, as well as events and factors of the tumor microenvironment (TME) that can alter the trajectory of the disease. That is one of the major advantages, since it can shed light on uncovering key molecular entities that can be identified as biomarkers and therapeutic targets, that were not easy to uncover using cell lines or *in vivo* models. Another critical advantage is that cancer organoids can preserve their genomic, epigenomic, and transcriptomic landscape of their in vivo counterparts, ensuring that these are biologically accurate models for studying cancer (LeSavage et al., 2022).

Personalized Medicine seems to be more reachable as patient-specific cancer organoids can be produced. Accomplishing this for every cancer patient at the moment, might not be feasible because of time and cost constraints, but it can be very promising for future research, since it can provide the accurate events and factors that caused tumorigenesis in each patient, since intra-patient heterogeneity exists. Hence, the therapeutic methods that will be followed for each case would be patient tailored. This can also contribute to creating "libraries" of several cases of tumorigenesis that can save time in researching the molecular identity of each cancer incident and the response in several treatment methods.

Finally, another advantage seems to be the relatively lower cost of producing and maintaining cancer organoids as well as the acquirement of more accurate results, in contrast

Maria Th. Pantelli

with other techniques used for studying cancer, such as Patient-Derived Xenografts (PDX-tumor tissue from patient engrafted into immunodeficient mice) or two-dimensional cell lines. This can be attributed to the fact that the TME of the injected mice, is different than the patient's TME, or to the fact that there is a limited number of cell lines that can be used for several types of cancer. In addition, in cell lines the effect of the TME and heterogeneity can not be studied (Guillen et al., 2022).

3.2. Main Limitations of Cancer Organoids Usage in Biological Research

Even though cancer organoids are one of the most accurate models for studying tumorigenesis, some limitations act as obstacles in establishing them as the primary model. The greatest problem is that the culture and expansion of the organoids might not go as planned, as it is yet an unpredictable process. The percentages of successful expansion are relatively low. This can be attributed to the fact that only a limited number of protocols exist. Also, for the development of an organoid, neoplastic cells are used. Hence, cell types that appear further on in the development of the disease, might not arise *in vitro*, resulting in losing critical information about the progress of the disease (Gao et al., 2014; LeSavage et al., 2022). Another problem that derives from the limited number of protocols, is that there is no exact medium that is being used, that reflects the exact conditions of the Extracellular Matrix (ECM) that are met *in vivo*. In addition to this, cell types that are found in the TME, such as fibroblasts, immune cells etc., also lack from the culture conditions. This can lead to misinformation about the interaction of the ECM with the organoid, as well as the TME's contribution to the heterogeneity (Barbáchano et al., 2021).

3.3. Comparing Cancer Organoids with *in vivo* Models

Animal models have been used in biological research for many years, as a mean of understanding molecular mechanisms, as well as testing drugs before human clinical trials. Ethics have been the central subject of debates about their use and the necessity of their sacrifice for scientific purposes. Thus, the scientific community is exploiting organoids in cases and projects that permit it, to retrieve more accurate results, as they represent human physiology better. This does not mean that animal models would be completely substituted, because in some projects these two models can be used in a complementary fashion to acquire better results.

Maria Th. Panteli

In practice, in cases where PDX experiments are being performed, the tumor is being engrafted in immunocompromised mice. This directly affects the accuracy of the retrieved results, as they do not recapitulate the exact TME that the tumor would have in humans.

Another important factor that promotes the development of organoids is that the expenses for maintaining animals in captivity are much higher than developing and preserving organoids cultures (Mukhopadhyay and Paul, 2023). Thus, more funding can be used in other aspects of their research.

4. Main Aim and Importance of the Project

This project is focused on identifying the main advances in the field of Prostate Cancer, especially regarding the development and use of Prostate Cancer Organoids. This review will focus on studying the current literature and the progress in the field, indicating the potential for identification of novel key molecular players that could serve as biomarkers for early prognosis. In addition, the existence of limitations and the requirement for improvements, will also be discussed.

The use of organoids brings research a step closer to precision medicine, as patient-specific prostate cancer organoids can be produced, to avoid the interpatient heterogeneity that stalls the treatment of the patients. Therefore, treatment and drug testing can be performed in a more patient-tailored manner.

Maria Th. Panteli

Overview

5. Development of Prostate Cancer Organoids

5.1. Generating and Culturing Prostate Cancer Organoids: The Challenges Behind These Techniques

A major challenge faced by researchers developing and maintaining Prostate Cancer Organoids (PCOs) is preservation of the tumor identity. Several techniques have been developed to address this issue, but they have not all proved successful (Zhou et al., 2021). In order to recapitulate the PCa epithelium in prostate cancer organoids, DMEM/F-1 was employed as the growth medium as it has been shown to permit the growth, differentiation, and viability of many mammalian cells along with several growth factors, such as Epidermal Growth Factor (EGF), Noggin to prevent undesirable, sudden differentiation, and R-spondin-1, a Wnt agonist, that promotes the expansion of the organoid. The specific combination of growth factors and their respective concentrations in the medium depend on the origin of the cells and the nature of the experiments to be conducted. For example, deprivation of Noggin and R-spondin-1 eliminated, AR expression (Karthaus et al., 2014). Moreover, elimination or decrease in the concentration of EGF, induced the AR pathway (Pappas et al., 2019). Thus, organoid culture conditions should be considered with great care and caution, so as to properly serve the experiments to be performed (Pappas et al., 2020).

Another reason for culturing organoids in proper medium, is to fully recapitulate the ECM interactions. The multifactorial ECM can alter the progression of tumorigenesis, because of the mechanical and biochemical interactions with the tumor. This could also contribute to uncovering its involvement in possible therapeutic approaches (LeSavage et al., 2022). A study was conducted that observed the contribution of integrins in relation with apoptosis. Scientists uncovered that its presence was responsible for the acquirement of chemoresistance and the inhibition of apoptosis. In follow up experiments, in which integrins could not bind to hemidesmosomes, apoptosis was induced, making the ECM an attractive target for the development of therapeutic techniques (Weaver et al., 2002).

Another important aspect when developing organoids was the origin of the cells used. These were pluripotent stem cells, such as Embryonic pluripotent Stem Cells (ESCs) or induced Pluripotent Stem Cells (iPSCs) (Calderon-Gierszal et al., 2015; Hepburn et al., 2020). Alternatively, they were cells from patient tissues such as cells of PCa biopsies, normal prostate epithelium or circulating tumor cells (Drost et al., 2016). Cells from several PCa cell lines and PDXs were also used (Ma et al., 2017). The organoids totally represented the identity of PCa

Maria Th. Panteli

cells that can be found *in vivo*. Most of the existing organoids represent AR-dependent and independent PCa, mCRPC and there are only few that represent NEPC (Zhou et al., 2021).

Two techniques were developed for the introduction of the cells into the medium. Some of the principles of the techniques were common, such as that the tissue had to be either dissociated to form single cells or minced using enzymatic activity or mechanical stress, to produce smaller fragments of the tumor. The single cells or the tumor fragments were then encapsulated into the medium and cultured for the appropriate amount of time that was needed for the 3D organoid to be produced. The latter technique seemed to hold the advantage of the preservation of the tumor's architecture (LeSavage et al., 2022).

Cancer Organoid development is a relatively novel technique; thus, many practical limitations arise, independently of the cancer's identity. It was previously mentioned that a common characteristic among cancers is the intra-tumoral heterogeneity. Thus, the source of the organoid is a major limitation. Usually, the organoids were made by only isolating a part of the tumor tissue, resulting in losing the effect that the cells left behind could have in tumorigenesis. Hence, what would happen *in vivo*, could not be recapitulated by the organoid. This was also proved by a study conducted by Roerink et al., where it was shown that different regions of the tumor were characterized by different methylation and transcription states (Roerink et al., 2018).

Practical limitations regarding the culture medium also arose that were crucial, especially in high-throughput experimental settings, that disfavored the use of PCa organoids. Firstly, most of the ingredients found in the medium were usually expensive. The fact that the PCa organoids depended on the constant supplementation of these ingredients, increased the expenses rapidly. Moreover, the proteinaceous nature of the medium came with storage and yield constraints, as it could easily be denatured or of low purification quality. Several proteins lost their activity when found in the medium, mainly because of their hydrophobic nature, adding up to the complexity of designing the proper culture medium (Tüysüz et al., 2017). Another practical obstacle is the processing of the tissue. The available techniques, such as the mincing of the specimen or its enzymatic digestion, did not always result in reproducible results. It was shown that the fragments of the tumor that were obtained were not always of the same size or the enzymatic cleavage sites were, sometimes, random (Driehuis et al., 2020).

Maria Th. Panteli

5.2. Improvements of Prostate Cancer Organoids Culture Conditions Aiming to Establish Them as an Accurate Model for Studying Tumorigenesis

To overcome the aforementioned limitations, major advancements are emerging. As for the tissue origin, scientists were sampling more regions of the tumor to capture as much of the heterogeneity as possible. Liquid biopsy was used instead, which is a novel technique that aims on both eliminating the invasive nature of standard biopsy and at the same time gathering as much cells as possible for establishing the organoid. This can also include cells and factors from the TME, which directly affects tumorigenesis (Crocetto et al., 2022). Scientists also came up with new mincing techniques to acquire as much as they could from the tumor, while on the same time, encapsulated the isolated part in the medium. For example, Horowitz et al., managed to come up with a microdissection technique that yielded in same sized fragments of the original tissue, after many repetitions of the technique. These fragments seemed to also be viable, since they were able to form organoids for a great period of time (Horowitz et al., 2021).

As for the ingredients of the culture medium, Tüysüz et al., designed lipid carriers derived from phospholipids and cholesterol, that were able to accompany hydrophobic proteins so that they maintained their activity in the culture medium. By applying such techniques organoids seemed to remain viable for a longer period of time and expand more successfully with higher rates (Tüysüz et al., 2017).

6. Employment of Prostate Cancer Organoids in Prostate Cancer Research

6.1. Studying the Molecular Mechanisms of Prostate Cancer in Prostate Cancer Organoids

Basic knowledge of PCa's molecular mechanisms already exists. But in order to establish PCOs as a model to study and treat PCa, the organoids must represent, at least, most of the pathways that are being hijacked *in vivo*. Only then, they can be used further on, for addressing prognostic markers, performing drug tests and studying novel, patient-specific therapies. So transcriptomic and genomic preservation from the original tumor to the organoid must be tested. In a study conducted by Karkampouna et al., a PCa organoid derived from two bone metastatic PCa PDXs was created. Immunofluorescence was performed, to verify the presence of all luminal markers of PCa. AR, CK8 and PSA were identified in both organoids, as well as in the PDX, from which they derived (Figure 3).

Maria Th. Panteli

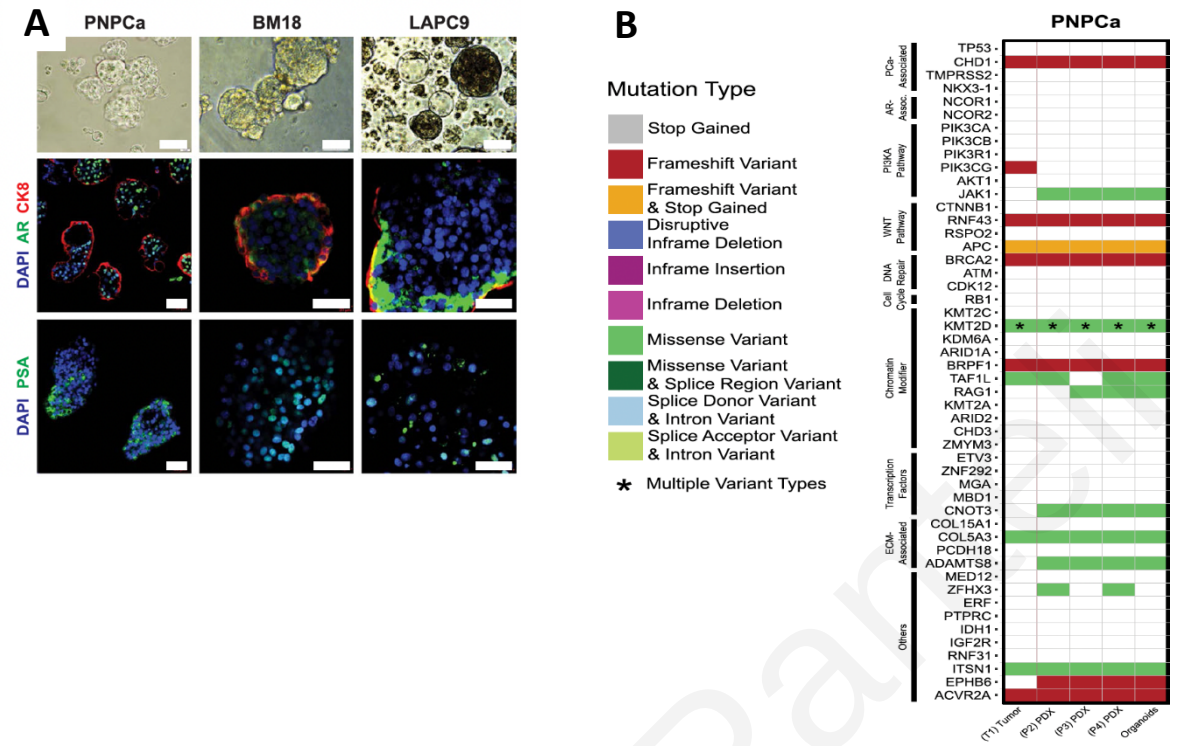


Figure 3: Prostate cancer organoids maintain their cell identity and their genomic profiles. In the leftmost panel a, the expression of luminal markers such as AR, CK8 and PSA was observed in both organoids. Genomic mutations were also studied, using WES. The mutation type is color-coded, and the genes under studied are listed in the rows of the chart. Each column represents the specimen that was observed. AR: Androgen Receptor; CK8: Casein kinase 8; PSA: Prostate-specific Antigen; WES: Whole exome sequencing. Modified and adapted from Karkampouna et al., 2021.

These verified that organoids' cells maintained their identity and their secretory function. After performing Gene Set Enrichment Analysis (GSEA) it was observed that several genes that were normally expressed in PCa *in vivo*, were also expressed in these organoids. These genes were involved in pathways related to cell growth, cell survival, AR signaling pathway etc. On the other hand, genes that were associated with immune system were highly under-expressed. This could explain the hijacking of immune system's surveillance, and the progression of the disease. The genomic states of the organoids were also examined using Whole Exome Sequencing (WES) (Figure 3). The presence of several copy number alterations, non-somatic mutations in preserved cancer genes such as BRCA2, and frameshift mutations in several chromatin modifiers, suggested that the organoids preserved these major genomic signatures very well. Since all mutations observed in PCa *in vivo* are also observed after the

Maria Th. Papadimitriou

development of the organoids, suggested that the molecular mechanisms responsible for tumorigenesis in vivo, are also exploited and used in PCOs, making them accurate models for studying the disease (Karkampouna et al., 2021).

PCOs can also be used to understand the exact process of lineage plasticity, which is the leading cause for drug resistance and the heterogeneity observed in CRPC. After observing the transcriptional status of PCa in animal models, Chan et al., exploited bioinformatic models to recapitulate and identify the onset for lineage plasticity in mice. It seemed that the first-formed adenocarcinoma population was the onset of lineage plasticity, as it acquired the expression of several TFs, that gave the ability to the cells to undergo Epithelial-to-Mesenchymal Transition (EMT) or acquire NEPC characteristics. It was shown that in the transition stage between adenocarcinoma and NEPC, JAK/STAT (Janus kinase/signal transducer and activator of transcription) signalling was enriched (Figure 4).

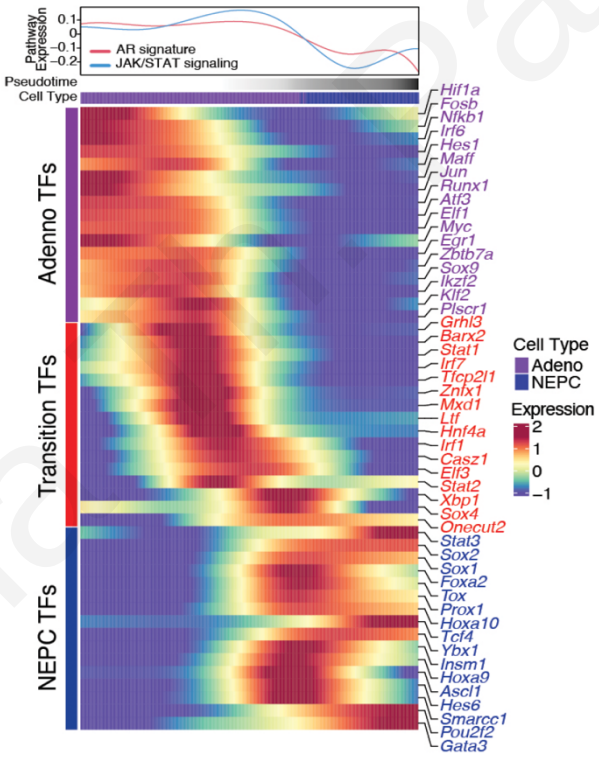


Figure 4: Increased dependency on JAK/STAT signaling while PCa transitions from adenocarcinoma to NEPC, in lineage plasticity. The heatmap is divided into three sections, in which the expression of TFs is observed in the three stages of undergoing lineage plasticity. In the transition phase, JAK/STAT pathway TFs and key molecular players are overexpressed. Modified and adapted from Chan et al., 2022.

Maria Spanti

In order to observe if the cells underwent lineage plasticity in a cell autonomous manner, or whether the TME was responsible, organoids were exploited because of the minimum effect of the TME on them. It was observed that only EMT occurred in a cell autonomous fashion after codeletion of Trp53 and Rb1 in the organoids. NEPC transition was only observed *in vivo*. This could be possibly attributed to the absence of stromal cells or other factors missing from the culture medium, because when scientists engrafted these organoids, within $Trp53^{+}$ and $Rb1^{+}$ mice, acquirement of NEPC-like characteristics, such as loss of AR dependency and the expression of synaptophysin (NEPC marker), were observed (Figure 5).

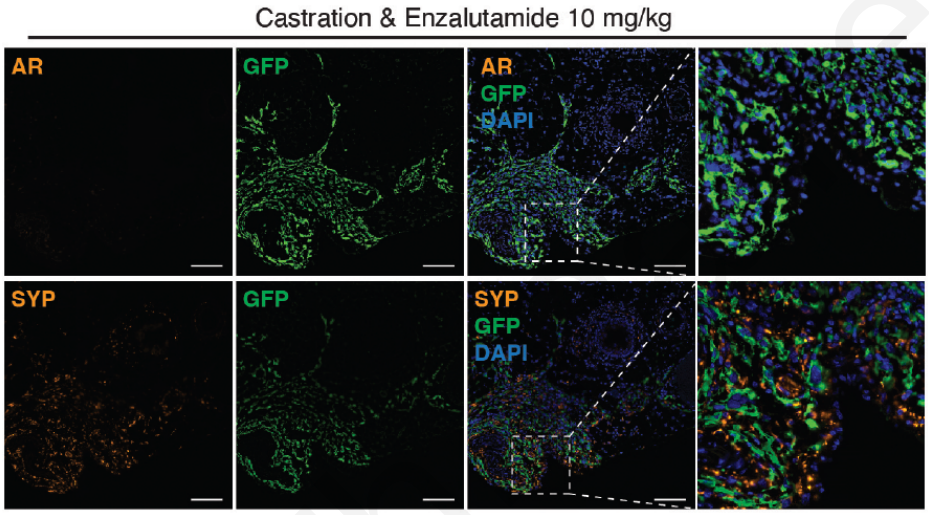


Figure 5: Loss of AR dependency and increased expression of Synaptophysin, in organoid cells after their engraftment in mice. The IF results indicate absence of expression of AR, along with high expression of Synaptophysin. These happened after the cells were engrafted in $Trp53^{+}$ and $Rb1^{+}$ mice. Modified and adapted from Chan et al., 2022.

Scientists also knocked down or overexpressed Jaks, using CRISPR-Cas9 and they observed that lineage plasticity, was reverted or enhanced respectively. Thus, the dependence on JAK/STAT is of major importance for the transition to the most aggressive type of PCa and might be leveraged for developing therapeutic strategies (Chan et al., 2022).

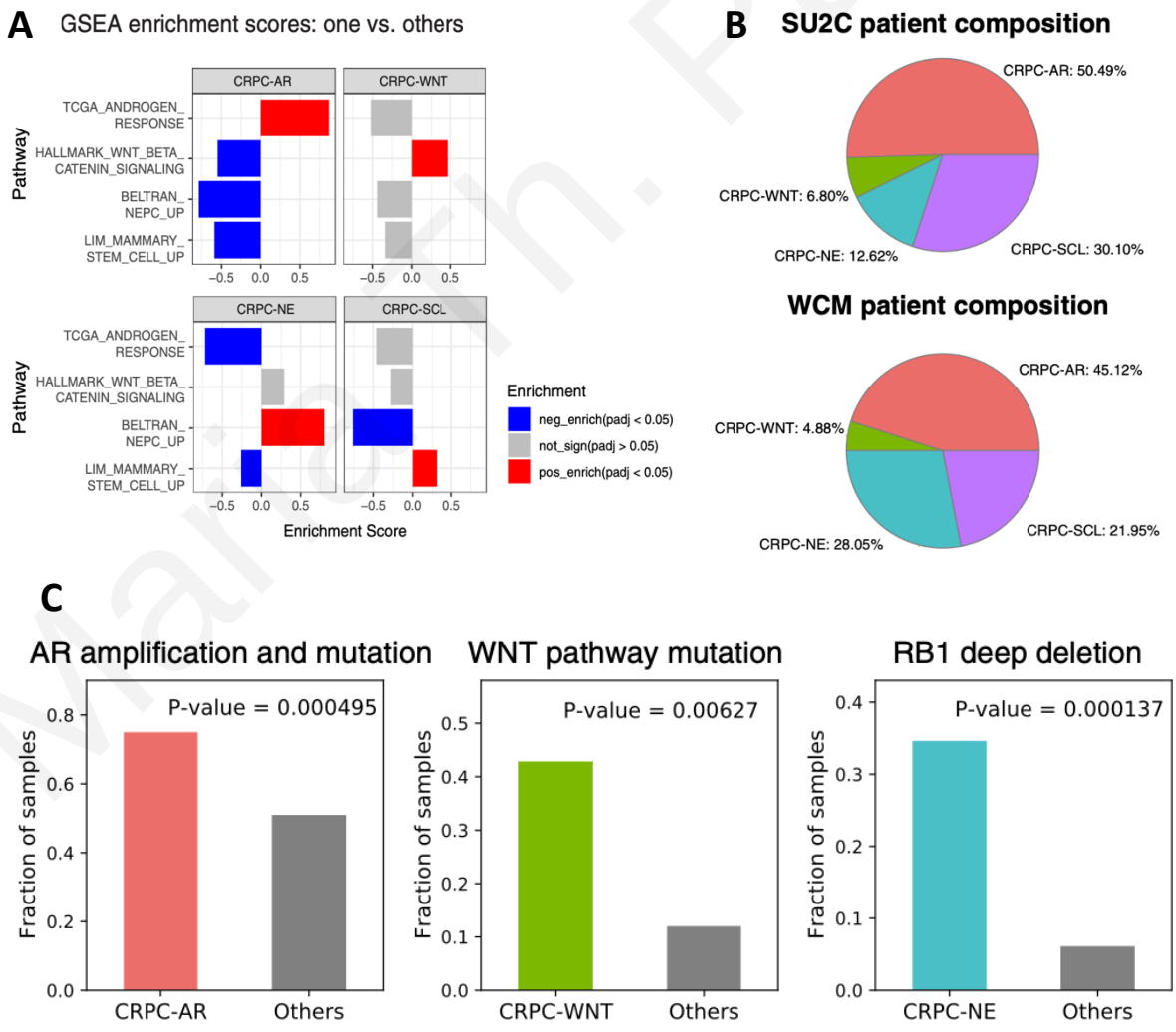
7. Prostate Cancer Organoids and Diagnosis

7.1. Exploitation of Prostate Cancer Organoids for the Identification of Diagnostic Biomarkers

By using PCOs and studying their genomic, epigenomic and transcriptomic profiles, novel biomarkers arose, that contributed to the onset and progression of PCa. As it was previously mentioned, JAK/STAT signaling was enriched in transition stages of the adenocarcinoma to

NEPC progression (Chen et al., 2022). So, TFs that promoted this event, such as STAT1/2 could be used as biomarkers of this transition when identified in cells, after ADT. Hence, PCOs can be used as a platform to collectively identify such biomarkers and be used to develop libraries and categorize patients depending on the subtype of PCa they have (Dutta et al., 2017).

Patient-derived PCOs were developed and analyzed using ATAC-seq assays (assay for transposase-accessible chromatin sequencing) (Tang et al., 2022). Based on the results of the assay, they managed to distinguish the subtypes of CRPC. GSEA and sequencing helped them categorize and identify each subtype, by observing the genes that were expressed. One of them was enriched in AR related genes (CRPC-AR), the second one was overexpressing Wnt pathway related genes (CRPC-WNT), the third subtype was expressing neuroendocrine markers (CRPC-NE), and the last group was identified for the first time (CRPC-SCL). The latter was expressing cancer stem cell markers, such as CD44 and TACSTD2 (Figure 6).



Marian. Patel

Figure 6: Identification of key signatures of four subtypes of CRPC and their use in categorizing patient samples. In panel A, the signaling pathways that are upregulated in each subtype are shown. In panel B the classification of the patient derived organoids using the key molecular signatures is presented in pie charts. The validity of the classification is presented in panel C, in which the classified subtypes were tested for common genomic mutations. Modified and adapted from Tang et al., 2022.

After grouping their organoids, scientists tried to address the TFs that were more highly expressed in each subtype. For CRPC-AR the most expressed TFs were AR and FOXA1, for CRPC-NE were the neurogenic differentiation factor 1 (NEUROD1) and achaete-scute homolog 1 (ASCL1), and for CRPC-WNT was the transcription factor 7–like 2 (TCF7L2). As for CRPC-SCL, the most expressed TF was CD44 (Figure 7).

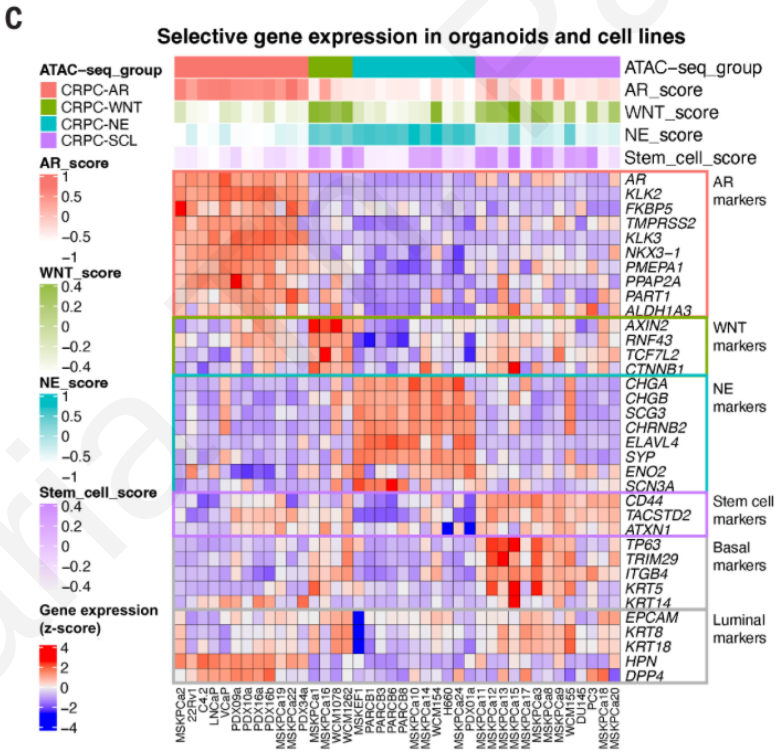


Figure 7: Identification of key transcription factors within each CRPC subtype. In light peach, high expression of AR is observed, which is a signature TF for CRPC-AR. In green high expression of TCF7L2 is observed which coincides with CRPC-WNT. As for CRPC-NE the TFs with the highest expression were SYP and SCG3. The TFs that were observed to be highly expressed for CRPC-SCL were CD44 and ATXN1. Modified and adapted from Tang et al., 2022.

In order to have more than one TF per subtype, scientists focused on the identification of unique key TFs for each subtype, in order to use them as signatures for classification. When they applied these in two patient cohorts, they managed to classify the patients' samples into the four subtypes of PCa. To test the validity of their classification, they performed genomic analysis on the patients' samples and observed that in each subtype the same mutations persisted. For example, most of the samples that were classified as CRPC-AR, had increased mutations and amplification of AR. CRPC-WNT samples had many mutations in Wnt-related genes, and CRPC-NE had deletions of RB1 (Figure 6). The last subtype, CRPC-SCL did not have consistent alterations in their genomic profiles, therefore scientists performed Chromatin Immunoprecipitation Sequencing (ChIP-seq), in order to observe which molecular factors interact with FOSL1, which is another TF that was enriched. YAP, TAZ and TEAD seemed to work together to promote tumorigenesis, and this was verified when they depleted either FOSL1 or YAP/TAZ, because the tumor regressed. In their absence chromatin accessibility is decreased and gene expression was disfavoring the progress of the disease (Tang et al., 2022).

The biomarkers do not always have to be genes or proteins. Epigenetic biomarkers can serve for the early detection as well.

Another study was conducted on prostate cancer organoids, but it was focused on the role of a long non-coding RNA (lncRNA) called H19. H19 seemed to be highly expressed in organoids that are characterized as NEPC, and it was less predominant in organoids of normal PCa biopsy and adenocarcinoma PCa. It was observed that its presence led to the overexpression of genes and markers of NEPC transition. In addition, scientists exploited knockdown experiments and observed that by knocking down H19, NEPC cells that were not responsible to enzalutamide (ENZA, which is used in ADT), regained their sensitivity to the drug [(Figure 8), (Singh et al., 2021)].

Maria Th. Partel

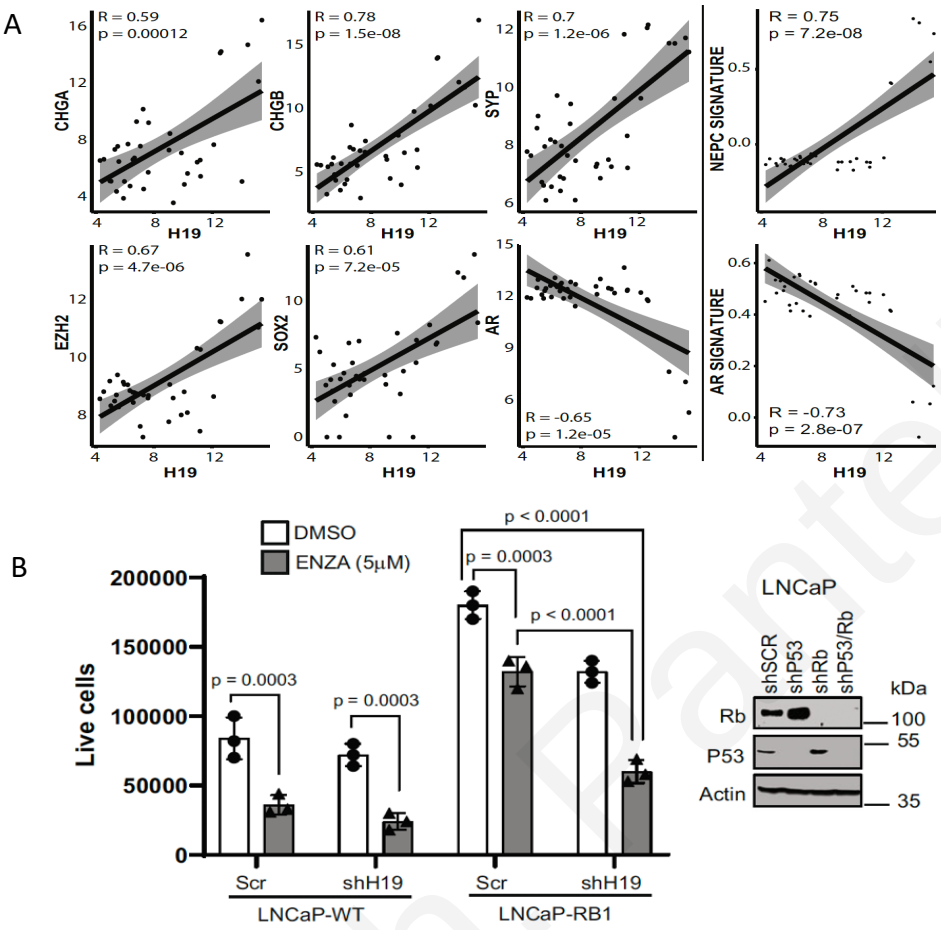


Figure 8: H19 is correlated with the appearance of NEPC character in cells and for the re-sensitization of cells in Enzalutamide. In panel A, the positive Pearson correlation of H19 with NEPC specific markers is shown. Negative correlation is observed between H19 and AR-related markers. In panel B, the increased sensitivity of NEPC cells is shown, in the ones with H19. The number of the live cells decreased when treated with enzalutamide in the presence of H19. Modified and adapted from Singh et al., 2021.

8. Prostate Cancer Organoids and Drugs

8.1. Drug Screening Using Prostate Cancer Organoids

Drug screening is a useful procedure that can uncover the effectiveness of drugs, massively, in large scale experiments, to navigate the focus of drug discovery only towards promising substances. Testing drugs on PCa organoids can serve as a useful analog to figure out how the drug would work *in vivo,* especially if the organoids are derived from patient tissue.

Maria Th Pantel

As it was previously discussed, not all culture conditions are suitable for all research applications on PCa organoids, thus Pappas et al., described exact protocols for developing organoids especially for drug screening (Pappas et al., 2020).

Other scientists were using the organoids to try and repurpose drugs that were used for the treatment of other types of cancer, and therefore, monitored the effectiveness of standard-of-care drugs. For example, Karkampouna et al., performed such tests, and only managed to identify 14 drugs that affected tumorigenesis and cell viability. These drugs mainly affected the AR pathway, HER2/EGFR activity and mTOR pathway [(Figure 9), (Karkampouna et al., 2021)].

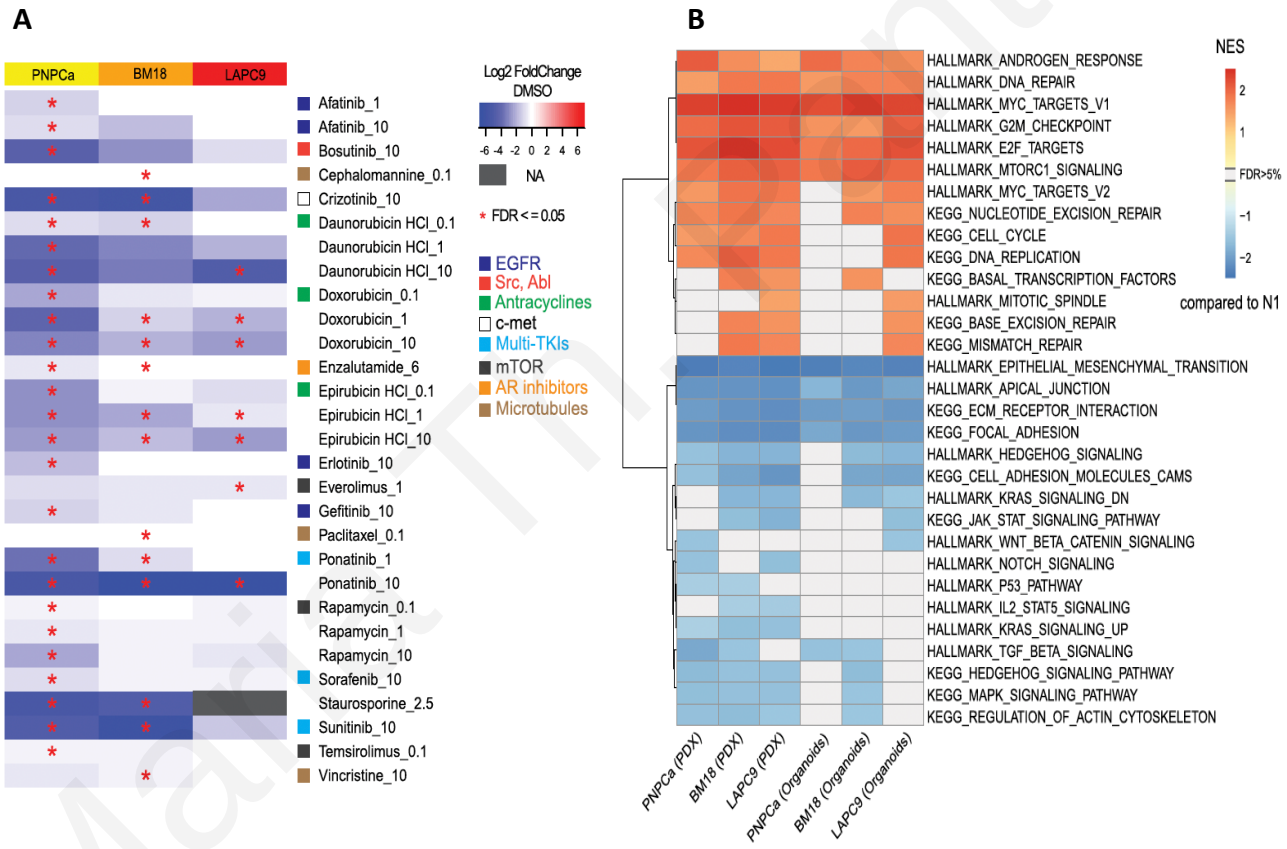


Figure 9: Fourteen drugs that affected cell viability of cultured organoids in drug screening tests. In panel A, a drug screening heat map of cell viability is shown after treating organoids with 14 standard-of-care drugs. Each pathway that these drugs affect is color-coded. In panel B, the altered expression of genes after treatment with the fourteen drugs is presented. Modified and adapted from Karkampouna et al., 2021.

Maria Mitsiadi

Results, like the ones presented above, underline the necessity of developing and identifying novel therapeutic strategies. Apart from these, most of the drugs are more effective in the case of AR-responsive PCa, resulting in shortage for drugs targeting CRPC.

A research team aimed on solving this problem by developing organoids, that derived from PDXs in order to use them as preclinical models for drug screening. These organoids captured all possible forms of PCa, as some of them were of AR-dependent PCa nature, and some other were of neuroendocrine nature. In addition, their method was able to capture differences in the morphology or in the composition of the organoid, something that previous researchers did not manage to do, because of the fact that results in organoids are not always reproducible, because of the variability of the organoids themselves. The scientists decided to test talazoparib, which was a compound undergoing phase 3 of clinical trials for the treatment of mCRPC. The organoids derived from both untreated and treated tumors. 287R was a castrate sensitive adenocarcinoma organoid, 224R-Cx and 305R-Cx derived from NE-PCa and 201.1-Cx was a metastatic CRPC with adenocarcinoma organoid. By increasing the doses of Tala, scientists observed that the metabolic rate and cells' viability of all organoids were decreasing [(Figure 10), (Choo et al., 2021)].

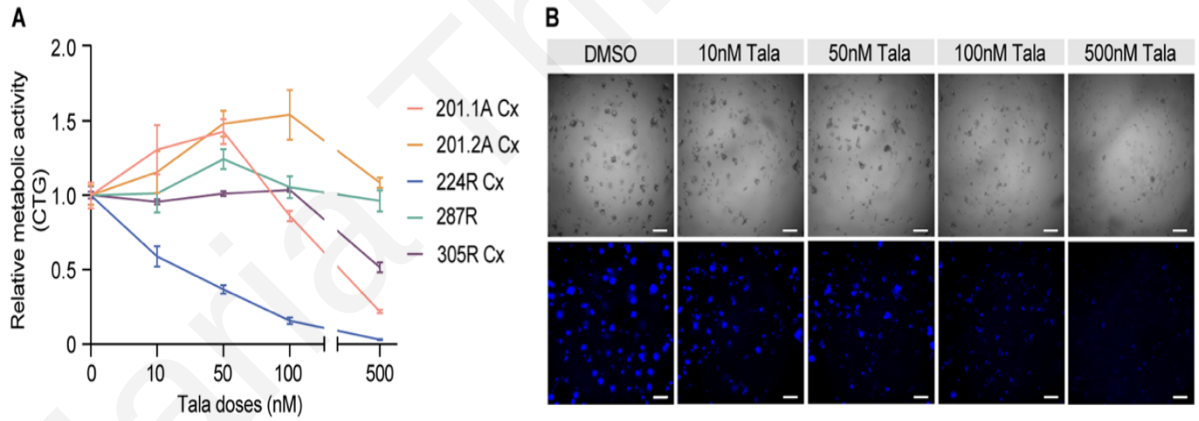


Figure 10: Increasing doses of Talazoparib seemed to inhibit viability of Prostate Cancer Organoids. In panel A, five types of organoids, with different PCa identity were subjected to increasing doses of Tala, and decreased metabolic rate was observed. In panel B, decreased cell viability was observed in 224R-Cx organoids, after Hoechst staining. Modified and adapted from Choo et al., 2021.

Maria Th. Pantelli

8.2. Studying Drug Resistance in Prostate Cancer Organoids

Since drug resistance is one of the major obstacles when it comes to PCa therapy, scientists exploited organoids to study the mechanisms in these platforms too, in order to elucidate any key major events that would help eliminate this problem. Apart from the JAK/STAT's contribution to lineage plasticity, there were also some molecular mechanisms that resulted in ADT resistance.

Tumor microenvironment can also contribute to drug resistance. Scientists discovered that certain cancer-associated fibroblasts, called CAFs, could interact with the tumor cells derived from PDX and promote resistance to bicalutamide and enzalutamide, way faster than when the cells were absent. Further on, they tested whether this was due to cell interaction or secretion of factors from the cells, thus they subjected PDX organoids to a media containing the secretion of CAFs' and they observed that cell number was drastically increased. To address which factor was responsible, the secretions of the CAFs were subjected in a series of fractionations and observed their resistance activation ability by identifying the activity of HER3 kinase. In the subfraction that activated HER3, they searched and found Neuregulin 1 (NRG1), which is a known ligand of HER3. They also observed that ADT therapy is what induces the expression of NRG1 in stromal cells [(Figure 11), (Zhang et al., 2020)]. Since it was shown that the TME can contribute to tumorigenesis, further studies must be performed.

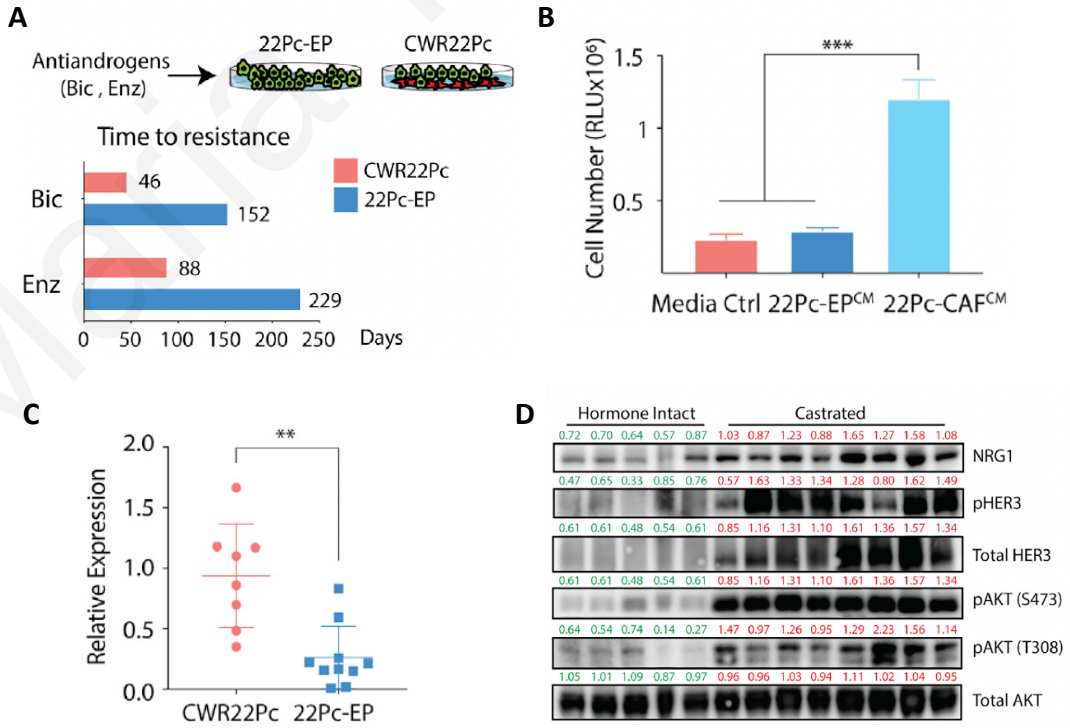


Figure 11: Identification of NRG1, a secreted protein of CAFs, as a factor that promotes drug resistance, by activating HER2 kinase. In panel A, the rapid acquire of resistance is presented in the co-cultures of the tumor cells, along with CAFs. In panel B the increase in the cell number of the culture organoids is presented, after introducing them to the secretions od CAF. In panel C, the increased expression of NRG1 in CAF cells is presented. In panel D, the NRG1 expression along with the HER3-AKT activation is presented, in both hormone intact and castrated tumors. Modified and adapted from Zhang et al., 2020.

9. Research for Therapeutic Strategies using Prostate Cancer Organoids

9.1. Development of Novel Therapeutic Strategies for Prostate Cancer Using Prostate Cancer Organoids

Biomarkers are not only useful for the early prognosis of PCa, as they also control functions of major significance when it comes to the progression of PCa. They can also serve as therapeutic targets, because from most of the experiments their depletion was associated with the regression of the disease. In the research conducted by Chan et al., scientists tried to reprogram patient derived organoids (PDOs) to acquire their luminal characteristics, using two kinase inhibitors, Ruxolitinib and Erdafitinib, in order to restore sensitivity in AR signaling pathway, and make them more responsive to treatment. What they observed was that most of the PDOs increased AR signaling, but a relatively high number of PDOs remained AR^-. This must be further studied, to address the reason that this happens. It could be either because of higher complexity in human cancers or because of the higher amount of time that human PDOs needed in order to be reprogrammed [(Figure 12), (Chen et al., 2022)].

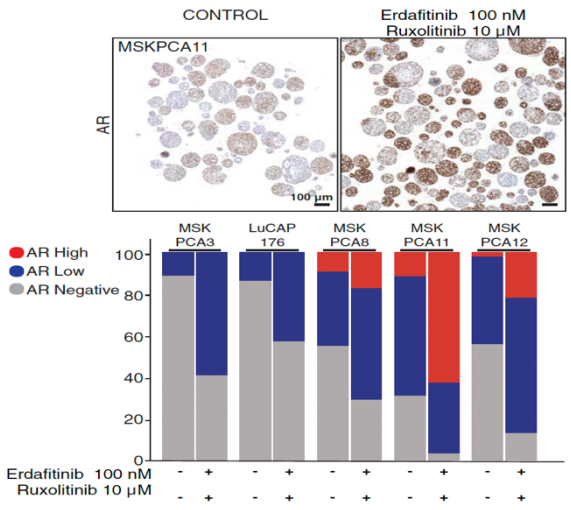


Figure 12: Reprogramming of NEPC patient-derived organoids, as a therapeutic attempt. In the uppermost panel, PDOs were stained for AR signaling pathway. Most of the control cells were negative, as opposed to organoids after undergoing treatment with Erda and Rux, in order to be reprogrammed. The bar chart indicated the sensitivity to AR signaling after treatment with the two drugs in PDOs. Erda: Erdafitinib; Rux: Ruxolitinib; PDOs: patient-derived organoids. Modified and adapted from Chen et al., 2022.

Another example of trying to affect the biomarkers as a therapeutic attempt, was performed in the research conducted by Tang et al., in which they tried to use Verteporfin, a drug known to inhibit the function of YAP/TAZ in macular degeneration. Two PDOs were used. One belonged to the CRPC-AR subtype (MSKPCa2) and the other one to the CRPC-SCL subtype (MSKPCa3). Verteporfin affected the latter PDO more than the first one (Figure 13), signifying the importance of firstly taking into consideration the heterogeneity of PCa and then choosing the most suitable treatment option (Tang et al., 2022).

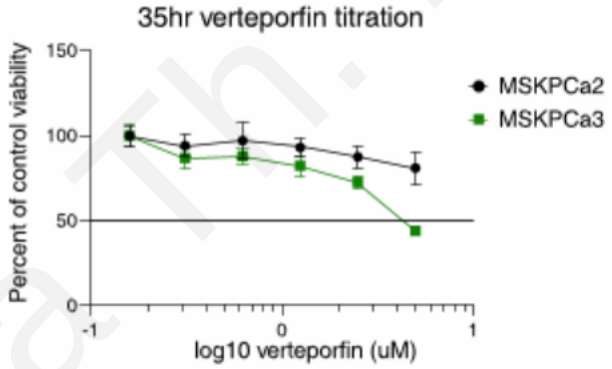


Figure 13: Subjecting PDOs in verteporfin to inhibit the effect of YAP/TAZ complex in CRPC. Two types of PDOs were used. One that was classified as a CRPC-AR subtype (MSKPCa2) and another one that it was classified as CRPC-SCL (MSKPCa3). As it is indicated by the graph, the latter responded better to verteporfin. PDOs: patient-derived organoids; CRPC: castration-resistant prostate cancer; AR: androgen receptor; SCL: stem cell-like. Modified and adapted from Chen et al., 2022.

Another therapeutic approach that can be followed is, firstly, to overcome drug resistance in CRPC, and, secondly, use already established drugs, to eliminate further progression of the disease. This was studied in a project carried by Xu et al., in which they observed the significance of Heat shock protein (HSP70) in the homeostasis of the CRPC-emergence

Maria Th. Panteli

responsible, AR-V7. Scientists incubated PDX organoids with JG98 (which is an inhibitor of HSP70) along with Enzalutamide and measured the viability of the cells within the organoid, as well as live and dead cells, using immunofluorescence. It was shown that the viability was decreased (Figure 14). These results were not observed in cells or PDXs that were AR resistant and of CRPC nature (Xu et al., 2023).

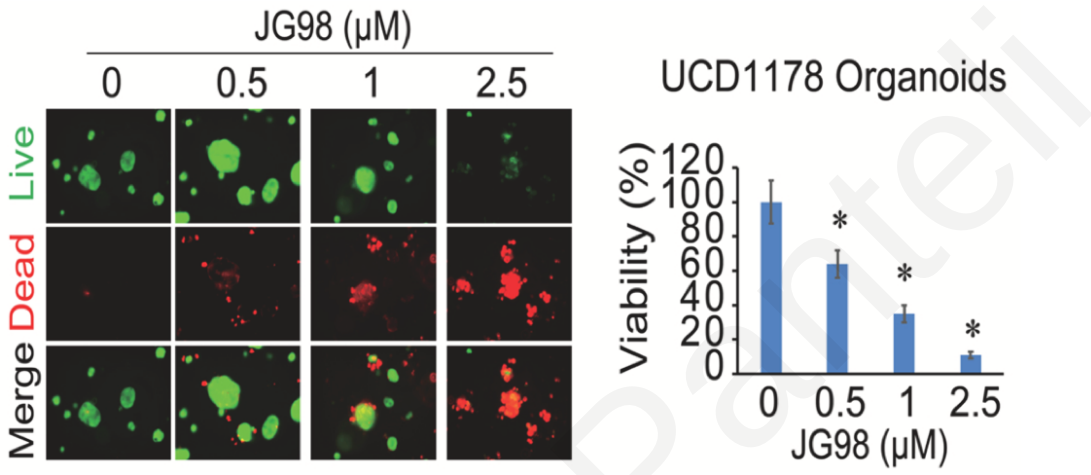


Figure 14: Decreased viability was observed in cells of PDX organoids after their incubation with JG98 and Enzalutamide. In the immunofluorescence panel, an increase in the dead cells (stained with red) was observed with the increase of JG98 and the presence of Enzalutamide. These was also observed and quantified after measuring the viability of the cells, using Cell-Titer Glo Luminescent Assay. Modified and adapted from Xu et al., 2023.

Combinatorial use of newly discovered inhibitors of pathways hijacked by PCa, along with utilization of conventional drugs, after restoring the sensitivity of the cells to them, is the epicenter of many studies. As this approach is really promising, more inclusive studies must be conducted to also emphasize the effect that the tumor microenvironment might hold, and maybe its utilization in therapeutic approaches.

Researchers along with the research of unraveling new therapeutic methods, were also using the organoids to test traditional therapeutic methods like irradiation or already approved drugs, to know whether their newly designed techniques were more effective, than already established ones. For example, Karkampouna et al., tested the effect that irradiation had on organoids, and observed that viability of cells was declining, as expected (Figure 15). Experiments like this also proved that organoids are suitable *in vitro* models for PCa, as they responded in the same way that in vivo tumors in patients, do.

Maria Th. Panteli

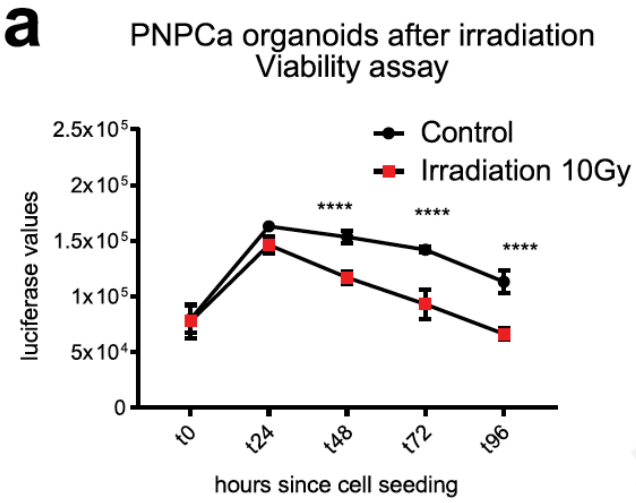


Figure 15: Standard-of-care irradiation of PDOs reduces cell viability. In the plot PDO is being irradiated and compared with a control PDO, to observe cell viability. Irradiation dramatically decreases cell viability. Modified and adapted from Karkampouna et al., 2021.

In this kind of research, downsides of using organoids can also observed. Organoids were developed from patients P82, who was in late-stage cancer and P134, who was in primary stages. Both of these organoids were resistant in treatment with enzalutamide. This observation did not coincide with the *in vivo* results, as P82 organoid seemed to be responsive to treatment, while P134 was not. So, in this case if the treatment followed, was only based on the experimental results on the organoids, it would not provide proper information for its efficacy when applied to patients (Karkampouna et al., 2021).

9.2. The Role of the Extracellular Matrix of Prostate Cancer Tumors in Various Treatment Procedures

Tumor microenvironment is of major importance when it comes to the development of the disease. Many scientists tried to decipher multiple mechanisms that are crucial for undergoing differentiation to NEPC in organoids, but this was difficult because culture medium does not fully represent the extracellular matrix, or the TME of the tumor. Recapitulating the TME is of major significance, since it was found that it is responsible for NEPC transition.

In a study conducted by Mosquera et al., scientists decided to study and analyze the microenvironment from patient tumor tissues and try to synthesize an ECM, that would resemble it as much as possible. Firstly, they had to analyze and identify all the components that took part in the formation of the ECM in the patient's tumor tissue. By exploiting RNA-

Maria Th. Panteli

seq and other genomic and proteomic techniques, it was observed that the ECM of both CRPC-AR and CRPC-NE was specifically enriched in proteins such as Collagen, Fibronectin, Vitronectin, and other cell adhesion proteins, in contrast with benign tumors (Figure 16).

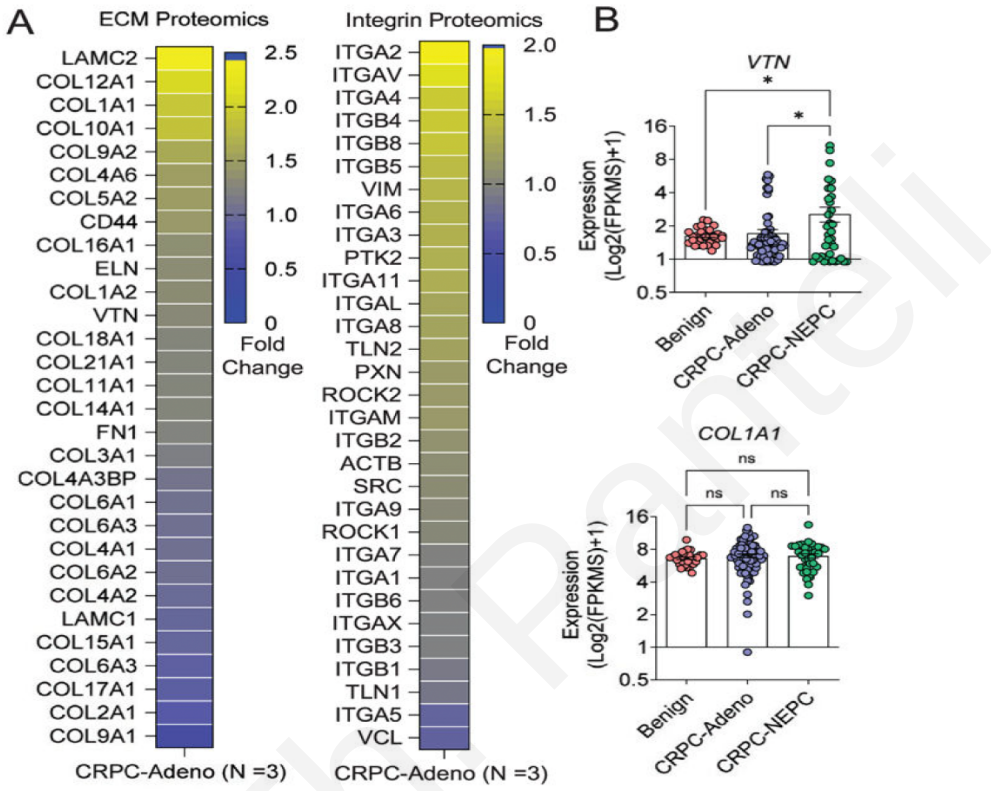


Figure 16: The ECM of the patient's tumor tissue is specifically enriched in cell adhesion proteins. In panel A, mass spectrometry results of the components of the extracellular matrix and integrin's signaling are presented. In panel B the increase in two of these components is shown, in both CRPC-AR and CRPC-NE, in contrast with the benign tissues. Modified and adapted from Mosquera et al., 2023.

After addressing the *in vivo* ECM's components, they managed to develop a synthetic hydrogel ECM, on which the organoids could grow. They observed that on this ECM, the organoids could behave normally and were able to undergo EMT and remodel the actin cytoskeleton.

One of the catalytic factors of NEPC transition is the epigenetic silencing that is caused by the Polycomb group proteins. In this case, epigenetic modifiers such as EZH2 (which promotes the methylation of H3K27Me2 to H3K27Me3) were overexpressed. This was also observed in this synthetic hydrogel-based PCOs.

María Tu Pantoja

Different synthetic subtypes of ECMs were created and seemed to differentially regulate gene expression. Each ECM was enriched in different peptides mimicking some of the cell adhesion proteins and their interactions with other accessory proteins. For example, RGD peptide mimicked Vitronectin, REDV mimicked fibronectin and GFOGER is a peptide that mimicked collagen. Each one drove the down- or up-regulation of different genes in both types of organoids (Figure 17).

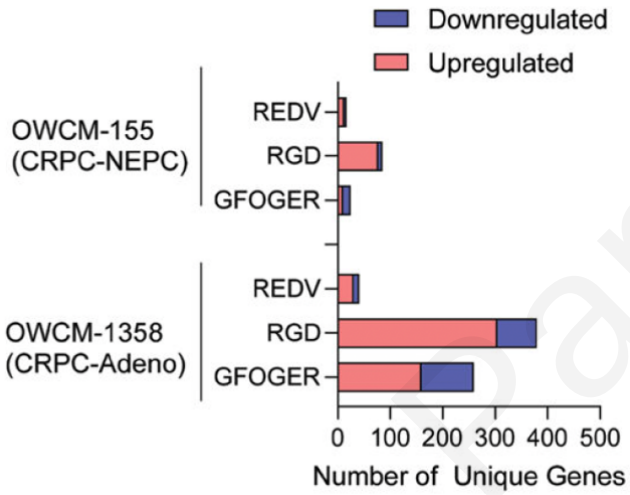


Figure 17: Unique gene expression is observed in different subtypes of synthetic ECMs. Each ECM is enriched in different peptides, that mimic the function of cell adhesion proteins. Each one promotes or obstructs the expression of different set of genes, in both CRPC-AR and CRPC-NE organoids. Mosquera et al., 2023.

These findings suggested that each of the ECM's components were crucial on the disease's trajectory. Results like this must be taken seriously into consideration because they might explain the diverse response to different therapeutic methods.

The diverse composition of these synthetic ECM's also affected EZH2's function, because the H3K27Me3 levels varied. The scientists took advantage of that and introduced an inhibitor of EZH2 (EZH2i) and observed how the organoids responded. In all three ECMs the organoid became smaller after treatment with the inhibitor, with bolder effects in GFOGER and REDV matrices (Figure 18). This indicated that ECM directly affected the effectiveness of the treatment.

Maria Th. Panteli

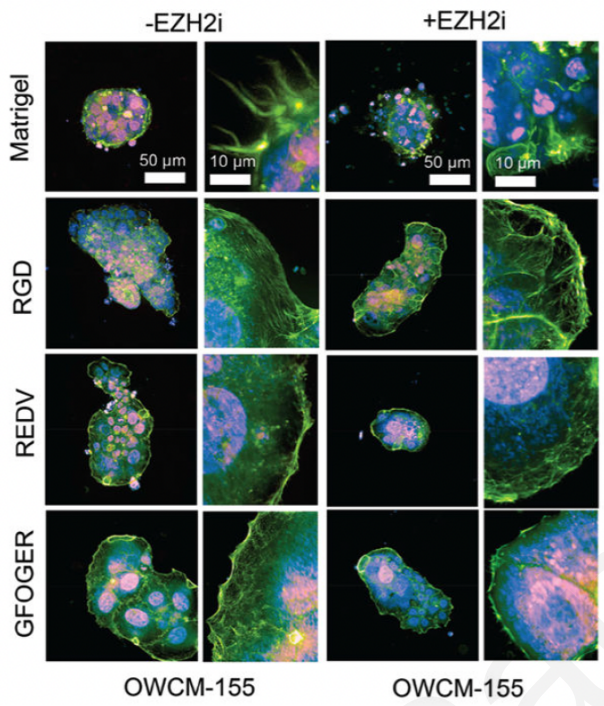


Figure 18: Decreased size of CRPC-NE organoids was observed after using an inhibitor of the EZH2. CRPC-NE organoids' size was reduced after treatment with EZH2 inhibitor. More profound effects were observed GFOGER and REDV organoids. Purple indicates the expression of EZH2, blue stains the nuclei of the cells and green stains the actin filaments. Modified and adapted by Mosquera et al., 2023.

To further investigate the effect that ECM had on therapeutic approaches, they decided to investigate another therapeutic approach by targeting the Dopamine Receptor D2 (DRD2), with two small molecule inhibitors (ONC201 and ONC206) that were used in glioblastoma treatments. They observed that the drugs altered the morphology of the organoids that were cultured in GFOGER and REDV ECM's but not in Matrigel or RGD (Figure 19).

Maria Th. Panteli

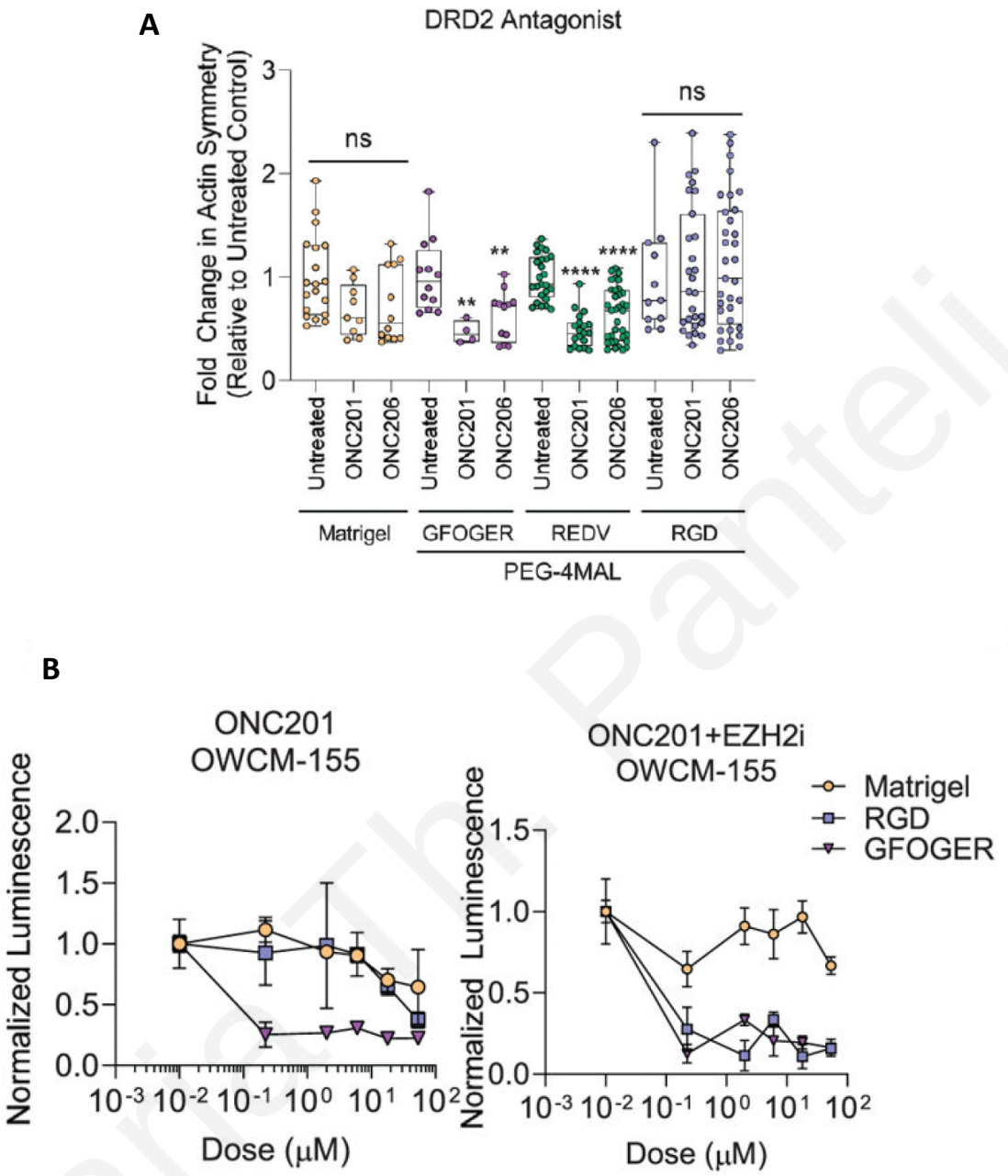


Figure 19: DRD2 inhibition directly affects the morphology of the organoids, and when combined with EZH2 inhibitors, the therapeutic effect of EZH2 inhibition is elevated. After inhibiting DRD2 the actin symmetry of organoids in GFOGER and REDV was altered, as opposed to organoids in Matrigel and RGD. Treatment of organoids with EZH2i, increased the response of organoids grown in RGD, and made them more responsive to EZH2 inhibition therapy. Modified and adapted by Mosquera et al., 2023.

Mario Th. Pantelj

They, then, tried to combine the two therapeutic approaches (EZH2i with ONC201) to observe the effect they had on the organoids. In the presence of both molecules the RGD-grown organoids became responsive to EZH2 inhibition, as opposed to the Matrigel organoids, that remained unresponsive to these approaches (Figure 19). Apart from the effect that the ECM had on the treatment effectiveness, this part of the research, also signified the importance of combinatorial therapeutic approaches.

By exploiting PDOs precision medicine and development of patient-tailored therapies is one step closer. On the other hand, new problems arise with this approach such as ethical problems and limited access in these diagnostic and therapeutic approaches.

Discussion and Conclusion

10. Summarizing the Major Findings of the Field

This project was focused on identifying the main advances in the field of Prostate Cancer, especially regarding the development and use of Prostate Cancer Organoids. By studying the current literature and the progress in the field, the potential for identification of novel key molecular players that could serve as biomarkers for early prognosis, was discussed. The existence of limitations and the requirement for improvements, were also presented.

As it is widely known, PCa is one of the leading diseases responsible for the death of a great percentage of men. In order to study the progression of the disease, several models are used such as PDXs implanted in immunocompromised mice, cell lines, and *in vivo* models such as mice. Because cell lines can not

recapitulate what happens in a 3D tumor, and PDXs are not grown in an environment, which is even remotely similar to that found in a patient, these models, can sometimes, be insufficient. Therefore, in the last decade, development of PCa organoids was invented. Several laboratories tried to identify the exact conditions under which an organoid should be grown, to represent as much of the primary tumor as possible. What remains unresolved is the need to recapitulate the exact ECM conditions of the organoid, as it was shown that ECM highly affects the process of tumorigenesis. Moreover, scientists focus on identifying new techniques that will allow them to capture as much information as they can from the primary tumor.

The anatomy and molecular mechanisms of the PG are often altered leading to the onset of PCa. These observations can also be made in organoids, since many molecular pathways can be altered, each one contributing to the events of tumorigenesis. Such pathways involve the AR-signaling pathway, the PI3K/Akt/mTOR signaling pathway, the Wnt signaling pathway and, last but not least, the JAK/STAT pathway. Genetics are also responsible because of the

Maria Th. Pantelli

differential expression of tumor suppressors or oncogenic genes (Vietri et al., 2021). Epigenetic factors, such as histone modifiers or chromatin remodelers, also impair the homeostasis of the metabolism of the cells and drive them towards a cancerous nature (Pardo et al., 2022).

As PCa progresses, lineage plasticity takes place and cells acquire different genotypic and phenotypic characteristics, resulting in the various forms of PCa. The major change that cells undergo, is the acquired independency from ARs, after ADT. In this case, cells start to express NE-specific genes, such as synaptophysin, and are considered to be more aggressive, because of their increased metastatic ability (Haffner et al., 2021). In that stage PCa is identified as CRPC, and organoids, are accurate models to capture this shift.

For the diagnosis of PCa, blood tests, biopsies and imaging techniques are commonly used, but it was observed that, in some cases these turned back false negative or false positive results. Thus, the organoids can serve as tools in order to identify biomarkers that might be useful for identifying more accurately the disease. Examples of such biomarkers are differentially expressed genes (such as AR related genes), epigenetic factors (such as lncRNAs) (Singh et al., 2021) and several promising transcription factors (such as FOSL1 for CRPC-NE and TCF7L2 for CRPC-WNT) (Tang et al., 2022). Based on the research conducted, the optimum way for identifying PCa, is to perform a combinatorial screening in order to be able to distinguish each tumor according to the subtype that it belongs. Addressing biomarkers that strictly characterize each stage of PCa, can be of major clinical significance, because the therapeutic approaches that will be followed are going to be tailored to each subtype.

When it comes to available treatments, the standard-of-care therapeutic techniques are ADT, surgical castration, chemotherapy and radiation. These techniques are effective only for a short period of time, because PCa progress to its AR-independent form, and becomes severely aggressive and metastatic. Thus, by research conducted on organoids, it was shown that only a limited number of drugs could work in this case, and no repurposed drugs seemed to be effective (Karkampouna et al., 2021). This is mostly attributed to the acquired drug resistance, that forces the cells to differentiate into a subtype that will not be affected from drugs. TME seemed to contribute to such events, as secretions of CAFs that contained NRG1 activated HER3 kinase (Zhang et al., 2020). This signifies the importance of not only studying the cells found within the tumor, but also cells that belong in the surrounding TME or ECM, which equally affect the progression of the disease.

Because of the fact that existing drugs are not a permanent therapy for this disease, scientists are trying to establish novel therapeutic techniques. Research is focused, especially, in mCRPC or NE-CRPC, as these two subtypes are more life threatening. The first approach was focused

Maria Th. Panteli

on identifying biomarkers as plausible targets for drugs. For example, by knocking down Jaks, scientists observed that lineage plasticity was reduced (Chan et al., 2022). Such results are promising for developing therapies that will aim on impeding the progression towards more aggressive subtypes. Moreover, scientists aimed on addressing ways to overcome already-acquired drug resistance to make cells sensitive to existing drugs. This approach will possibly eliminate the shortage of chemical compounds that can target several subtypes of PCa, and it will also minimize the effect that drug resistance has in the quality of patients' lives. Additionally, by creating organoids derived from patients' an indicative response can be seen, in order to predict how the drug would affect the tumor *in vivo.* Combinatorial use of existing and newly discovered therapies can be the setting stone towards minimizing the health implications of PCa.

Some repurposed drugs showed promising results in reverting the acquired resistance of CRPC. One example was the enzalutamide which was able to target the AR pathway and revert the resistance to it. A few more examples were afatinib, erlotinib and gefitinib which were able to act against the EGFR/HER2 pathways. The other drugs that showed promising results as well, affected the DNA replication mechanism and several pathways in which Tyrosine Kinases had a major role (Karkampouna et al., 2021). All the aforementioned drugs, aim to hijack the mechanisms of the cancerous cells, and reprogram them, in order to acquire their initial, luminal characteristics.

One example of a chemical compound that could be used as a putative novel drug in CRPC was Verteporfin since it was shown that it could affect organoids belonging both to the CRPC-AR subtype and to the CRPC-SCL subtype. Verteporfin affected the latter PDO more than the first one signifying the importance of firstly taking into consideration the heterogeneity of PCa and then choosing the most suitable treatment option (Tang et al., 2022).

Since ECM and TME were shown to affect tumorigenesis and PCa's progression, both of them might serve as targets for therapies. The composition of both matrices is proved to alter the response to therapy, thus by addressing the factors that cause these events, inhibiting them might lead to the increase of the therapy's effectiveness.

To conclude, major advancements were made in the development of this field. Biomarkers were identified and possible therapeutic targets were established, in order to minimize the effects of this disease. Despite this fact, the current literature still has some gaps that are essential to be filled in order to perform bigger leaps in the advancement of the field, regarding both PCa and its study using PCOs.

Maria Th. Pantelli

11. Future Perspectives

Further research for both PCa and the development of organoids must be conducted because there are several gaps in the literature that must be filled. The most important gap was the lack of literature and research regarding mCRPC. It is a subtype which is characterized by a continuously changing phenotypic and genomic nature. Nevertheless, is of major clinical significance because it costs the lives of many patients, since it remains mostly untreated.

As for a possible therapeutic approach that can be further studied, is to target PCa from as soon as its initiation, as a preventive measure. This is what ADT was trying to do, but it was proved that it was not effective in the long-term. This will result in reducing the risk for the tumor to differentiate into a more aggressive subtype.

As for the organoids, another gap that was observed, was the organoid development and culture. Maybe, in the future, scientists will be able to replicate the exact environment that is found *in vivo*, by designing culture conditions that will include stromal and immune cells, or maybe even the blood supply or the tumor architecture. This will be initiated, only after the standardization of the culture conditions, in order for the results of several laboratories to be comparable and reproducible. This will also result in increasing their efficacy, for using them as drug testing platforms, in order to better mimic clinical responses.

By being able to image the organoids in real time, further information can be gathered in order to monitor the exact changes they undergo, as they are influenced by drugs or as they interact with other cells from their surroundings.

Last but not least, by identifying ways to reduce the cost of developing and maintaining PCa organoids, will result in the broader use of this model. In order for all of the above improvements and future studies to happen, collaboratively work within the scientific community must be made.

Maria Th. Pantel

Abbreviations

ADT: Androgen Deprivation Therapy

AR-V7: Androgen Receptor splice Variant 7

AR: Androgen Receptors

ASCL1: Achaete- Scute Homolog 1

ATAC- seq assays: assay for transposase-accessible chromatin sequencing

AVPCa: Aggressive Variant Prostate Cancer

BRCA1: Breast Cancer 1

BRCA2: Breast Cancer 2

CAFs: cancer-associated fibroblasts

CD44: Acidic Cell surface adhesion protein

ChIP- seq: Chromatin Immunoprecipitation Sequencing

CK1: Casein Kinase 1

CoA: Co- Activator

CoR: Co- Repressor

CRISPR- Cas9: Clustered Regularly Interspaced Short Palindromic Repeats- Caspase 9

CRPC: Castration- Resistant Prostate Cancer

CYP17A1: 17–20 lyase and 17-α hydroxylase of the cytochrome P450 family

DHT: Dihydrotestosterone

DNMTs: DNA methyltransferases

DRD2: Dopamine Receptor D2

DRE: Digital Rectal Examination

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

EMT: Epithelial- To- Mesenchymal Transition

EMT: Epithelial- to- Mesenchymal Transition

ENZA: Enzalutamide

ESCs: Embryonic pluripotent Stem Cells

ETS: Erythroblast Transformation Specific

EZH2: Enhancer of Zeste Homolog 2

EZH2i: inhibitor of Enhancer of Zeste Homolog 2

FOSL1: AP-1 transcription factor subunit

FOXA1: forkhead box A1

GFRs: Growth Factor Receptors

Maria Th. Panteli

GSEA: Gene Set Enrichment Analysis

GSK3: Glycogen Synthase Kinase 3

H19: Imprinted Maternally Expressed Transcript

H3K18Ac: Acetylation of the eighteenth lysine residue of histone 3

H3K27Me2: di-methylation of the 27th lysine in Histone 3

H3K27Me3: tri-methylation of the 27th lysine in Histone 3

H3K4me: Methylation of the fourth lysine residue of histone 3

HER2/EGFR: Human Epidermal Growth Factor Receptor Type 2

HER3 kinase: Human Epidermal Growth Factor Receptor Type 3 kinase

HPSE: Heparanase

HR: Homologous recombination

Hsp: Heat shock protein

HSP70: Heat Shock Protein

iPSCs: induced Pluripotent Stem Cells

JAK/ STAT: Janus kinase/ signal transducer and activator of transcription

JG98: inhibitor of HSP70

LncRNA: long non- coding RNA

mCRPC: Castration-Resistant Prostate Cancer

MMR: mismatch repair

MRI: Magnetic Resonance Imaging

MSH: mutS Homolog

mTORC1: Mammalian target of rapamycin complex 1

mTORC2: Mammalian target of rapamycin complex 2

NCOA1/2: Nuclear Receptor Co- Activator

NCORI1/2: Nuclear Receptor Co- Repressor

NE: Neuroendocrine markers

NEPC: Neuroendocrine Prostate Cancer

NEUROD1: neurogenic differentiation factor 1

NLS: Nuclear Localization Signal

NRG1: Neuregulin 1

P13K/Akt/ mTOR: Phosphatidylinositol-3-kinase/Protein kinase B/ Mammalian target of rapamycin

PCa: Prostate Cancer

PCOs: Prostate Cancer Organoids

Maria Th. Panteli

PDK1: Pyruvate Dehydrogenase Kinase 1

PDOs: Patient Derived Organoids

PDX: Patient- Derived Xenografts

PG: Prostate Gland

PIN: Prostate Intraepithelial Neoplasia

PSA: Prostate-specific antigen

PTEN: Phosphatase and Tensin Homolog

Rb: Retinoblastoma Protein

RB1: Transcriptional Corepressor 1

SCL: Stem Cell- like

TACSTD2: Tumor Associated Calcium Signal Transducer 2

TAZ: Transcriptional Coactivator with PDZ- binding motif

TCF7L2: Transcription factor 7- like 2

TEAD: Transcriptional Enhanced Associated Domain

TF: Transcription Factor

TME: Tumor Microenvironment

Trp53: Transformation- related protein 53

TSC2: Tuberous Sclerosis Complex 2

WES: Whole Exome Sequencing

YAP: Yes- associated Protein

Maria Th. Panteli

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Maria Th. Pantelli