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MicroRNA profile associated with enzalutamide resistance in Prostate Cancer: an opportunity for biomarker and therapeutic target identification

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MICRORNA PROFILE ASSOCIATED WITH ENZALUTAMIDE RESISTANCE IN PROSTATE CANCER: AN OPPORTUNITY FOR BIOMARKER AND THERAPEUTIC TARGET IDENTIFICATION

Dissertação de Candidatura ao Grau de Mestre em Oncologia – Especialização em Oncologia Laboratorial submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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Abbreviations

#

2D - Two-dimensional

3D - Three-dimensional

A

ADT - Androgen Deprivation Therapy

AGO2 - Argonaute 2

AKT - AKT serine/threonine kinase

AR - Androgen Receptor

ARE - Androgen response elements

ATCC - American Type Culture Collection

ATP - Adenosine Triphosphate

B

B2M - Beta-2 microglobulin

BER - Base Excision Repair

BRAF - v-raf murine sarcoma viral oncogene homolog B

BSA - Bovine serum albumin

C

C1 – Spheroid cell density of 5 000 cells

C2 – Spheroid cell density of 10 000 cells

C3 – Spheroid cell density of 20 000 cells

cDNA - complementary Deoxyribonucleic Acid

cfDNA - circulating free Deoxyribonucleic Acid

CLSM - Confocal laser scanning microscopy

CO2 - Dioxide Carbon

CRPC - Castration resistant prostate cancer

CT - Cycle Threshold

CTC - Circulating Tumor Cell

D

DAPI - 4′,6-diamidino-2-phenylindole

DBD - DNA-binding domain

DGCR8 - Double stranded RNA binding protein DiGeorge syndrome critical region

DHT - Dihydrotestosterone

DNA - Deoxyribonucleic Acid

DPBS - Dulbecco's Phosphate-Buffered Saline

DRE - Digital rectal examination

DROSHA - Double stranded RNAse III enzyme

E

EAU - European Association of Urology

ECM - Extracellular Matrix

EDTA - Ethylenediamine tetraacetic acid

EGF - Epidermal Growth Factor

EGFR - Epidermal Growth Factor Receptor

EMT - Epithelial mesenchymal transition

ENZ – Enzalutamide

ERK - Extracellular signal-regulated kinase

ESR - Estrogen Receptor

EVs – Extracellular vesicles

F

FBS - Fetal Bovine Serum

FDR - False discovery rates

G

GAPDH- Glyceraldehyde-3-Phosphate Dehydrogenase

GnRH - Gonadotropin-releasing hormone

GO - Gene Ontology

GR - Glucocorticoid Receptor

GTP - Guanosine Triphosphate

GUSB - Glucuronidase beta

H

HDI - Human Development Index

I

IARC - International Agency for Research on Cancer

IC50 - Half maximal inhibitory concentration

ISUP - International Society of Urological Pathology

K

KEGG - Kyoto Encyclopedia of Genes and Genomes

KRAS - Kirsten rat sarcoma virus

L

LBD - Ligand-binding domain

LNCaPER - LNCaP enzalutamide- resistant (30 μM)

M

MAPK - Mitogen-activated protein kinase

MEK - Mitogen-activated protein kinase kinase

miRNA - microRNA

MMR - Mismatch repair

mRNA - messenger Ribonucleic Acid

mTOR - mammalian Target of Rampamycin

MVBs - Multi-vesicular bodies

N

ncRNA - non-coding

NE - Neuroendocrine

NER - Nucleotide-excision repair

NTA - Nanoparticle Tracking Analysis

NTD - N-terminal domain

P

PC - Prostate cancer

PEN-STREP - Penicillin-Streptomycin

PFA - Paraformaldehyde

PI - Propidium iodide

PI3K - Phosphoinositide 3-kinase

PIN - Prostatic intraepithelial neoplasia

PPI - Protein-protein interaction

Pre-miRNA - precursor-microRNA

Pri-miRNA - primary-microRNA

PSA - Prostate-specific antigen

PTEN - Phosphatase and Tensin Homolog

Q

qPCR - quantitative Polymerase Chain Reaction

R

RISC - RNA-induced silencing complex

RNA - Ribonucleic Acid

RPMI - Roswell Park Memorial Institute

S

Sph - spheroid

STAT - Signal Transducer and Activator of Transcription

STRING - Search Tool for the Retrieval of Interacting Genes

T

TEM - Transmission Electron Microscopy

TGF- α -Transforming Growth Factor Alpha

TGF-β - Transforming Growth Factor Beta

TME- Tumor Microenvironment

TNF - Tumor necrosis factor

TRBP - Transactivation Responsive RNA Binding Protein

TRUS - Transrectal ultrasound

U

UTR - Untranslated Region

W

WST - Water-soluble tetrazolium salt

X

XPO5 - Exportin 5

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Resumo

O cancro da próstata (CP) é um dos tumores malignos mais frequentemente diagnosticado e a quinta principal causa de morte por cancro no homem. Uma das principais estratégias de tratamento do CP avançado e metastático é a terapia de bloqueio de androgénico (TBA). Contudo a maioria dos doentes acaba por desenvolver resistência a esta forma de tratamento, desenvolvendo o CP resistente à castração (CPRC). Dentro da TBA, os antiandrogénios de segunda geração, onde se inclui a enzalutamida (ENZ), contribuem positivamente para o aumento da sobrevivência global dos doentes com CRPC. No entanto, cerca de 20-40% apresenta resistência primária a estes agentes, e os restantes ao longo do tratamento acabam também por desenvolver resistência secundária a estes. A aquisição de resistência à ENZ é atualmente um importante problema clínico, pois não só não existem biomarcadores preditivos que permitam a deteção precoce destas formas de resistência, como as abordagens terapêuticas alternativas são limitadas.

O desenvolvimento e a progressão do cancro estão associados a modificações do microambiente tumoral, sendo as vesículas extracelulares (VEs) importantes mediadores e contribuidores para a modulação deste, influenciando a comunicação intercelular. As VEs podem transportar diferente informação, destacando-se a sua capacidade de conterem como carga os microRNAs (miRNAs) que são pequenas moléculas de RNA não codificante responsáveis pela regulação genética pós-transcricional. Os miRNAs têm sido amplamente estudados em oncologia, estando a sua desregulação associada a diferentes processos que contribuem para a progressão tumoral e aquisição de fenótipos de resistência a tratamentos, apresentando elevado potencial de serem usados como biomarcadores de prognóstico e preditivos de resposta a tratamentos anti-neoplásicos. Deste modo, no presente estudo estabelecemos um perfil de miRNAs derivado de VEs associado à aquisição de resistência à ENZ em CP, clarificando a sua dinâmica durante o processo de aquisição desta resistência, de forma a identificarmos potenciais novos biomarcadores preditivos de resistência e futuros alvos terapêuticos.

Numa primeira fase, estabelecemos um modelo *in vitro* da resistência à ENZ através da continua exposição das células LNCaP a concentrações crescentes de ENZ (2,5 μM, 5 μM, 10 μM, 20 μM e 30 μM (LNCaPER)), tendo a aquisição de resistência sido confirmada por avaliação da atividade metabólica, do ciclo celular e quantificação relativa nos níveis de expressão de mRNA do recetor de androgénios (RA) e do antigénio específico da próstata (PSA). Adicionalmente, o perfil de expressão de miRNAs das células LNCaP *versus* LNCaPER foi comparado com recurso ao nCounter® Human v3 miRNA Expression Assay (NanoString®), tendo-se verificado que a linha celular LNCaPER apresentava a sobreexpressão de 12 miRNAs e a subexpressão de 13 miRNAs comparativamente com

as LNCaP. A análise *in silico* permitiu concluir que os miRNAs diferencialmente expressos se encontravam envolvidos na proliferação celular, regulação do ciclo celular, migração e metabolismo, influenciando vias de sinalização como a PI3K/AKT e a via de sinalização mediada pelo TGF-β. Subsequentemente, definimos os 5 miRNAs sobreexpressos (hsa-miR21-5p, hsa-miR-22-3p, hsa-miR-200a-3p, hsa-miR-221-3p e hsa-miR-222-3p) com mais genes alvo, como o perfil de miRNAs a ser analisado no modelo *in vitro* 3D do tipo esferoide, avaliando-se o seu potencial como biomarcador preditivo de desenvolvimento de resistência à ENZ, assim como a sua dinâmica ao longo do processo de resistência.

Inicialmente, testamos diversas condições para formar esferoides 3D, pela medição do diâmetro e avaliando a atividade metabólica. A condição de maior densidade celular (C3) foi escolhida para a formação de esferoides a partir de células resistentes. Usando imunofluorescência, analisamos a organização estrutural e molecular do esferoide. Destaca-se a redução gradual das células proliferativas, pela diminuição da expressão de Ki-67, e um aumento de células apoptóticas no centro do esferoide, pelo aumento da marcação de caspase 3, particularmente acentuada nos esferoides LNCaPER.

Relativamente à quantificação da expressão dos miRNAs selecionados observamos, associado à concentração mais baixa de ENZ, uma tendência de aumento da expressão dos níveis intracelulares do perfil de miRNAs, o que sugere um efeito agudo do agente. Adicionalmente, verificamos que associado à administração de concentrações mais elevadas de ENZ, ocorreu uma tendência de diminuição dos níveis intracelulares do hsa-miR-21-5p, hsa-miR-22-3p e hsa-miR-200a-3p, contudo um aumento estatisticamente significativo do nível de hsa-miR-22-3p derivado de VEs na condição de 20 μM (11,08 *fold-increase,* p=0.047). Relativamente aos níveis intracelulares do cluster hsa-miR-221/222 verificou-se um aumento gradual de forma estatisticamente significativa com o aumento da concentração de ENZ (hsa-miR-221-3p: 2.5 μM ENZ p=0.031; 5 μM ENZ p<0.001; 10 μM ENZ p=0.001; 20 μM ENZ p<0.001; hsa-miR-222-3p : 2.5 μM ENZ p=0.022; 5 μM ENZ p<0.001; 10 μM ENZ p<0.001; 20 μM ENZ p<0.001), verificando-se também um aumento estatisticamente significativos nos níveis de hsa-miR-221/222 derivados de VEs na condição experimental de 20 μM ENZ (hsa-miR-221-3p: 37,53 *fold-increase,* p=0.02; has-miR-222-3p 64.45 *fold-increase*, p=0.004).

Os resultados obtidos sugerem o envolvimento do hsa-miR-221 e o hsa-miR-222 no processo de aquisição de resistência à ENZ, através da sua incorporação em VEs, o que potenciará a modificação do microambiente tumoral, apresentando assim potencial de serem usados como biomarcadores preditivos. De facto, admite-se que estes miRNAs estão envolvidos em processos celulares como a divisão celular, modulando

negativamente genes envolvidos na regulação do ciclo celular, assim como apresentam a capacidade de modular importantes vias de sinalização celular, de que é exemplo a via de sinalização PI3K/AKT, através da inibição do PTEN. Futuramente, é fundamental o estudo de mais miRNAs de modo a melhorar a capacidade preditiva do perfil de miRNA derivado de EV e, de seguida, observar a dinâmica deste perfil em doentes que recebem terapia com ENZ. Além disso, seria importante investigar o potencial destes miRNAs como potenciais alvos terapêuticos para reverter a resistência à ENZ.

Abstract

Prostate cancer (PC) is one of the most frequently diagnosed malignant tumors and the fifth leading cause of cancer death in men. One of the main treatment strategies for advanced and metastatic PC is androgen deprivation therapy (ADT). However, most patients eventually develop resistance, developing castration-resistant PC (CRPC). Within ADT, second-generation antiandrogens, including enzalutamide (ENZ), make a positive contribution to increasing the overall survival of patients with CRPC. However, around 20-40% show primary resistance to these agents, and the rest develop secondary resistance over the course of treatment. The acquisition of ENZ resistance is currently a major clinical problem, since there are no predictive biomarkers that allow early detection of these forms of resistance, neither alternative therapeutic approaches.

The development and progression of cancer are associated with changes in the tumor microenvironment, and extracellular vesicles (EVs) are important mediators and contributors to its modulation, influencing intercellular communication. EVs can carry different types of information, especially microRNAs (miRNAs), which are small non-coding RNA molecules responsible for post-transcriptional gene regulation. MiRNAs have been widely studied in oncology, and their deregulation is associated with tumor progression and the acquisition of treatment-resistant phenotypes, with high potential for use as prognostic biomarkers and predictors of response to anti-neoplastic treatments. Therefore, in this study we established a profile of miRNAs derived from EVs associated with the acquisition of resistance to ENZ, clarifying their dynamics during the process of resistance acquisition to identify potential new predictive biomarkers of resistance and future therapeutic targets.

We started by establishing an *in vitro* model of ENZ resistance by continuously exposing LNCaP cells to increasing concentrations of ENZ (2.5 μM, 5 μM, 10 μM, 20 μM and 30 μM (LNCaPER)). The acquisition of resistance was confirmed by evaluating metabolic activity, the cell cycle and relative quantification of androgen receptor (AR) and prostate-specific antigen (PSA) mRNA expression levels. In addition, the miRNA expression profile of LNCaP versus LNCaPER cells was compared using the nCounter® Human v3 miRNA Expression Assay (NanoString®), and it was found that the LNCaPER cell line overexpressed 12 miRNAs and underexpressed 13 miRNAs compared to LNCaP. Through an *in silico* analysis we concluded that the differentially expressed miRNAs were involved in cell proliferation, cell cycle regulation, migration and metabolism, influencing signaling pathways such as PI3K/AKT and the TGF-β-mediated signaling pathway. Subsequently, we determined the 5 overexpressed miRNAs (hsa-miR21-5p, hsa-miR-22-3p, hsa-miR-200a-3p, hsa-miR-221-3p and hsa-miR-222-3p) with the most target genes, as the miRNA profile to be analyzed in the *in vitro* 3D spheroid model, assessing their potential as a predictive

biomarker of ENZ resistance development, as well as their dynamics throughout the resistance process.

Initially, we tested different conditions to form 3D spheroids, measuring their diameter and evaluating their metabolic activity. The condition with the highest cell density (C3) was chosen for the formation of spheroids from resistant cells. Using immunofluorescence, we analyzed the structural and molecular organization of the spheroid. Noteworthy was the gradual reduction of proliferative cells, due to the decrease in Ki-67 expression, and an increase in apoptotic cells in the center of the spheroid, due to the increase in caspase 3 marking, which was particularly pronounced in LNCaPER spheroids.

Regarding the quantification of the expression of selected miRNAs, we observed a tendency for increased expression of intracellular levels of the miRNA profile associated with lower concentrations of ENZ, which suggests an acute effect of the agent. In addition, we found that associated with the administration of higher concentrations of ENZ, there was a tendency for the intracellular levels of hsa-miR-21-5p, hsa-miR-22-3p and hsa-miR-200a-3p to decrease, although there was a statistically significant increase in the level of hsa-miR-22-3p derived from EVs in the 20 μM condition (11.08 fold-increase, $p=0.047$). With regard to intracellular levels of the hsa-miR-221/222 cluster, there was a statistically significant gradual increase with increasing ENZ concentration (hsa-miR-221-3p: 2.5 μM ENZ $p=0.031$; 5 μM ENZ $p<0.001$; 10 μM ENZ $p=0.001$; 20 μM ENZ $p<0.001$; hsa-miR-222-3p: 2.5 μM ENZ $p=0.022$; 5 μM ENZ $p<0.001$; 10 μM ENZ $p<0.001$; 20 μM ENZ $p<0.001$), there was also a statistically significant increase in the levels of hsa-miR-221/222 derived from EVs in the 20 μM ENZ experimental condition (hsa-miR-221-3p: 37.53 fold-increase, $p=0.02$; has-miR-222-3p 64.45 fold-increase, $p=0.004$).

The results obtained suggest the involvement of hsa-miR-221 and hsa-miR-222 in the process of acquiring resistance to ENZ, through their incorporation into EVs, which will potentiate the modification of the tumor microenvironment, thus presenting the potential to be used as predictive biomarkers. In fact, these miRNAs are thought to be involved in cellular processes such as cell division, negatively modulating genes involved in cell cycle regulation, as well as having the ability to modulate important cell signaling pathways, such as the PI3K/AKT signaling pathway, by inhibiting PTEN. In the future, it is essential to study more miRNAs in order to improve the predictive capacity of the EV-derived miRNA profile and then observe the dynamics of this profile in patients receiving ENZ therapy. Furthermore, it would be important to investigate the potential of these miRNAs as potential therapeutic targets to reverse ENZ resistance.

Introduction

1. Introduction

1.1. Cancer: general concepts

Nowadays, cancer is an important health issue worldwide, being responsible for a large number of deaths every year [1]. GLOBOCAN estimated more than 19.2 million new cancer cases (Figure 1- A) and nearly 10 million deaths in 2020, globally (Figure 1- B) [2].

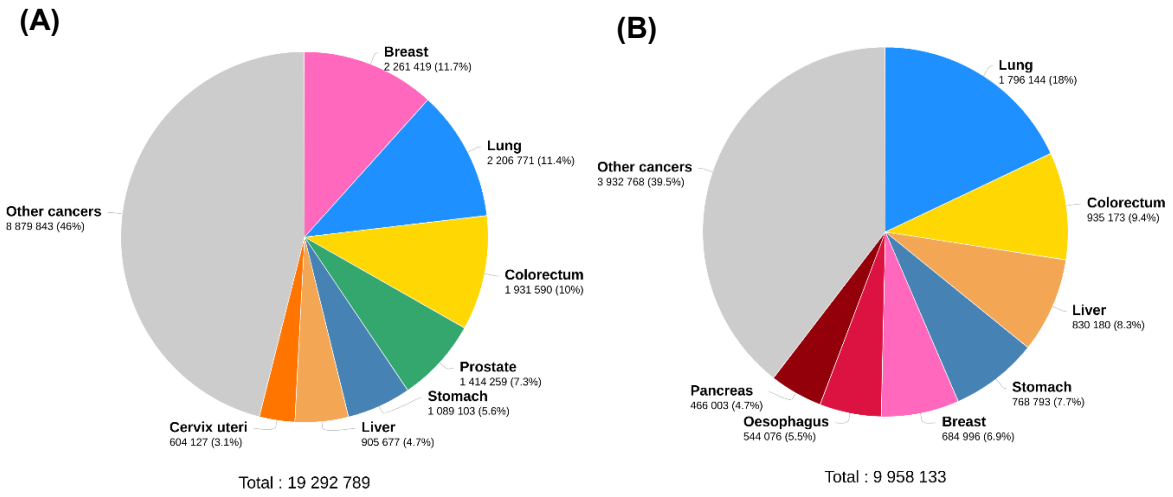


Figure 1: Cancer incidence (A) and cancer mortality (B) worldwide in both sexes and considering all ages (adapted from GLOBOCAN 2020, IARC) [2].

Europe accounted for 22.8% of the cancer cases registered in 2020 and for 20% of the deaths by cancer [2, 3]. Out of this, more than 60 thousand new cases and 10 thousand deaths were registered in Portugal [2].

The global burden of cancer incidence and mortality is rapidly increasing. However, the impact of the various types of cancer is not evenly distributed among the world population due to evident disparities in incidence and mortality rates, which can be attributed to socioeconomic, ethnic, and lifestyle factors [3-5]. The incidence and mortality rates of most cancers rise as the Human Development Index (HDI) increases [6]. Age stands as a crucial risk factor for the development of cancer, as it is a genetic disease caused by the accumulation of DNA (Deoxyribonucleic Acid) alterations [7]. Hence, the increase of the number of new cases, mostly in industrialized and developed countries, can be partially attributed to the overall increase in life expectancy [8]. Moreover, the level of development in a country significantly influences various risk factors that contribute to the likelihood of developing cancer, such as tobacco smoking, obesity, and sedentary behaviors [8]. Another factor contributing to this disparity is the implementation of screening and early detection methods, which are more prevalent in developed countries, enabling early diagnosis and contributing to the rising number of cases [8]. Due to the considerable impact

of demographic parameters in cancer incidence and mortality, such as population growth and ageing, in 2070 the incidence of all malignancies combined is expected to double compared to 2020 [4].

Cancer is described as a genetic disease originated by the accumulation of alterations in the cell genome [7]. Therefore, it can be defined as a group of diseases characterized by the abnormal and uncontrolled proliferation of genetically altered cells, capable of migrating to distant regions and initiating metastasis formation. Moreover, it is a heterogeneous disease with multiple etiologies and development patterns [7].

Carcinogenesis is the complex process by which normal cells undergo genetic and epigenetic changes that lead to the development of cancer [9, 10]. This process includes three key stages: initiation, promotion and progression [9, 10]. Carcinogenesis begins with the initiation stage which involves the exposure of normal cells to certain carcinogenic agents, such as chemicals, radiation, or biologic agents, like virus [9-11]. These agents can cause DNA damage in the cells. If the DNA damage is not repaired, it can lead to permanent genetic alterations in the cells' DNA [9-11]. In the promotion stage, promoter agents like nicotine and phorbol esters enhance the proliferative capacity of initiated cells, leading to more cumulative genetic and epigenetic alterations, which will lead to the appearance of characteristics of malignant cells in a gradual manner [9-11]. Finally, the tumor progression stage is irreversible and it is characterized by additional genetic alterations leading to uncontrolled growth and local invasion [9-11]. This culminates in the formation of a malignant tumor composed of several malignant cell subclones derived and characterized by different genetic and chromosomic mutations patterns. These cells are genetically unstable and tend to become more aggressive with the cumulative genetic and epigenetic alterations [12].

The progressive malignant transformation of cells primarily occurs due to alterations in two principal classes of genes: proto-oncogenes and tumor suppressor genes [13-15]. Proto-oncogenes, like *EGFR* and *MYC,* encode proteins that regulate cell growth and differentiation. The occurrence of genetic alterations such as chromosomal translocations, amplifications and mutations in proto-oncogenes convert them into oncogenes and lead to its constitutive expression and abnormal cell proliferation [13-15]. Its only necessary that alteration occur in one of the alleles, to change the cellular behavior [13-15]. On the other hand, tumor suppressor genes, such as *PTEN*, *TP53* and *RB1*, regulate cell cycle and inhibit cell proliferation. For the inactivation of tumor suppressor genes, it is usually necessary that both alleles of the gene must be mutated to change gene activity [13-15]. In addition, alterations in genes involved in DNA repair and chromosomal segregation during

mitosis, such as mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) are also involved in the development of cancer [13, 14].

In late stages, cancer can spread to anatomically distant regions of the body, forming metastasis, which is the process responsible for most cancer related deaths [16]. The classic hypothesis of the metastatic process is based on Stephen Paget's "Seed and Soil" model [17, 18]. This model suggests that cancer metastasis can occur when cells from the primary tumor detach and migrate to distant organs, with favorable microenvironments, through blood and lymphatic circulation [10, 17, 18]. Since then, several hypotheses, such as the progression model and the metastasis by cellular fusion, have emerged to expand this classical model. The majority of modern metastasis research focuses on the properties of the metastatic "seed and soil" model and provide a more detailed understanding about the biological and genetic complexities that modulate the capacity of cells to undergo the general sequential steps of the metastatic process: detachment from the basement membrane, local invasion, intravasation into the blood and lymphatic vessels, dissemination, cellular extravasation and metastatic colonization [17, 19, 20]. Moreover, nowadays it is understood that the phenotype of stromal, immune and cancer cells are altered by the complex and dynamic crosstalk stablished between them, in which extracellular vesicles (EVs) are important players and influence the cellular transformation, proliferation, vascular permeability and the preparation of distant tissues for tumor cells colonization, i.e. contributing for the establishment of pre-metastatic niches, and favoring the development of metastasis [20-22].

Tumor cells have phenotypic and molecular characteristics arising from the process of neoplastic transformation and progression that allow them to be distinguished from the normal cells that have originated them [12]. Hanahan and Weinberg, in the year 2000, first proposed six major alterations in the physiology of cells that were essential for the development of the malignant phenotype, being known as *Hallmarks of Cancer*: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, unlimited replicative potential, sustained angiogenesis and tissue invasion and metastasis [23]. Later, in 2011, with a better understanding of cancer biology, as well as of the metabolic and genetic changes suffered by cells during carcinogenesis, the same authors updated this panel with two new *Hallmarks* and two enabling characteristics: evasion of the immune system, abnormal metabolism, genome instability and inflammation [24]. Recently, Hanahan has updated the list and incorporated the phenotypic plasticity, epigenetic reprogramming, polymorphic microbiomes, and senescent cells (Figure 2) [25].

Hanahan and Weinberg have also recognized the involvement of tumor microenvironment (TME) in cancer development since *Hallmarks of Cancer*, such as the cell senescence and metabolic and epigenetic reprograming arise from aberrant physical properties of the TME [24, 25]. Indeed, significant advances have been made in recent years in our understanding of the components of the TME and their interactions. In this context, EVs carry oncogenic molecular information and lead to phenotypic changes in recipient cells. This cross-talk is particularly important for tumor development, progression, therapy resistance and metastasis [26].

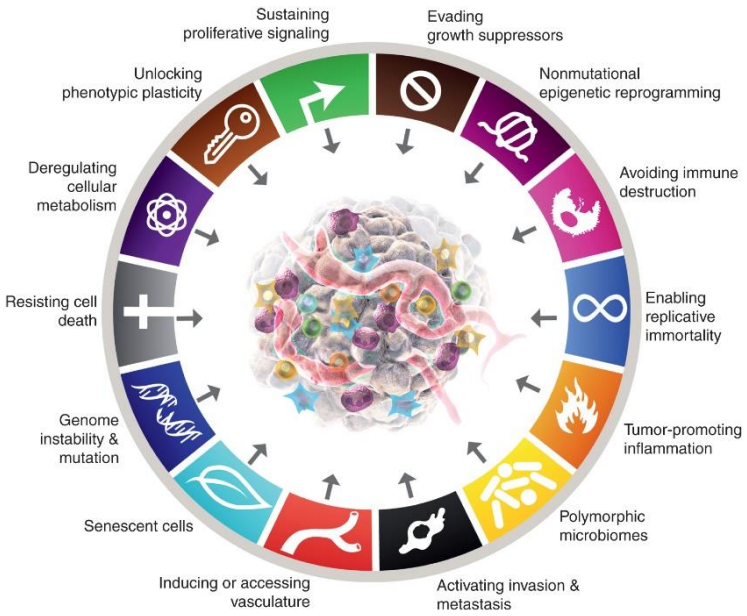


Figure 2: The *Hallmarks of Cancer* with the new proposed cancer features in 2022 by Hanahan (adapted from Hanahan, D. [25]).

1.2. Intercellular communication in the tumor microenvironment - extracellular vesicles role

The TME is a complex network composed of immune cells, blood vessels, fibroblasts, components of the extracellular matrix, such as collagen, fibronectin and signaling molecules, and the tumor cells [27, 28]. Tumor cells and the other elements of the tumor microenvironment constantly interact and influence each other affecting cancer development and progression [27, 28].

Current research has shown that cancer formation, progression and treatment failure are critically dependent on TME remodeling and the cross-communication between its components [29]. Tumor cells, through intricate signaling networks, can regulate both cellular and non-cellular components, controlling them for their own advantage through the promotion of cellular heterogeneity, clonal evolution and drug resistance [29]. This

communication can occur through several mechanisms, including direct cell-to-cell contact through cell junctions, which allow the transference of nucleic acids, growth factors and metabolites, and by classical paracrine signaling of cytokines or growth factors. Communication can also occur through circulating tumor cells (CTCs) and cell-free DNA (cfDNA) [29, 30]. Some tumor cells can also enter the bloodstream, circulate as CTCs and interact with distant cells, promoting signaling cascades and horizontal transfer of genetic information, potentially lead to the establishment of secondary tumors [29, 31, 32].

Furthermore, non-tumor and tumor cells can release DNA fragments as a result of cell death and alter the biology of recipient cells, thus being involved in horizontal gene transfer and oncogenic transformation of normal cells [29]. Recently, EVs have emerged as important mediators of cellular communication through the interchange of bioactive molecules, being important mediators of TME modulation [29, 30, 32]. The EVs are released by mechanisms that remain conserved through evolution and by both procaryotic and eucaryotic cells, including tumor cells [30, 33-36]. EVs are small particles delimited by a lipid bilayer that are naturally released into the extracellular space [37-39]. Cells can secrete three main subtypes of EVs, which are differentiated based upon their biogenesis, release pathways, size and content: exosomes (~30–150 nm), microvesicles (~100–1000 nm), and apoptotic bodies (~500–2000 nm) (Figure 3) [37-39]. Exosomes are produced through inward budding of the plasma membrane, originating multivesicular bodies (MVBs), which are secreted when these compartments fuse with the plasma membrane (Figure 3). Microvesicles are produced by direct budding from the plasma membrane (Figure 3). In accordance with their biogenesis, the membrane composition of microvesicles closely reflects that of the parent cell's plasma membrane. Both exossomes and microvesicles carry proteins, including cytokines, chemokines, proteins involved in cellular signaling, lipids, carbohydrates, and genetic material [40]. Apoptotic bodies are obtained by cell lysis during the late stages of apoptosis [37, 38]. Unlike exosomes and microvesicles, apoptotic bodies contain intact organelles and chromatin [37, 38].

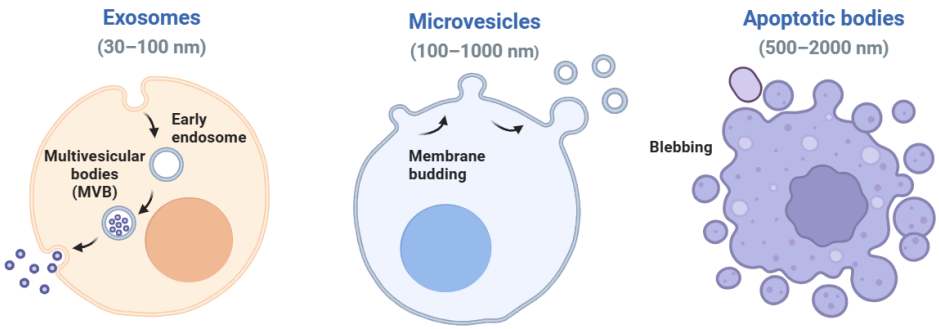


Figure 3: Schematic representation of the origin and secretion of the main classes of EVs (BioRender).

EVs carry several biological active molecules from parental cells, including proteins, lipids, DNA and RNA (Ribonucleic Acid) molecules, including non-coding RNAs (ncRNAs) [37-39]. However, the cargo composition of EVs is diverse and dynamic and can be influenced by factors such as the cellular state and the microenvironment [37, 38].

As mentioned, EVs have been recognized as potent vehicles of local and/or systemic intercellular communication in TME and metastatic niches, therefore being responsible for tumor progression, metastasis, and development of therapy resistance, which is currently one of the main barriers in patient cancer management [33, 37, 41]. EVs-mediated communication includes the direct activation of cell-surface receptors, inducing intracellular signaling cascades in target cells and transfer of their cargo by endocytosis, phagocytosis, or even by fusion with the target cell's membrane, thereby influencing various physiological and pathological functions of the recipient cells [33, 36, 37, 41, 42]. They can also modify the cellular physiology of distant non-tumor cells, to allow the dissemination of cancer cells and the establishment of pre-metastatic niches in distant organs [33-35].

In this context, it has been suggested that EVs also play an important role in epigenetic regulation. Through the transmission of ncRNA molecules, particularly microRNAs (miRNAs), EVs can modulate the expression of target genes associated with tumorigenesis, thereby influencing the phenotype of recipient cells (Figure 4) [43]. In fact, miRNAs, constituting a significant proportion (76%) of the RNA content in EVs, are recognized as highly promising and extensively studied EVs-derived biomolecules in the pursuit of novel cancer biomarkers [44, 45].

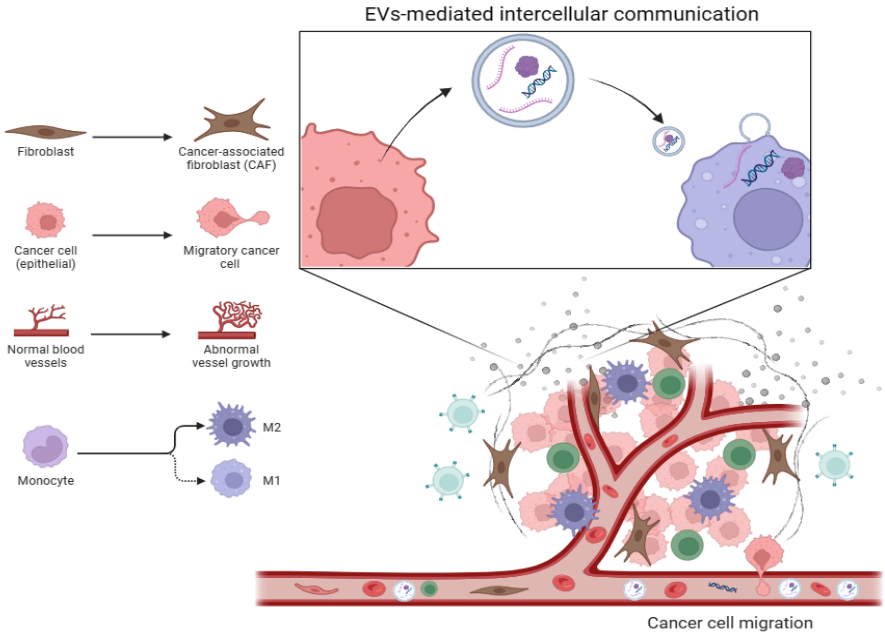


Figure 4: Tumor microenvironment (TME) communication through extracellular vesicles. Interactions between the tumor and surrounding cells in the TME promote immune suppression, angiogenesis, metastasis, and therapeutic resistance (BioRender).

These findings, combined with EVs' high stability in several biological biofluids such as blood and urine, woke up a great interest in the scientific community about their potential as new non-invasive prognosis and predictive cancer biomarkers [44, 46, 47].

1.3. MicroRNAs

MiRNAs are a class of small non-coding and single-stranded RNA molecules of approximately 21–23 nucleotides in length that regulate gene expression at the post-transcriptional level [48-50]. Several studies have established that miRNAs deregulated expression is significantly associated with cancer initiation and progression [51]. MiRNAs comprise approximately 1-5% of all predicted human genes and play a crucial role in regulating the expression of about 30% of all protein-coding genes [49].

MiRNA biogenesis involves multiple steps: transcription in the nucleus, export to the cytoplasm, followed by processing and maturation (Figure 5) [48-50]. Initially, RNA polymerase II or III transcribes primary miRNA transcripts (pri-miRNAs) in the nucleus [48-50]. Pri-miRNAs present a hairpin structure, containing the sequence for the mature miRNA. A Microprocessor complex, comprising double stranded RNase III enzyme (Drosha) and DiGeorge syndrome critical region 8 (DGCR8), recognizes and cleaves pri-miRNA, generating hairpin-shaped precursor miRNAs (pre-miRNAs) [48-50]. Pre-miRNAs are transported by exportin 5 (XPO5) to the cytoplasm [48-50]. In the cytoplasm, RNase endonuclease III, Dicer, processes pre-miRNAs by recognizing the phosphate at their 5' end and 3' overhang near the terminal loop, creating a short double-stranded RNA duplex [48-50]. This duplex consists of the mature miRNA strand (guide strand) and its complementary passenger strand (miRNA*) [48-50]. Dicer works with transactivation-responsive RNA-binding protein (TRBP) to enhance its cleavage efficiency through helicase binding [48-50]. TRBP also helps Dicer interact with Argonaute proteins (AGO2), facilitating the assembly of the miRNA-induced silencing complex (RISC). The guide strand is retained in the RISC complex, while the passenger strand is typically degraded [48-50]. The mature miRNA within the RISC complex binds to target messenger RNA (mRNA) molecules by complementary base pairing [48-50]. The miRNA can interact with the target mRNA's 3' untranslated region (UTR), leading to mRNA degradation or translational repression, depending on the level of complementarity (Figure 5) [48-50]. When the miRNA has a high level of complementarity with the target mRNA, it can trigger mRNA degradation (Figure 5) [48-50]. If the complementarity between the miRNA and the target mRNA is partial, the

miRNA can inhibit the translation of the mRNA without causing its degradation (Figure 5) [48-50]

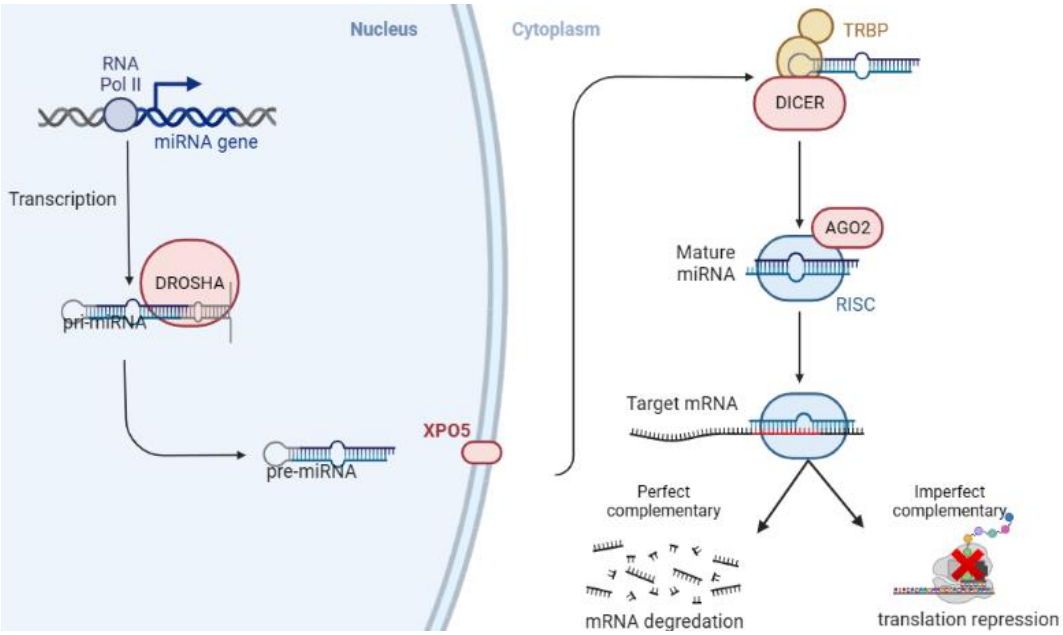


Figure 5: MiRNA biogenesis and mRNA translation repression (BioRender).

Each miRNA may regulate the expression of different mRNAs and conversely, each mRNA may be targeted by multiple miRNAs [52]. This creates a complex regulatory network where miRNAs can fine-tune gene expression by controlling the abundance and translational activity of multiple mRNAs. The deregulated expression of miRNAs plays a crucial role in cancer, leading to their classification into two subclasses: oncomiRNAs or tumor suppressor miRNAs [53]. OncomiRNAs target tumor suppressor genes by binding to their mRNA, and subsequently exhibiting overexpression in cancer. On the other hand, tumor suppressor miRNAs are usually downregulated in the context of cancer, since they target mRNA of oncogenes, inhibiting their expression [53].

MiRNAs have specific characteristics that define them as promising biomarkers. They are ubiquitously produced by all cells and show distinct expression patterns in normal *vs* pathological conditions. This dual behavior not only allows early diagnosis but also holds prognostic value, enabling the identification of potentially aggressive tumors [54]. Furthermore, miRNAs are present and can be reproducibly and accurately quantified from a wide variety of biologic samples, including plasma, serum and urine, that can be easily obtained through minimal invasive procedures [54, 55]. Moreover, they are stable in biological fluids and resistant to various storage conditions, since they are secreted into the extracellular space encapsulated in EVs, which protect them from degradation [54].

Recent studies have revealed important functions of several miRNAs in the pathogenesis of human diseases. One example, it is the hsa-miR-21 that is one of the most studied miRNAs in cancer and presents an oncogenic function, since it is overexpressed in several cancers, such as breast, pancreatic, prostate, and colorectal cancers [56, 57]. Hsa-miR-21 has been presented as a plausible diagnostic and prognostic biomarker, as well as a therapeutic target [56, 57]. It has been reported that higher circulating levels of hsa-miR-21 are significantly associated with poor survival outcomes and therapy resistance in a vast diversity of cancers including breast, pancreatic and prostate cancers [58]. Anwar and colleagues work indicates that upregulation of circulating hsa-miR-21 is an important marker for diagnosis and therapeutic response in breast cancer patients [59]. Additionally, Lee and colleagues detected higher hsa-miR-21 expression levels in the enzalutamide-resistant EVs isolated from enzalutamide-resistant LNCaP cells and sera of enzalutamide-resistant prostate cancer (PC) patients [60]. On the other hand, hsa-miR-141 and hsa-miR-375 were two of the first reported circulating miRNAs in PC [61, 62]. In fact, Watahiki and colleagues demonstrated that plasma levels of hsa-miR-141 and has-miR-375 are potential biomarkers to differentiate localized PC and metastatic castration resistance PC (CRPC) [62]. Moreover, plasma levels of has-miR-141 and has-miR-375 may predict time to progression in metastatic CRPC patients treated with docetaxel, abiraterone or enzalutamide, thus demonstrating the involvement of both miRNAs in the development of therapy resistance in PC [63, 64]. Thus, researchers have been interested in studying miRNAs in PC, mostly due to the lack of reliable biomarkers for risk stratification and for therapy response monitoring [2, 65].

1.4. Prostate cancer

Prostate cancer (PC) is one of the most incident cancers in men worldwide, being the 2nd most common cancer among the male population [2, 66]. In 2020, GLOBOCAN reported 1 414 259 new cases of PC, which represents 14.1% of all cancers in men [2]. With an estimated death number of 375 304 worldwide, PC contributes to 6.8% of all cancer-related deaths in men, representing the 5th leading cause of cancer death in the male population in 2020 [2]. However, PC prevalence exhibits considerable geographical variation, being more frequent in regions with a high HDI, namely Northern and Western Europe, Australia/New Zealand, North America and Japan (Figure 6) [2, 67, 68].

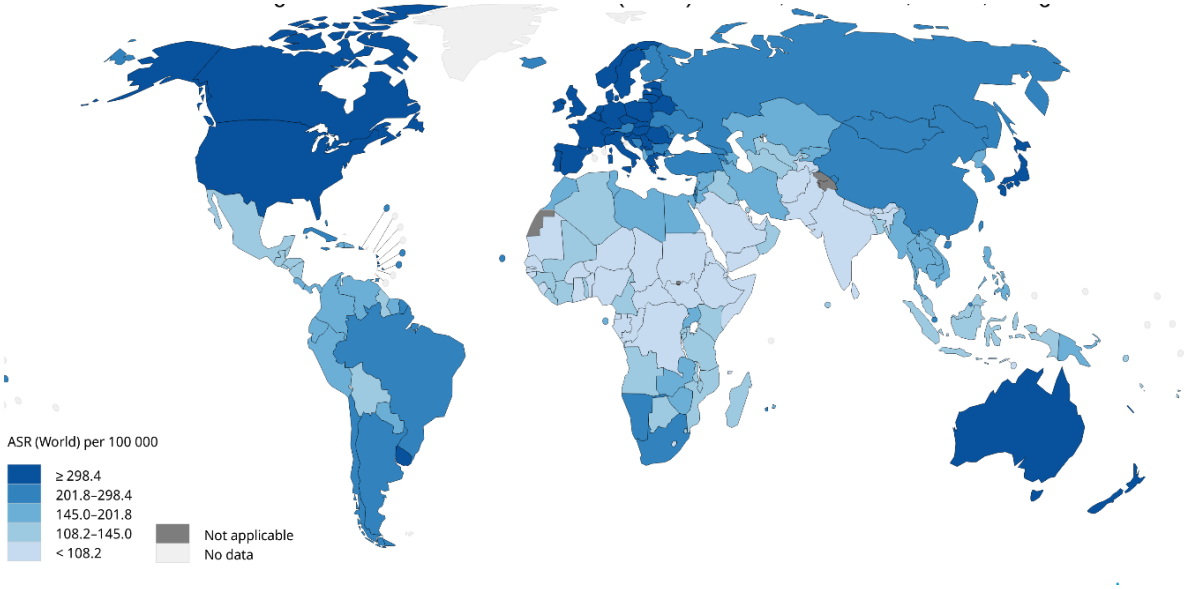


Figure 6: Estimated age-standardized incidence rates (World) in 2020, all cancers, males, all ages (adapted from GLOBOCAN 2020, IARC) [2].

Several factors contribute to the heterogeneity in PC incidence rates. In developed countries, improved access to healthcare facilities and higher rates of prostate-specific antigen (PSA) screening plays a significant role in the early detection and the increase of reported cases [67-69]. Additionally, these countries tend to have longer life expectancies and a larger elderly population, leading to a higher proportion of individuals within the age group where PC is more prevalent [67-69]. In Portugal, PC is the most frequent neoplasia in men, accounting for 20% of new cases and standing as the 3rd cause of cancer-related death in men. The etiology of PC is multifactorial since age, family history and ethnicity being well-established non-modifiable risk factors [70]. However, the literature suggests several exogenous and behavioral risk factors associated with an increased risk of PC including diet, obesity, smoking, and exposure to pesticides [70].

PC usually develops very slowly and is often preceded by dysplastic lesions several years earlier [67, 71, 72]. Nearly all PC diagnosed are adenocarcinomas, which are neoplasms with malignant differentiation of the prostate glandular epithelium [67, 72]. The process of malignant transformation of the prostate follows several stages. It begins with the establishment of a premalignant state characterized by a lack of cell differentiation and the emergence of anaplasia, called prostatic intraepithelial neoplasia (PIN) [67, 72]. This stage, which is still pre-malignant, may progress to localized PC in cases with high-grade PIN (Figure 7) [67, 72, 73].

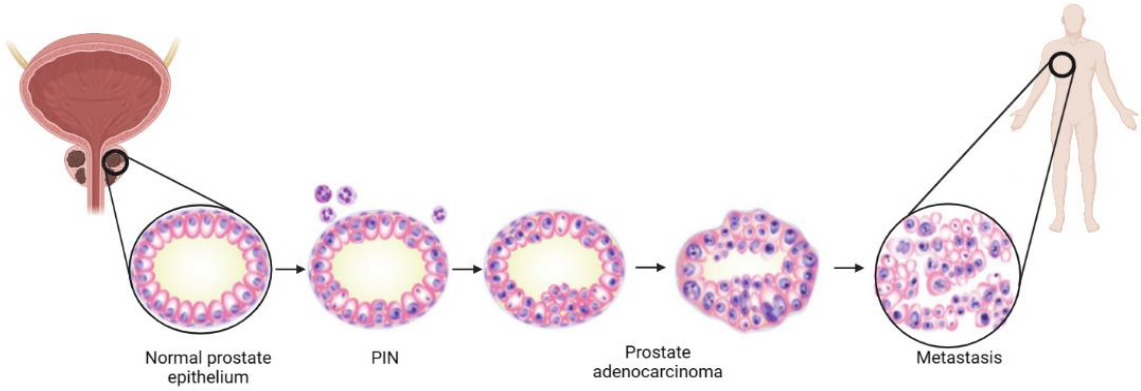


Figure 7: Model of PC carcinogenesis (adapted from Anya J Burton and colleagues [73] and adapted with BioRender).

Despite major advances in cancer biology and imaging, PC has been traditionally diagnosed by digital rectal examination (DRE) and PSA testing, followed by a prostate biopsy for diagnosis confirmation [74-76].

PSA testing consists in measuring circulating levels of PSA, a protein from the kallikrein family produced by both normal, benign prostate hyperplasia, and malignant prostatic tissues. PSA is commonly expressed at higher levels in men with poorly differentiated tumors [74-76]. The wide prescription of PSA screening and the establishment of screening programs have been linked to a notable increase in PC incidence, mostly in developed countries [69, 74-76]. PSA testing has contributed to improved PC survival rates by allowing the earlier detection of local and asymptomatic diseases, ultimately preventing metastasis [77, 78]. However, it is important to note that high PSA levels can be also caused by non-malignant conditions [75, 76, 79, 80]. Therefore, although PSA testing is valuable, it can yield false-positive results, potentially leading to the diagnosis of indolent disease and subjecting men to unnecessary biopsy and overtreatment [75, 76, 79, 80]. Indeed, an estimated 20–50% of men diagnosed with PC through PSA testing have indolent tumors that might never progress to a clinically significant disease [75, 76, 79, 80]. Thus, PSA measurement shows a lack of specificity and accuracy.

The stage and degree of neoplasia is evaluated by analyzing the histological characteristics of the tumor and the degree of cellular differentiation. The Gleason classification system, created by Donald Gleason, categorizes the histological characteristics of prostate adenocarcinoma into five architectural patterns ranging from 1 to 5, where higher grades are associated with more undifferentiated forms and worse prognosis [81, 82]. Since most PC cases exhibit multiple growth patterns, the Gleason Grading System was modified so the two most dominant patterns were combined to form a

single Gleason score, resulting in a grading system with nine tiers (ranging from 2 to 10) [81, 82]. However, over the years, the Gleason grading system underwent modifications and in 2014 the International Society of Urological Pathologists (ISUP) introduced a five-tier grade grouping (ranging from 1 to 5) [81, 83]. Gleason scores of ≤6 were combined into ISUP grade (group) 1, while Gleason scores of 9 and 10 were combined into ISUP grade (group) 5. Gleason score 7 was split into ISUP grade (group) 2 and ISUP grade (group) 3. This modified grading method was also adopted by the European Association of Urology (EAU) guideline committee for PC [81, 83].

The great majority of men are diagnosed with either localized or locally advanced disease, with a good prognosis. [84, 85]. For low-risk PC patients, an approach involving active surveillance is often adopted. This strategy involves a series of PSA testing, physical examinations, prostate biopsies, or a combination of these to monitor for progression with an intent to cure those who develop a more significant disease [84-86]. On the other hand, intermediate-risk patients with higher PSA levels often undergo radical prostatectomy, external beam radiation or brachytherapy [84-86]. Since androgen is needed for the growth and progression of PC, androgen deprivation therapy (ADT), has been the gold standard treatment for high-risk, advanced and metastatic PC [84-86]. High-risk PC is traditionally treated with a combination of radiotherapy and ADT [84-87].

1.4.1. Androgen Deprivation Therapy

The initial discovery by Charles Huggins and colleagues that PC is an androgen-dependent malignancy, whose onset and progression are closely related to the androgen receptor (AR) signaling pathway provided the basis for ADT implementation as the primary strategy for the treatment of advanced and metastatic PC [88-90].

Androgens, primarily produced in the testes with a smaller amount synthesized in the adrenal glands, play a pivotal role in the normal development and functioning of the prostate gland [88, 91]. However, in the context of PC, androgens can stimulate the growth and division of cancer cells, thereby driving disease progression [88]. Of these androgens, dihydrotestosterone (DHT), the most potent form, exerts its biological effects by directly binding to the AR [88].

AR is a member of the nuclear receptor superfamily, functioning as a transcription factor [88]. It consists of several domains: the N-terminal domain (NTD), which is responsible for transactivation and recruitment of coactivators/corepressors; the central DNA-binding domain (DBD), facilitating AR's binding to specific DNA sequences known as androgen response elements (AREs) in the promoter regions of target genes; and the C-

terminal ligand-binding domain (LBD), which interacts with androgens and regulates AR activation [88, 92, 93]. When androgens bind to the AR, it triggers a conformational change that enables the receptor's translocation to the nucleus [88, 93]. In the nucleus, AR forms dimers and binds to specific target gene promoters, enhancing the transcription of genes, such as *CAMKK2, IGF1R* and *FKBP5*, involved in cellular metabolism, growth, and proliferation, respectively [88, 93].

Depending on the ADT agent used, the goal of ADT is to either decrease the androgens levels or inhibit their activity within prostate cells, by preventing their binding to AR. The purpose is to interrupt the androgen signaling pathway since PC relies on androgen signaling for growth [88, 94].

The inhibition of androgen synthesis can be accomplished by suppressing gonadal androgen secretion through surgical castration (orchiectomy), pharmacological castration using gonadotropin-releasing hormone (GnRH) agonists and GnRH antagonists [88, 94]. The inhibition of androgen binding in prostate cells can be accomplished by antiandrogens administration [88, 94]. The first therapeutic attempt to inhibit AR activation was through the development of first-generation antiandrogens, such as bicalutamide [95]. The antiandrogens compete with endogenous androgens for AR binding and block its activation and translocations to the nucleus [88, 94].

ADT initially proves to be an effective therapeutic approach, since over 80% of PC are androgen dependent at initial diagnosis [92]. However, despite initial remission achieved with ADT, within 2-3 years one-third of patients undergoing ADT experience disease relapse, and inevitably progress to CRPC [92].

CRPC occurs when PC progresses to a castration-insensitive phase of disease after developing ADT resistance. This advanced form of PC is associated with poor survival rates and an average of 16-18 months survival time from the beginning of progression. In response to this clinical problem, second-generation antiandrogen therapies, such as enzalutamide (ENZ), were developed and approved.

The second-generation antiandrogens, which include enzalutamide, apalutamide, darolutamide, and abiraterone acetate, have significantly improved the management of CRPC. They have increased the median overall survival, reducing levels of androgens in the bloodstream and within the tumor, as well as lowering serum PSA. [95-97]. Second-generation antiandrogens act as a more potent AR antagonist, binding to the AR with higher affinity and effectively blocking AR activity to greater extent than first-generation antiandrogens [95, 96].

ENZ was firstly synthesized by Charles Sawyers and Michael Jung in 2009 and was firstly approved by FDA in 2012, as a therapeutic agent for metastatic CRPC who have previously undergone docetaxel-based chemotherapy [98]. Later, ENZ was also approved for non-metastatic CRPC and metastatic castration-sensitive PC [99, 100].

ENZ exerts its antitumor effects by targeting the AR signaling pathway in multiple levels. It operates through competitive inhibition of androgen binding to AR. In contrast to first-generation antiandrogen, ENZ further blocks nuclear translocation of the AR, impedes its DNA binding capacity, and inhibits coactivator recruitment. This leads to apoptosis and inhibition of cell proliferation (Figure 8) [101, 102].

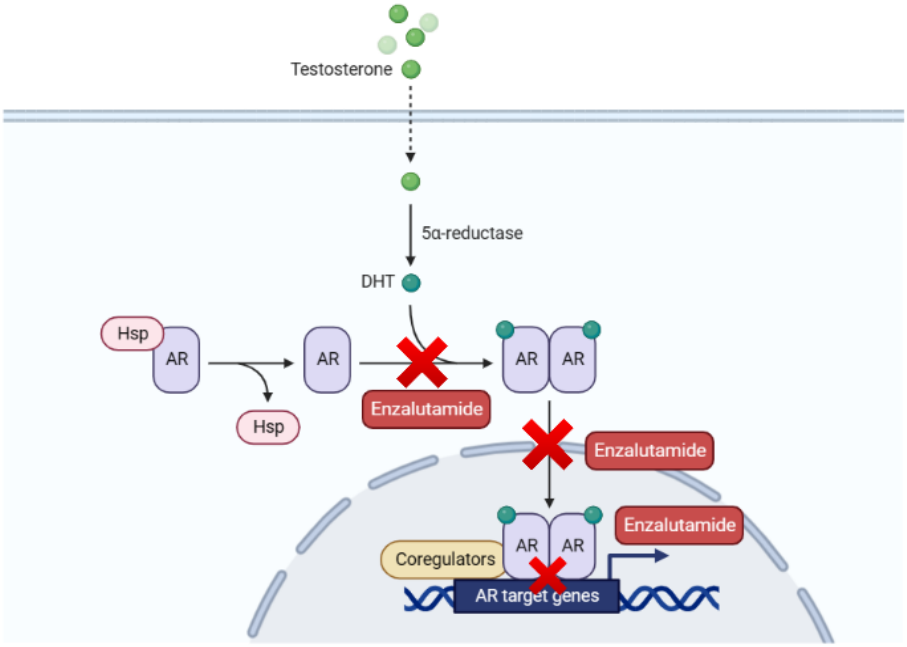


Figure 8: Mechanism of action of enzalutamide in the androgen receptor signaling pathway (BioRender).

Despite the high efficacy and overall survival increase of CRPC patients, there is still a fraction of patients of 20-40% who remains unresponsive to this therapy. Furthermore, even those patients that initially respond eventually acquire resistance. This resistance ultimately leads to the clinical progression of the disease and, unfortunately, often results in death [95, 103].

According to the literature, there are several AR- dependent and independent mechanisms that trigger ADT resistance [104, 105]. They arise from the intratumoral heterogeneity and disease progression brought on by the treatment's selective pressure on the TME. It can emerge from alternative mechanisms of AR activation, including AR mutations, amplification or alternative splicing variants [92, 105, 106]. Amplification of the AR gene is one of the most frequent genetic alterations observed, typically resulting in elevated AR mRNA expression and, consequently, increased levels of the AR protein. This

alteration is associated with a poor response to ENZ treatment, being more frequent in patients progressing on ENZ than in treatment-naïve patients [107]. AR mutations may lead to a decrease in the AR ligand specificity. For instance, the T878A point mutation in the LBD leads to an AR activation in response to androgens, but also to non-androgen hormones and is associated with poor response to ENZ. These alterations allow activation of the AR pathway even in the presence of low levels of androgens [92, 106]. The somatic mutation F877L/F876L is one of the most often observed alterations in patients treated with ENZ [92, 95, 102-104, 108]. When this mutation is present, ENZ acts as an agonist rather than an antagonist promoting tumor progression and cause itself resistance to antiandrogen therapy [92, 95, 102-104, 108]. PC can also shift from an endocrine-driven to a paracrine/ autocrine-driven disease in which the cancer cells produce androgens leading to persistent AR activation [109]. Besides point mutations, alternative splice variants result from different exome combinations and originate different versions of a gene's mRNA transcript, which culminate in different protein isoforms that can present different functions. In this context, these alterations are also important in the development of resistance. For instance, AR variants that lack the LBD are constitutively active proteins and, therefore, sustain the activation of the androgen pathway. One of the variants most involved in the development of ADT resistance and progression to CRPC frequently detected and currently studied is AR-V7 [105, 108, 110, 111]. Indeed, Antonarakis and co-workers demonstrated a significant overexpression of AR–V7 in ENZ-treated patients and observed an association with resistance to ENZ treatment [108, 110].

As previously mentioned, CRPC can also be driven by mechanisms not related to AR, including activation of glucocorticoid receptor (GR), impairment of DNA repair pathways, and acquisition of characteristics and functions of a different cell lineage [92, 104, 106]. The hyperactivation of alternative signaling pathways, for instance, the PI3K/AKT/mTOR pathway, can also promote cell survival and growth independently of AR signaling and is also involved in second-generations antiandrogen drug resistance, like ENZ [104, 108]. Wnt/β-Catenin signaling is frequently activated in the late stage of PC and contributes to the development of therapy resistance [112]. Moreover, a recent study identified Wnt/ β-Catenin signaling as a major mechanism for the development of ENZ resistance in PC [111, 113].

Additionally, cancer cells can bypass androgen dependence and continue to grow and survive despite low androgen levels through aberrant Epidermal Growth Factor Receptor (EGFR) pathway activation [114]. EGFR, a transmembrane glycoprotein, is a member of the ErbB receptor family. Its activation is initiated by ligand binding, which results in the dimerization of the receptor. Several ligands are recognized as activators of EGFR,

including Epidermal Growth Factor (EGF) and Transforming Growth Factor alpha (TGF-α). The activation of EGFR is an important oncogenic pathway that contribute to CRPC progression [114]. Previous studies carried out in our group have shown that different levels of EGFR ligands can influence the response to ADT, confirming the hypothesis that modulation of the EGF/EGFR pathway could affect the development of CPRC [115, 116]. EGFR contributes to cell survival by activating intracellular signaling pathways, including the KRAS-BRAF-MEK/ERK, PI3K/AKT/mTOR, and STAT (Signal Transducer and Activator of Transcription) pathways [117, 118]. These intracellular signaling pathways regulate gene expression, thereby promoting cellular processes like cell proliferation, angiogenesis, migration, and the suppression of apoptosis [117, 118]. More recently, Thomas M. Steele and colleagues demonstrated that ENZ treatment increases EGFR ligands, which result in the phosphorylation of EGFR and ERK activation. They also conclude that EGFR inhibitors that prevent ERK phosphorylation were effective in suppressing viability of ENZ-resistant CRPC cells [119, 120].

Epithelial plasticity, in which PC cells lose epithelial differentiation, express less AR and AR targets, and exhibit neuroendocrine (NE) characteristics, is an increasingly recognized mechanism for ADT resistance in PC [92]. NE differentiation in PC is frequent following ADT failure and is also a common mechanism of ENZ resistance [111].

Despite the significant progress made in the last decade with the development of more effective second-generation anti-androgens and an important understanding of the driving mechanisms behind CRPC and ADT resistance, metastatic CRPC remains an uncurable global cancer burden [121]. This is also driven by the lack effective therapeutic approaches and is exacerbated by the challenge of early detection of relapses and non-responsiveness to therapies [121].

In fact, early and accurate prediction biomarkers for ENZ resistance are essential for clinicians to understand the disease's evolution and tailoring a more personalized patient follow-up [122]. However, the prognosis and therapy response biomarkers currently used in the clinic, like PSA test, often lack the specificity required for accurately assessing the disease [123]. As described, a growing body of evidence highlight the significance of cell communication mediated by EVs and the miRNAs' role in tumor progression and the development of resistance phenotypes to therapies [122, 123]. The study of miRNAs derived from extracellular vesicles associated with the response to enzalutamide assumes importance because it can provide valuable information into the molecular mechanisms underlying this response. This, in turn, can contribute to the identification of new therapeutic targets. The discovery of miRNAs that are correlated with either sensitivity or resistance to ENZ holds the potential to stratify patients who could benefit from treatment: moreover, it

aids in monitoring the therapy responses and the early identification of resistance development. [121, 122]. This approach can help clinicians to personalize treatment schemes, avoiding ineffective therapies and reducing unnecessary side effects [121, 122]. Thus, the analysis of EVs in liquid biopsies holds great potential for the definition of biomarkers of therapy response in context of ENZ-resistant. This is particularly noteworthy given that ENZ is one of the most potent and commonly prescribed antiandrogens [121, 122].

1.5. Deregulated microRNAs in enzalutamide resistant prostate cancer

Currently, several studies have reported deregulated miRNAs in context of ENZ resistance, however most of the efforts made focused on the expression of a limited number of selected miRNAs [124]. In fact, hsa-miR-20b, hsa-miR-106a and hsa-miR-363, that target different genes involved in cell cycle control, differentiation and survival, including *MYCN, E2F1, STAT3, AURKA, KLF4,* are reported as underexpressed in ENZ resistant LNCaP cell line [125]. Several other miRNAs have been shown to be underexpressed in ENZ resistant VCaP, LNCaP, PC3 and DU-145 cell lines, including hsa-miR-1 which regulates the expression of *ZBTB4,* promoting androgen-independent proliferation [126]. Downregulation of hsa-miR-215 is correlated with overexpression of *KDM1B*, which promotes AR-dependent transcription and ENZ-resistance in prostate cancer cells [127]. Additionally, hsa-miR-361, whose expression is lower in patients with recurrent PC, increases Enz sensitivity via targeting the ARv7 and *MKNK2*, which are oncogenes involved in the development of and progression of PC through mediating cell growth, proliferation and invasion [128]. Also, hsa-miR-628-5p acts as tumor suppressor miRNA, being downregulated in PC3 cell line comparatively to RWPE, and reported to decrease resistance of ENZ in LNCaP cells [129]. Through bioinformatic prediction, hsa-miR-628-5p directly targets *JAG1*, a ligand of Notch signaling [129].

On the other hand, overexpression of hsa-miR-346, hsa-miR-361-3p, and hsa-miR-197 through miRNA mimics increase PC cell proliferation under ENZ treatment [130]. Moreover, hsa-miR-let7a and hsa-miR-32 overexpression promote NE differentiation and ENZ resistance [131]. In contrast, overexpression of hsa-miR-644a potentiates ENZ effects on prostate cancer cells, through direct targeting of *AR, ZEB1, CDK6, SNAIL, MYC* [132].

Additionally, since AR pathway is key in the development of therapy resistance, the induced overexpression of certain miRNAs involved in AR pathway can regulate the sensitive/resistant phenotype. Indeed, hsa-miR-30c-1-3p, hsa-miR-103a-2-5p, hsa-miR-

124, hsa-miR-181c-5p and hsa-miR-8080 overexpression directly suppresses ARv7 expression, which is one of the most described AR variants in advanced and ENZ-resistant PC [133-135]. Thus, these miRNAs, when overexpressed, decrease ENZ resistance in C4-2, VCaP, and 22RV1cell lines and in nude mice [133-135].

Lei and colleagues reported that hsa-miR-198 is expressed in ENZ resistant cells, thus inhibiting autophagic cell death, through direct target of *AMBRA1* [136]. Moreover, overexpression of hsa-miR-125b-2-3p and hsa-miR-4736 in C4-2 cells inhibited ENZ-induced prostate cell invasion. Also, overexpression of hsa-miR-99b decreased survival of LNCaP cells under ENZ treatment.

Regarding biomarker potential of circulating miRNAs in ENZ-resistant PC, only a few miRNAs have been studied in blood samples from ENZ-resistant PC patients. Hsa-miR-21 levels are increased in exosomes isolated from ENZ- resistant patients' sera. Additionally, hsa-miR-21 and hsa-miR-141 present higher levels in mCRPC plasma samples of patients undergoing ENZ-treatment compared with healthy control [60]. Patients undergoing this same treatment that present high levels of hsa-miR-375 or hsa-miR-3687 show a shorter time to progression [63]. However, despite promising, these miRNAs were studied in small cohorts and their value as resistance biomarker needs further validation in larger cohort studies.

Despite the great interest in the study of EVs in cancer, there is still limited experimentally validated evidence and several challenges associated to the use of EVs, that are mostly related to the difficulties in standardizing the isolation and characterization procedures [124, 137]. Also, the study of miRNAs has some limitations that still need to be overcome due to its lack of specificity, and the lack of sensitive and specific detection methods to accurately measure miRNA expression levels [124, 137, 138].Thus, to detect early emergence of resistance phenotypes before disease progression can be detected clinically, it is crucial to define a profile of deregulated miRNAs in the context of ENZ-resistant PC, mostly in study models that, at the greatest extent possible, mimic the physiological conditions of the tumor.

1.6. *In vitro* 3D spheroid models applicability in the study of extracellular vesicle-derived miRNAs

Most research in the oncology field relies on the use of two-dimensional (2D) *in vitro* cell cultures, since they are relatively simple and cost-effective to establish and maintain [139]. Moreover, 2D cell culture is a well-established and versatile cellular model, with a diverse range of available cell lines that can mimic different cancer types and stages [139].

However, 2D cultures have many limitations. First, 2D cultures do not mimic the natural structures of tissues or tumors. In the 2D culture method, cell-cell and cell-extracellular environment interactions are not represented as they would be in the tumor mass [139-141]. These interactions are responsible for cell differentiation, proliferation, expression of genes and proteins, responsiveness to stimuli, drug metabolism and other cellular functions [139-141]. So, 2D cell culture can alter cell morphology, expression profiles and functionality, ultimately failing to mimic the true in vivo phenotype of cancer cells. Another disadvantage of 2D culture is that cells have unlimited access to oxygen, nutrients, metabolites and signal molecules, which does not translate the real conditions due to inherent in vivo tumors natural architecture and physical properties [139-141]. In addition, 2D cultures usually lack the TME components and interactions, which are essential for tumor progression [139-141].

On the other hand, three-dimensional (3D) *in vitro* models provide significant advantages over 2D models, since they better replicate cell-cell interactions, the three-dimensional architecture and molecular conditions found in *in vivo* [139, 142, 143]. Furthermore, 3D models also recreate oxygen and nutrient gradients, all of which have a significantly impact on cellular behaviors and responses [139, 142, 143]. 3D cell culture can be divided into spheroid and organoid models and currently, there are also available several scaffold or scaffold-free 3D cell culturing techniques, with inherent advantages and disadvantages (Figure 9) [144-146]. Despite the diverse 3D culturing methods available, the micromolded agarose (non-adhesive hydrogel) technique presents numerous advantages. In this technique, cell-cell interactions dominate over cell-substrate interactions, enabling cells to self-organize and undergo morphological changes. Importantly, it maximizes the communication between cells and the extracellular matrix (ECM) [147].

This spheroid culture system emerges as an especially valuable 3D cell culture method, with broad applications applied in life science research [144]. In cancer-related research, tumor spheroids have gained widespread popularity due to their ability to promote cell-cell interactions and recreate the *in vivo* TME [133]. Notably, larger spheroids can mimic the hypoxic conditions within the tumor masses [133]. Additionally, several studies have highlighted the importance of 3D cultures in the study of EVs in cancer research since recent observation demonstrates that EVs' cargo markedly depends on culture conditions [148-150]. The TME is known to influence EVs biogenesis and cargo loading. By culturing cancer cells in 3D, researchers can better understand how EVs are released within a more relevant tumor-like context [148-150]. Since EV biogenesis and cargo loading is mediated by many of the biological processes that are not physiologically represented in 2D cultures, EVs

derived from 3D cultures can present a more similar cargo to that found in patients derived EVs [148-150]. Understanding the specific content of EVs and their dynamics in 3D culture settings provides essential information about the functional role of EVs in cancer progression, metastasis and drug resistance [148-150].

In attempts to overcome some of the undesirable limitations associated with 2D culture, the adoption of a 3D model could contribute to better understand the resistance mechanisms and to identify circulating molecular biomarkers involved in the development of ENZ resistance. These identified biomarkers may hold the potential for future inclusion in clinical guidelines.

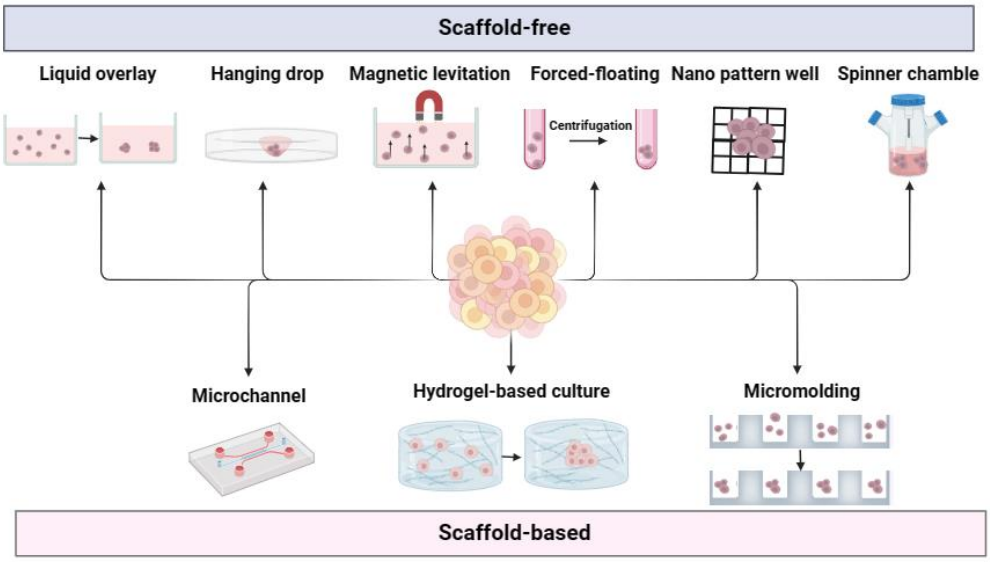


Figure 9: Most common scaffold-free and scaffold-based techniques for 3D cell culture (BioRender).

Objective

2. Objective

The main aim of this study is to identify an EV-derived miRNA profile associated with the acquisition of ENZ resistance in PC. Moreover, we intend to clarify the dynamics changes of this profile during the process of ENZ resistance acquisition, in order to identify potential new predictive biomarkers that may find application in the future in clinical practice.

2.1. Specific objectives

- Establish and characterize a cell line resistant to ENZ (LNCaPER). The resistance will be induced by continuous exposure of LNCaP cells to successively higher concentrations of ENZ (2.5 μM; 5 μM; 10 μM; 20 μM) until reaching the maximum ENZ concentration of 30 μM.

- Define a miRNA profile associated with ENZ resistance acquisition. This will be assessed by comparing the miRNAs levels of LNCaP *vs* LNCaPER cells using a high throughput approach;

- *in silico* identification of molecular targets and the involved signaling pathways of the miRNA profile identified associated with resistant to ENZ;

- Establish 3D spheroid models of LNCaP, LNCaP resistant to intermediate ENZ concentrations and LNCaPER cell line;

- Analyze the dynamic expression of the top 5 overexpressed miRNAs associated with resistant to ENZ acquisition in 3D spheroid-derived EVs.

Material and Methods

3. Material and Methods

3.1. Cell line characterization and maintenance

The LNCaP cell line used in the present study was acquired from the American Type Culture Collection (ATCC) biobank and was derived from a biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male with confirmed diagnosis of metastatic prostate carcinoma. These cells express AR and are responsive to 5-alpha-dihydrotestosterone [151].

Firstly, a cryopreserved vial of LNCaP cells was thawed and cells were kept in RPMI 1640 (*PAN-Biotech™*), supplemented with 10% of FBS (Fetal Bovine Serum) (*Gibco®*) and 1% of Pen-Strep (*Gibco®*). Cells were kept in an incubator under 37ºC of temperature, at 5% CO_2 in a humid atmosphere. The authenticity and purity of the cell vial used was confirmed by cell genotyping using *POWERPLEX 16 HS kit* (*Promega*) in Genomics platform (*GenCore*) from I3S. A sample of the cultured cells' medium was collected and tested for mycoplasma infection weekly.

When cells had reached 80-90% confluence, they were trypsinized using trypsin-EDTA (1X) (*Gibco®*). The detached cells were resuspended in fresh growth media. The concentration of viable cell in cell suspension was determined using trypan blue solution (*VWR™*) and the *EVE™ Automated Cell Counter* (*NanoEnTek®*) in order to plate and obtain cell pellets with the desired number of cells.

3.2. Determination of IC50 of ENZ

To determine the half maximal inhibitory concentration (IC_{50}) of ENZ in LNCaP cells, the WST-1 cell viability assay (*Abcam®*) was performed. In a 96-multi well plate, 8 wells were cultured with 100 µL of a 200 000 cells/ml cell suspension, per each ENZ concentration studied: 0 µM; 10 µM; 20 µM; 30 µM and 40 µM. 48h after cell culture, WST-1 assay reagent (tetrazolium salt) (*Abcam®*) was added to the cell culture media, at a concentration of 10% (V/V). After 2h, the amount of formazan dye produced, from the mitochondrial cleavage of the tetrazolium salt, was quantified by measuring the absorbance at 450nm and 650nm using the multi-plate reader EZ Read 400 (*Biochrom®*). The proportion of cleaved tetrazolium salt into formazan was quantified in relation to the control (0 µM). The IC_{50} was calculated using IC_{50} calculator (AAT Bioquest), according to the following equation [152]:

$$Y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + \left(\frac{X}{IC50}\right)^{\text{Hill coefficient}}}$$

$$Y = -22.0571 + \frac{103.7985 + 22.0571}{1 + \left(\frac{X}{34.8249}\right)^{3.8049}}$$

The maximum concentration of ENZ to be studied was defined by IC_{50} results.

3.3. Development and characterization of the LNCaPER cell line

To establish a cell line resistant to 30 μM derived from the LNCaP cells (LNCaPER), cells were exposed to increasing concentrations of ENZ, for ten months, at concentration endpoints of 2.5 μM; 5 μM; 10 μM; 20 μM and 30 μM ENZ.

Before increasing ENZ concentration, cells were photographed to check for morphological changes using Olympus IX51 microscope. The metabolic capacity of the cells was evaluated, and a cell cycle analysis was performed to assess cell cycle distribution of the whole cell population and, therefore, assure that cells were proliferating and not quiescent, to prove that the resistance phenotype had been established (Figure 10). Additionally, the mRNA levels of PSA and AR were quantified in LNCaP and LNCaP resistant to all ENZ concentration end points (Figure 10).

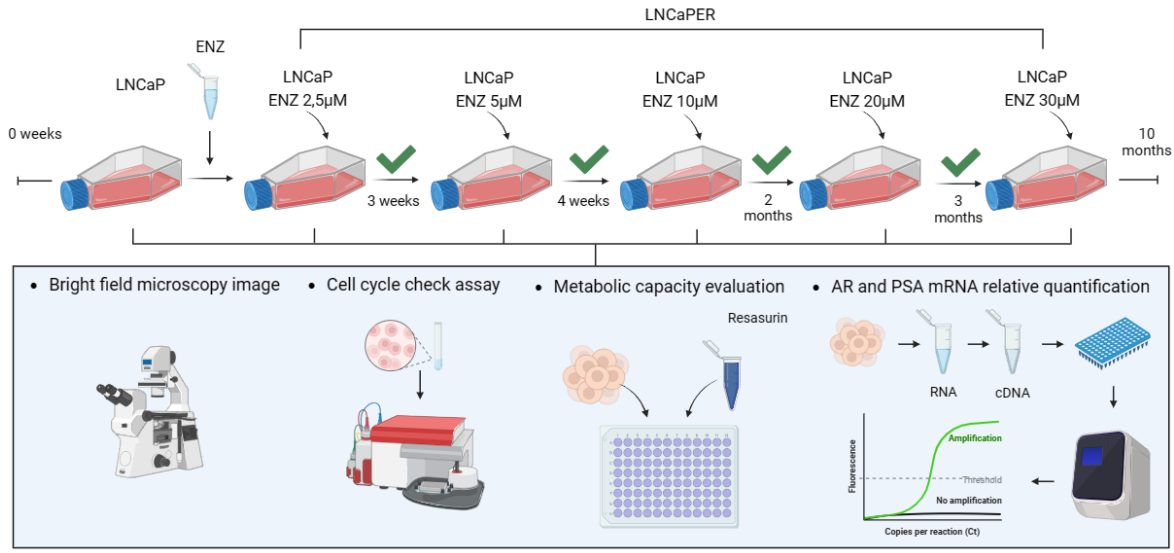


Figure 10: Process of developing the ENZ-resistant cell line, LNCaPER, from the LNCaP cell line. The LNCaP cells were continuously treated with ENZ for 10 months. The concentration of ENZ administered was increased after AR pathway evaluation and when the cells reached a similar metabolic capacity and cell cycle distribution to the LNCaP cells, i.e., when a resistance phenotype was established at each end point (Created with BioRender).

3.3.1. Cell cycle check

The cell cycle was analyzed using Propidium Iodide (PI) Staining. For that 2 ml of 200 000 cells/ml suspension were cultured per well in a 6-multi well plate (3 wells per condition), in the presence of the respective ENZ concentration. After 48h, cells were detached with trypsin-EDTA (1X) (*Gibco*®) and fixed with 70% ethanol for 15 minutes. The cells were then washed with DPBS (*Gibco*®) and DNA was stained with a 5% PI, 1% Triton and 1% RNase A solution. The cells were incubated for 15-30 minutes and analyzed by flow cytometry using Beckman Coulter Cytomics FC500 (*Beckman Coulter*®). The results were analyzed in and the CXP software. The percentage of cells in each cell cycle phase were calculated from the total of 10 000 cells (100%), per each condition.

3.3.2. Metabolic capacity measurement

The metabolic capacity was assessed using the resazurin assay. In a 96-multi well plate, 100 µL of 200 000 cells/ml suspension were cultured in each well. In each experiment cells were cultured in 8 wells with ENZ, at each one of the defined concentrations; and 8 wells cultured with original LNCaP cells (control). After 48h, Resazurin (*ACROS Organics*®) was added to each well at 10% (V/V). To calculate the percentage of resazurin metabolized, absorbances were then measured at 570 nm and 600 nm using the multi-plate reader EZ Read 400 (*Biochrom*®) and applied the following equation:

$$\%\ Metabolized\ Resazurin = \frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} * 100$$

O1- Extinction coefficient of oxidised Alamar Blue at 570 nm (80586)

O2- Extinction coefficient of oxidised Alamar Blue at 600 nm (117216)

A1 - Abs $_{570\ nm}$ sample

A2 - Abs $_{600\ nm}$ sample

R1 - E from reduced Alamar Blue at 570 nm (101088)

R2 - E from reduced Alamar Blue at 600 nm (9514)

N1 - Abs $_{570\ nm}$ blank

N2 - Abs $_{600\ nm}$ blank

The percentage of resazurin metabolized of the ENZ-treated cells was compared to the non-treated cells when the LNCaP cell line reached 100% of resazurin metabolized in each assay performed.

3.4. AR and PSA mRNA levels relative quantification by real-time qPCR

To quantify the expression levels of AR and PSA mRNAs, RNA was extracted from 3 replicates of 1.5 million cells from each ENZ concentration administered, using the *GSB Total RNA kit-Blood and Culture cells* (*GRiSP*®). Concentration and purity of the isolated RNA was then assessed by absorbances measurement at 260 and 280 nm using the *NanoDrop® Lite Spectrophotometer (Thermo Scientific™).*

The complete RNA sequence was used for cDNA synthesis using *High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®)*. The reverse transcription reaction was performed according to the following conditions: 10 minutes at 25ºC, followed by 120 minutes at 37ºC and, finally, 5 minutes at 85ºC.

The relative quantification of the mRNA molecules of interest was performed by real-time PCR using StepOne™ PCR (*Applied Biosystems®*). The amplification reaction was performed using 1x Master mix (*Applied Biosystems®*) with 1x specific *TaqMan® mRNA Expression Assays* (B2M (beta-2-microglobulin): Hs00187842_m1; AR: Hs00171172_m1; PSA: Hs02576345_m1 (*Applied Biosystems®*)) and cDNA sample.

For each sample the CT (Threshold Cycle) value was determined using the StepOne™ v2.2 software (*Applied Biosystems®*). All samples were quantified in duplicate and the relative expression was quantified by the Livak method ($2^{-\Delta\Delta CT}$). A negative control was always included in each reaction plate, for each assay used.

3.5. MiRNA profiling through NanoString Analysis

To establish the profile of miRNAs associated with ENZ resistance, 3 replicates of 2 million cells from LNCaP and LNCaRER were used for total RNA extraction with *RNA Purification kit (Norgen Biotek Corp.)* according to the manufacturer instructions.

The total RNA extracted from LNCaPER and LNCaP cells, were quantified in the *nCounter® Pro Analysis System using nCounter® Human v3 miRNA Expression Assay Kit (NanoString®)*. This assay is designed to provide an ultra-sensitive, reproducible, and highly multiplexed method for detecting miRNAs in total RNA samples. It contains 799 probes, six positive controls, eight negative controls, six ligation controls, and five mRNA reference controls [153]. This hybridization-based system uses molecular barcodes called *nCounter Reporter Probes* for detection and quantification of each miRNA, allowing a multiplexed detection of multiple microRNAs without the need of reverse transcription or amplification.

For sample preparation there is a step of multiplexed annealing of the specific tags to their target miRNA, followed by a hybridization reaction. Then excess probes are washed away using a two-step magnetic bead-based purification and an enzymatic purification to remove the unligated tags. After washing, the Capture Probes and target/probe complexes are eluted off the beads and are hybridized to magnetic beads complementary to sequences on the Reporter Probe. An additional wash is performed to remove excess Capture Probes. Finally, the purified target/probe complexes are eluted off the beads and immobilized on the cartridge for data collection. The assay was performed according to the protocol provided by the manufacturer and all sample guidelines/ recommendations.

The data was analyzed using ROSALIND software. Normalization, fold changes and ps were calculated using criteria provided by *Nanostring*®. Following background subtraction based on POS_A probe correction factors, normalization was performed in two steps: positive control normalization and codeset normalization. During both steps the geometric mean of each probeset was used to create a normalization factor. ROSALIND calculated fold changes and ps for comparisons using the t-test method. P adjustment was performed using the Benjamini-Hochberg method of estimating false discovery rates (FDR). The significantly up-regulated and down-regulated miRNAs were selected considering a $p < 0.05$ and a cut off of ≥ 1.5 or ≤ -1.5 expressional fold change difference, respectively. For graphical representation the results were converted considered Log_2 (fold change) value>0.6 or<-0.6 and a $-Log_{10}$(adjusted p)>1.3.

3.6. *In silico* analysis

The target mRNAs of the identified miRNA profile were determined using miRTarBase v9.0 database, which compiles information relatively to miRNA-mRNA interactions from reviewed literature published until 2021. It was also performed a literature review of the relevant papers published from 2021 to 2022 to retrieve the information relative to mRNA targets of the selected miRNAs not included in the version of the database used. It was only considered miRNA-target interactions information provided from strong evidence methods (Western blot, qPCR and luciferase assay).

Subsequently, protein-protein interaction (PPI) networks for the potentially upregulated and downregulated proteins that were targeted by more than one of the determined miRNAs were created using the Search Tool for the Retrieval of Interacting Genes (STRING) app in the Cytoscape software. The proteins that didn´t stablished any interaction were excluded. The interactions with a combined score of >0.4 were selected as significant. The functional enrichment analysis of Gene Ontology (GO), Kyoto Encyclopedia

of Genes and Genomes (KEGG) and Reactome pathways was performed with the STRING enrichment analysis tool, considering a with a false discovery rate (FDR) of $p<0.01$.

3.7. Spheroids assemble and characterization

3.7.1. Spheroid formation

For the preparation of agarose molds, 1g of pure agarose powder (*SeaKem™ LE Agarose, Lonza™*) was measured and autoclaved in dry cycle. Subsequently, a 0.9 (v/v) NaCl solution was prepared and filtered in aseptic conditions. The autoclaved agarose was then dissolved in 50 ml of the sterile saline solution. In aseptic conditions, 550µL of molten agarose was pipetted into pre-autoclaved #12-81 micro-molds (*MicroTissues®*). After the agarose had solidified, the agarose counter-mold (*3D Petri Dish®*) was gently removed and placed in a 12-well plate. Before cell culture agarose molds were equilibrated with RPMI 1640 culture medium supplemented with 1% Pen-Strep.

The tripsinized LNCaP and ENZ-treated LNCaP cells were resuspended in RPMI 1640 supplemented with 10% exosome-Depleted FBS (*Gibco®*) and 1% of Pen-Strep (*Gibco®*). Finally, cells were seeded in each agarose mold at densities of 5 000 cells/sph, 10 000 cells/sph or 20 000 cells/sph.

3.7.2. Morphology and metabolic activity

Spheroids morphology was assessed during each of the timepoints previously defined using microscopy (Olympus IX51) and ImageJ (National Institutes of Health) was used to determine the diameter.

In parallel, metabolic activity of 3D cultured cells was performed using *CellTiter-Glo® 3D Cell Viability Assay* at each of the predefined timepoints. From each mold 5 to 6 spheroids were collected and transferred into a maximum of 100 µL of medium to an opaque-walled 96-well plate. Subsequently 100 µL of *CellTiter-Glo® 3D reagent* (*Promega*) was added to each well. The plate was vigorously shacked for 5 minutes in the dark and then incubated at room temperature for an additional 25 min. The luminescence was measured using *GloMax® Multi Detection System (Promega®)*. Three replicates were conducted for each cell line in each condition. For graphical representation of the variation of metabolic activity of LNCaP spheroids and ENZ-treated spheroids over time the measured luminescence values at 72h and 7 days were divided by the value obtained at 24h (fold change).

3.7.3. Cell spheroids fixation

To fix the cell spheroids, the cell culture media was removed and washed with DPBS. Subsequently, 1,5 ml of a 4% paraformaldehyde (PFA) (*EMS*®) solution was added to each well following a 20 min incubation period. After incubation, PFA solution was removed and the spheroids were washed with DPBS and 200 µL of molten agarose was pipetted to seal the agarose mold.

3.7.4. Immunohistochemistry

The fixed spheroids in the agarose molds were embedded in paraffin and sectioned. The paraffin sections underwent deparaffinization with xylene and rehydrated through a series of ethanol solutions of decreasing concentrations and finally immersed in PBS. The rehydrated sections were transferred to a rack with 10 mM citrate buffer with a pH of 6.0. This buffer facilitated antigen retrieval by subjecting the samples to heat at 95ºC for 30 min. After cooling down, the slides were washed with PBS and immersed in a 0.25% Triton solution. After a 10 min incubation period, the slides were washed and subsequently treated with 0.25% ammonium chloride solution. For effective blocking of non-specific binding sites, the sections were treated with a 5% FBS and 1.5% BSA solution. After blocking, samples were incubated overnight at 4ºC in humid and dark conditions with the following primary antibodies: Ki-67 rabbit (*Abcam*®); vimentin mouse (*Santa Cruz*®); Fibronectin rabbit (Sigma); β-catenin mouse (*BD Biosciences*®); caspase 3 rabbit (*Cell signaling technology*®) and collagen type IV mouse (*Dako*®). After sections were washed and the secondary antibody and DAPI were added. Finally, the slides were mounted using *VECTASHIELD*® Antifade Mounting Medium and prepared for imaging by confocal laser scanning microscopy (CLSM, Leica SP2 AOBS SE). The acquired images were visualized and processed using Fiji software.

3.8. EVs isolation

The EVs were precipitated from cell spheroids culture media 72h and 7 days after spheroid assembly. The EVs were isolated using an adapted protocol previously optimized by the research group for EVs extraction from 2D cell culture [154]. Spheroids cell medium was collected and centrifuged during 30 min, at 3500 rpm and 4ºC, to remove cells and debris. After centrifugation the culture medium was filtered using a 0.22 µm filter (*GE Healthcare Whatman*™). Following, the Total Exosome Isolation Reagent from cell culture media (*Invitrogen*™) was mixture with the cleared medium in a proportion of 1:2. The mixture was incubated at 4 °C overnight and then centrifuged for 1 hour at 10 000 g. The

pellet, which contained the pre-enriched EVs, was then resuspended in filtered DPBS and kept at -80 °C until further analysis.

3.9. EVs characterization

The EV samples were analyzed for particle concentration and size distribution by the NS300 Nanoparticle Tracking Analysis (NTA) system (*Malvern Panalytical*). In order to obtain the proper concentration for the best NTA measurement, samples were pre-diluted in filtered DPBS (1:100). Video acquisitions were carried out using a camara level of 16 and a threshold of 5. Analysis of particle concentration per mL and size distribution was performed with NanoSight NTA software v3.2 (*Malvern Panalytical*).

Additionally, EV samples were also imaged by transmission electron microscopy (TEM) at I3S Histology and Electron Microscopy facility, in order to obtain an accurate characterization of EV samples, including information about their morphology, size distribution, and the presence of aggregates and non-vesicular particles.

3.10. MiRNA quantification from cell spheroids and respective EVs

The identified miRNAs profile was quantified in cells and in the secreted EVs. The RNA extraction from cell spheroids and from isolated EVs in 3D culture was performed in the automatic extractor *KingFisher™ Duo Prime Purification System (Thermo Scientific™)* using *MagMAX™ mirVana™ Total RNA Isolation Kit (Thermo Scientific™).*

RNA samples served as templates for cDNA synthesis for each studied miRNAs using a *Taqman®MicroRNA Reverse Trancription kit (Applied Biosystems®)* and sequence-specific stem-loop primers for hsa-miR-21-5p, hsa-miR-22-3p, hsa-miR-200a-3p, hsa-miR-221-3p, hsa-miR-222-3p and RNU44, which was used as endogenous control for miRNA quantification in cell pellets. The reverse transcription reaction was performed according to the following conditions: 30 minutes at 16ºC, followed by 60 minutes at 42ºC and, finally, 10 minutes at 85ºC.

The mRNA samples were also used as templates for complementary cDNA synthesis for GAPDH mRNA relative quantification, which was used as endogenous control miRNA quantification in EVs, following the previously described methods and conditions.

The relative quantification of the mRNA and miRNA molecules of interest was performed by real-time PCR using StepOne™ PCR (*Applied Biosystems®*), following the

previously described methodology and conditions. The reaction was performed using 1X probes (*TaqMan® microRNA Expression Assays*, has-miR-21-5p: TM000397, hsa-miR-22-3p: TM000398, has-miR-200a-3p: TM000502, has-miR-221-3p: TM000524 and RNU44: TM001094. For GAPDH mRNA quantification it was used the *TaqMan® mRNA Expression Assay*: Hs00187842_m1.

3.11. Statistical analyses

The significance of the differences observed in metabolic activity assays and between spheroid diameters was evaluated using GraphPad Prism 8. It was performed the Shapiro-Wilk normality test. When the results presented a normal distribution the existence of significant differences between groups was evaluated using one-way-ANOVA and Tukey's multiple comparisons test. When the results were not normally distributed the nonparametric Kruskal-Wallis test and Dunn's multiple comparisons test were applied.

The statistical analysis of the differences observed between the miRNAs and mRNAs expression levels was performed using *IBM® SPSS® Statistics*, using t test analysis and performing the Livak method ($2^{-\Delta\Delta Ct}$).

Results

4. Results

4.1. Characterization of an Enzalutamide-resistant cell line

To determine the inhibitory effect of ENZ in LNCaP cells, the IC_{50} value was calculated. Figure 11-A shows the percentage of viable LNCaP cells treated with different ENZ concentrations and Figure 11-B shows the dose-response curve. The half inhibitory concentration obtained was 34.8 µM. Based on the IC_{50} value obtained, the concentration of 30 µM of ENZ was defined as the maximum concentration to be administered to establish the LNCaPER cell line.

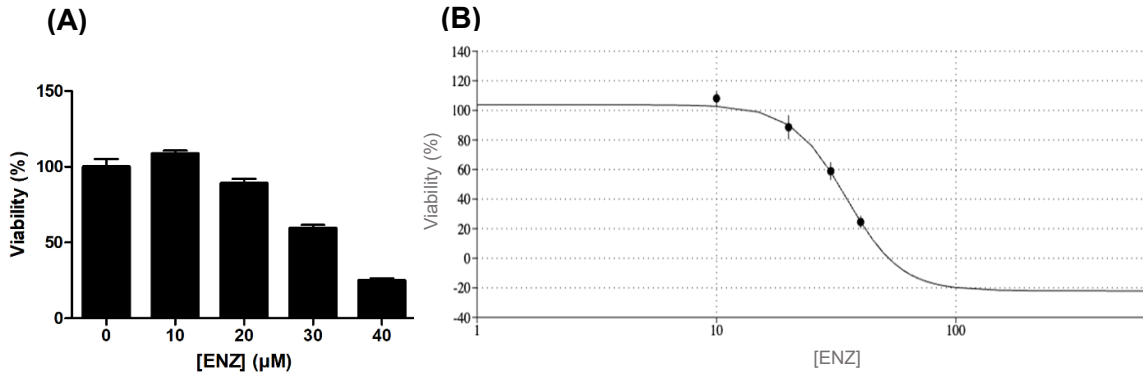


Figure 11: (A) Graphical representation of cell viability with different ENZ doses. (B) Graphical representation of the dose-response curve for ENZ in LNCaP cells.

Figure 12 represents bright-field microscopic images of LNCaP cells that have developed resistant to varying ENZ concentrations, namely 2.5 µM, 5 µM, 10 µM, 20 µM and 30 µM. During the process, some changes were observed in the morphology of cells. The ENZ treated cells exhibited a tendency to form more aggregates with less spindle-shaped morphology characteristic of LNCaP cells. Moreover, there was an increase in the presence of apoptotic bodies.

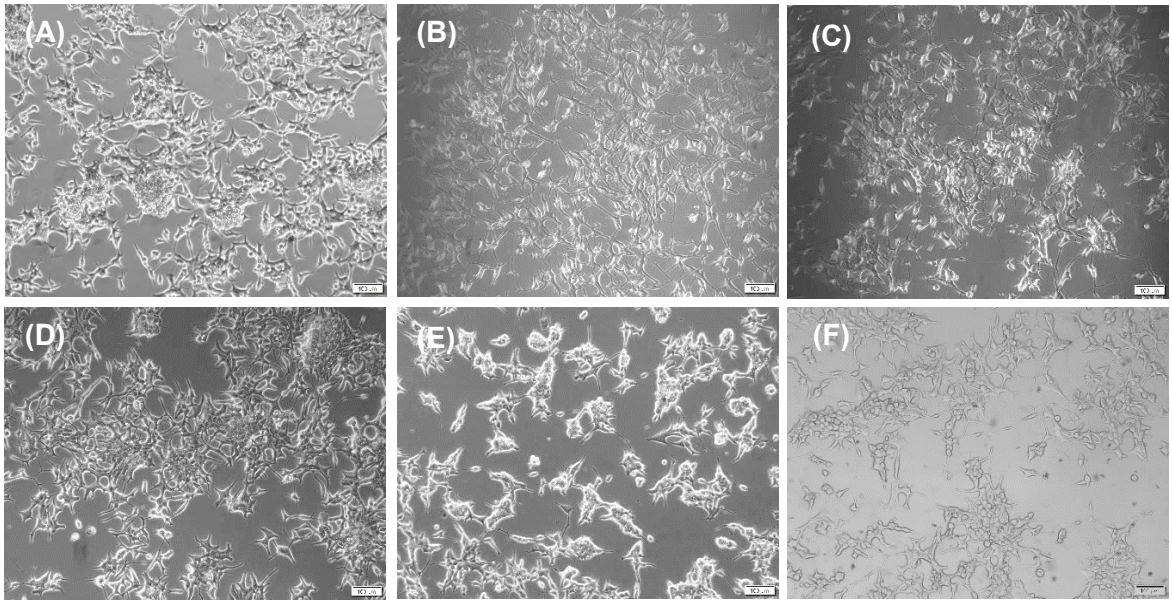


Figure 12: Bright-field microscopic image (100x) of LNCaP and LNCaP treated with different ENZ concentrations (A) LNCaP; (B) LNCaP 2.5 μM; (C) LNCaP 5 μM; (D) LNCaP 10 μM; (E) LNCaP 20 μM; (F) LNCaP 30 μM (LNCaPER). Scale bar: 100 μm.

The results of the metabolic capacity performed for each ENZ concentration studied are represented in figure 13. To facilitate this assessment, the metabolic capacity of ENZ-treated LNCaP cells was compared to that of the untreated LNCaP cells, which was considered as a baseline of 100%. Before increasing the concentration of ENZ administered, we assured that the metabolic capacity of the cells treated with ENZ in each concentration was close to the metabolic capacity of the LNCaP cells, always less than 25% difference in metabolized resazurin. It is important to notice that as the concentration of ENZ administered increases, the metabolic capacity of ENZ-treated LNCaP cells approaches that of untreated LNCaP cells (Figure 13).

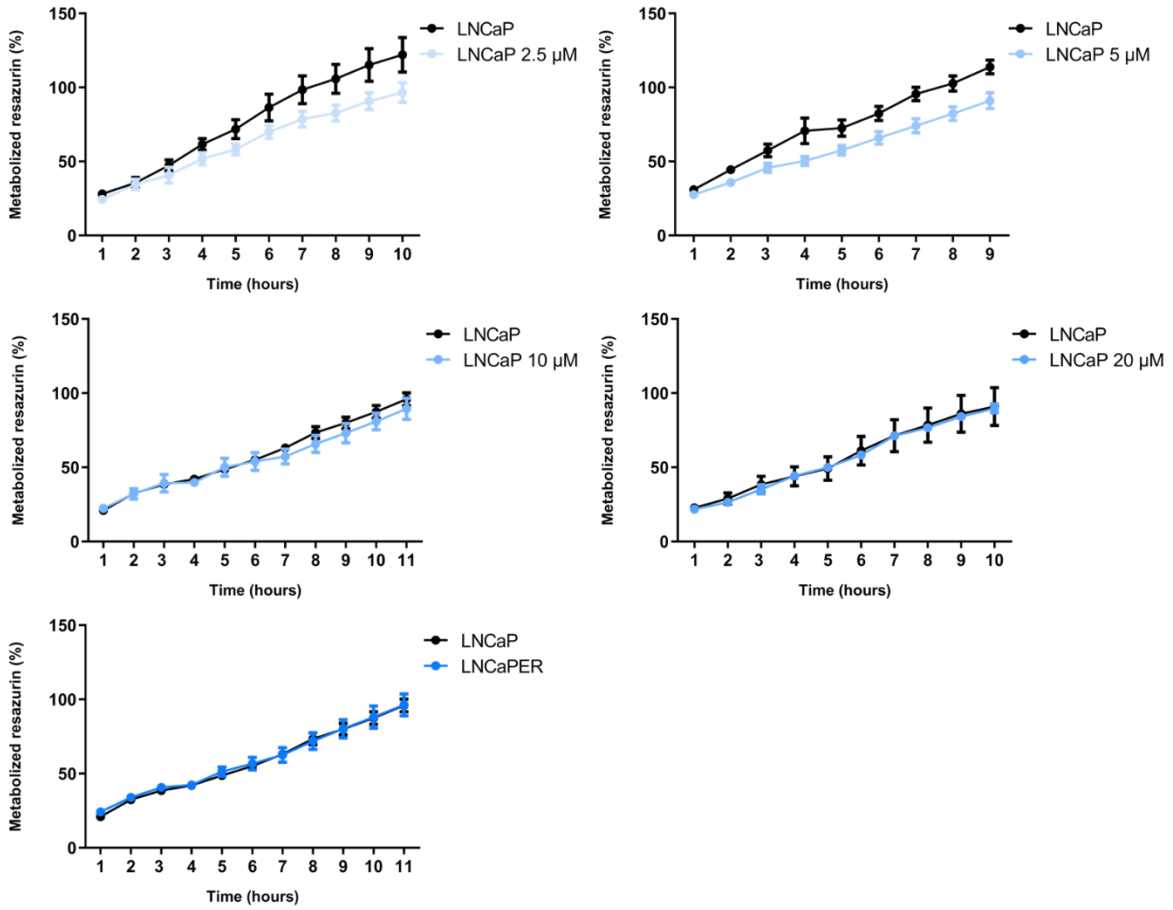


Figure 13: Graphical representation of the metabolic capacity of LNCaP 2.5 µM, LNCaP 5 µM, LNCaP 10 µM, LNCaP 20 µM and LNCaPER cells.

Figure 14 shows the results of the cell cycle assay of the LNCaP and LNCaPER cells. Comparing the distribution of cells in each phase between ENZ-treated cells and original LNCaP cells, we observed that proportions of cells in phase G_0/G_1 were similar (LNCaP: 72.47%; LNCaP 2.5 µM: 77.24%; LNCaP 5 µM: 79.49%; LNCaP 10 µM: 84.01%; LNCaP 20 µM: 73.35%; LNCaPER: 77.01%), as well as in phase S (LNCaP: 7.99%; LNCaP 2.5 µM: 4.37%; LNCaP 5 µM: 3.38%; LNCaP 10 µM: 2.20%; LNCaP 20 µM: 7.06%; LNCaPER: 6.96%) (Figure 14). Similarly, no differences were observed between the proportion of cells in the G_2/M phase (LNCaP: 19.06%; LNCaP 2.5 µM: 12.70%; LNCaP 5 µM: 12.69%; LNCaP 10 µM: 10.08%; LNCaP 20 µM: 16.74%; LNCaPER: 15.79%) (Figure 14).

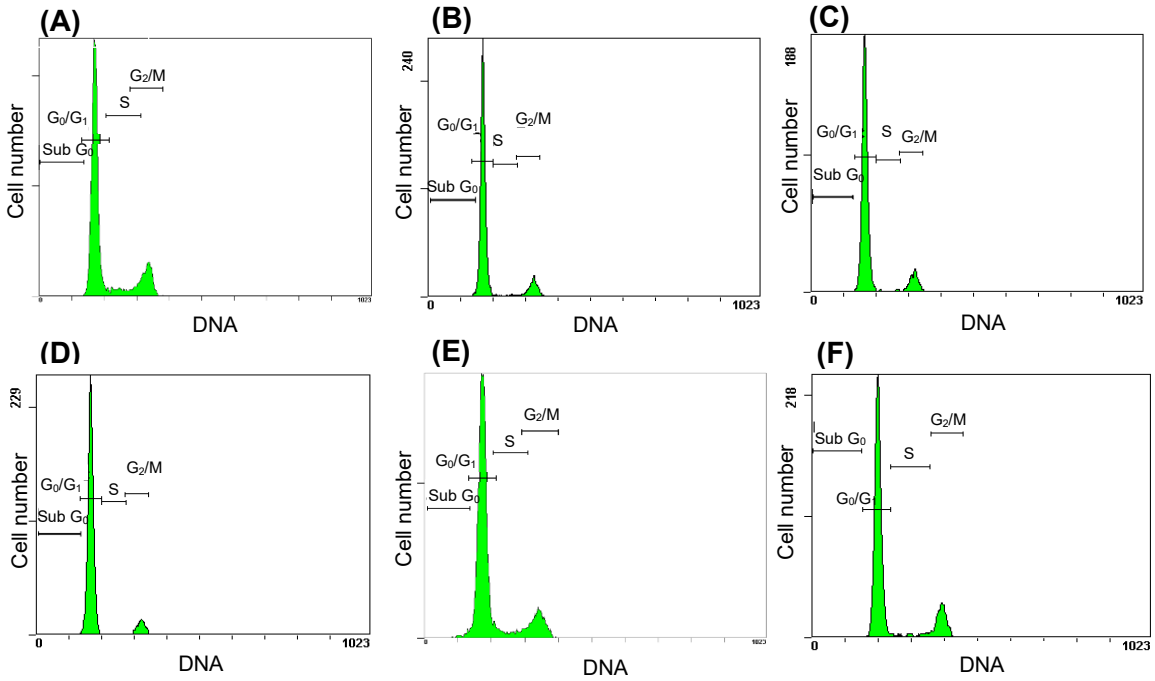


Figure 14: Cell cycle analysis through PI staining and flow cytometry of (A) LNCaP; (B) LNCaP 2.5 μM; (C) LNCaP 5 μM; (D) LNCaP 10 μM; (E) LNCaP 20 μM; (F) LNCaPER.

The continuous addition of ENZ in increasing doses was also reflected in the mRNA levels changes of *AR* and *PSA*. According to the results, *PSA* mRNA expression was significantly decreased during the whole process of resistance establishment, comparatively with LNCaP cells. Indeed, LNCaP 2.5 μM showed a decrease in *PSA* mRNA expression of 94% ($2^{-\Delta\Delta Ct}$=0.065, p=0.001); LNCaP 5 μM showed a decrease of 91% ($2^{-\Delta\Delta Ct}$=0.095, p=0.002) and LNCaP 10 μM presented a decrease of 78% ($2^{-\Delta\Delta Ct}$=0.22, p=0.009) (Figure 15). Both LNCaP 20 μM and LNCaPER presented a decrease of 96% in *PSA* mRNA expression ($2^{-\Delta\Delta Ct}$=0.040, p<0.001) (Figure 15).

On the other hand, the AR mRNA expression levels were only significantly decreased in LNCaPER when compared with LNCaP, with a reduction of 38% ($2^{-\Delta\Delta Ct}$=0.62, p=0.009) (Figure 15).

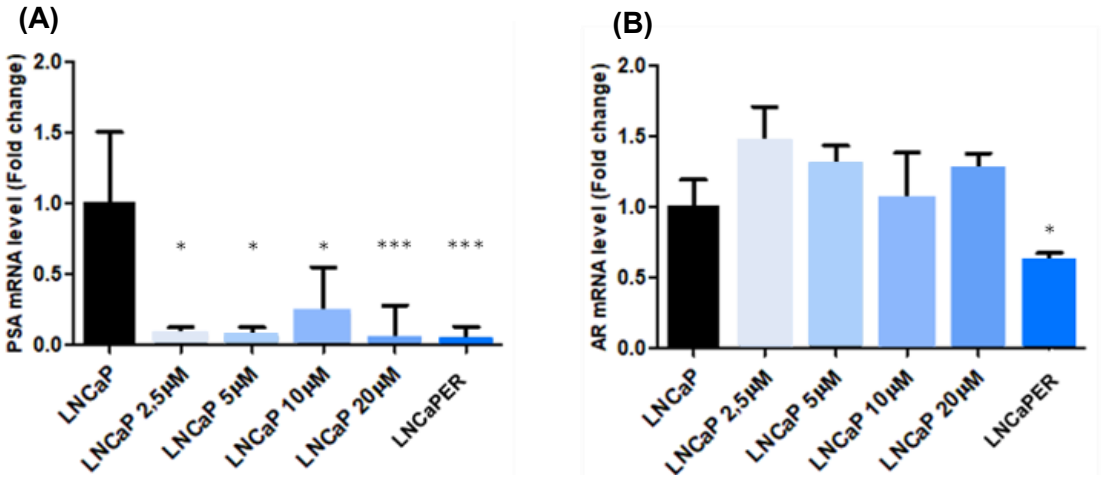


Figure 15: Graphical representation of the relative quantification of the expression levels of (A) PSA mRNA and (B) AR mRNA by qPCR in LNCaP and cells resistant to each of the established concentrations of ENZ (*: p<0.05; ***: p <0.001).

Taken together, the metabolic capacity, the cell cycle distribution results as well as the relative quantification of *AR* and *PSA* mRNAs levels support the cells ENZ acquisition phenotype.

4.2. MiRNA deregulation profile associated with ENZ resistance acquisition and in silico mRNAs targets identification

In order to define the miRNA profile associated with the acquisition of ENZ resistance, we compare the miRNA expression profile of LNCaPER *vs* LNCaP.

We found thirteen underexpressed and twelve overexpressed miRNAs in LNCaPER compared to LNCaP cells, which are represented in figure 16 and table 1 (Supplementary tables 1 and 2).

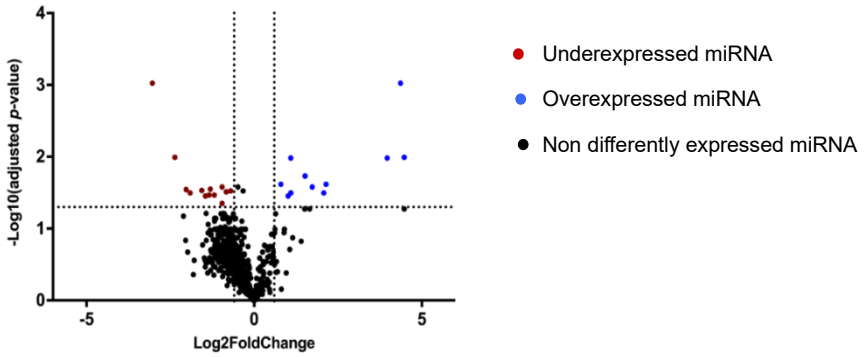


Figure 16: Volcano Plot of miRNAs quantified in LNCaP vs LNCaPER. Significantly overexpressed miRNAs are represented as blue dots (Log_2 (Fold change)>0,6) and significant underexpressed genes are represented as red dots (Log_2 (Fold change)<-0,6) (Log_{10} (adjusted p)>1,3).

Table 1: MiRNA significantly underexpressed and overexpressed in LNCaPER comparatively to LNCaP.

Underexpressed miRNAs	Overexpressed miRNAs
hsa-miR-7-5p	hsa-miR-22-3p
hsa-miR-18a-5p	hsa-miR-30d-5p
hsa-miR-1973	hsa-miR-1269b
hsa-135a-5p	hsa-miR-200a-3p
hsa-miR-19a-3p	hsa-miR-30e-5p
hsa-miR-15b-5p	hsa-miR-21-5p
hsa-miR-20a-5p+hsa-miR-20b-5p	hsa-miR-30a-5p
hsa-miR-93-5p	hsa-miR-221-5p
hsa-miR-421	hsa-miR-15a-5p
hsa-miR-940	hsa-miR-221-3p
hsa-miR-25-3p	hsa-miR-196b-5p
hsa-miR-570-3p	hsa-miR-222-3p
hsa-miR-328-3p	

Using the miRTarBase v9.0 database and miRNA-mRNA interaction data published from 2021 to 2022, we identified a total of different 335 validated target genes for the thirteen underexpressed miRNAs (Supplementary table 1) and 464 validated target genes for the twelve overexpressed miRNAs (Supplementary table 2).

Additionally, we found that 84 mRNAs were targeted by at least two overexpressed miRNAs and 56 mRNAs were targeted by at least two underexpressed miRNAs. *BCL2L11, PTEN and TGFBR2* mRNAs were the most common targets of underexpressed miRNAs, each being targeted by 5 miRNAs. Other potentially upregulated genes involved in PC included *BCL2* and *SMAD7*, both regulated by 4 underexpressed miRNAs, and VEGFA regulated by 3 underexpressed miRNAs. *TP53* was the most common target of the overexprexpressed miRNAs, being regulated by 7 miRNAs. Also, *RECK* and *CDKN1B* are potentially downregulated proteins being targeted by 5 and 4 overexpressed miRNAs, respectively.

These previously determined target genes were used to construct the protein interaction network for the potentially downregulated and upregulated proteins (Figure 17 and 19). For the potentially downregulated proteins, 8 did not establish any PPI (HOXB5, CERS2, BCL9, SSX2IP, CORO1A, SLC25A, SSSCA1 and DKK2). Therefore, the main cluster was formed by 76 nodes, 709 edges and the p for PPI was $1x10^{-16}$ (Figure 17).

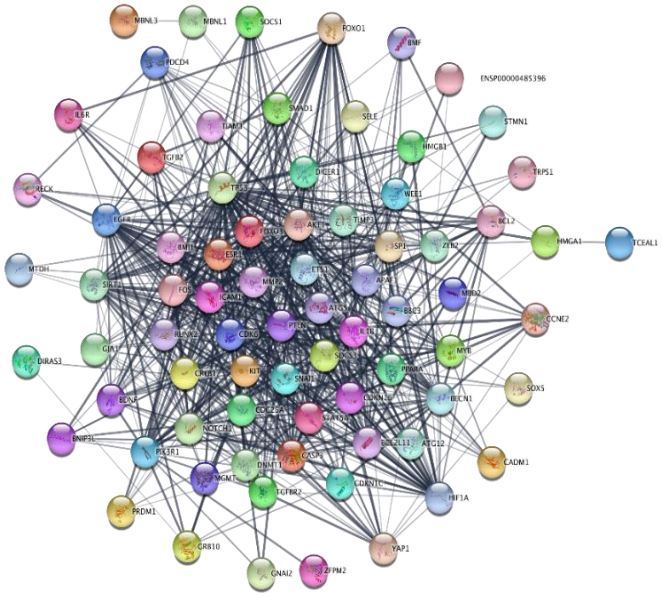


Figure 17: Protein-protein interaction network (PPI) for the potentially downregulated proteins.

For the potentially upregulated proteins, 7 did not establish any PPI (ATXN1, ATG16L1, TCEAL1, RGS5, CRIM1, MTSS1, TUSC2). The main cluster was formed by 49 nodes, 325 edges and the p for PPI was $1x10^{-16}$ (Figure 18).

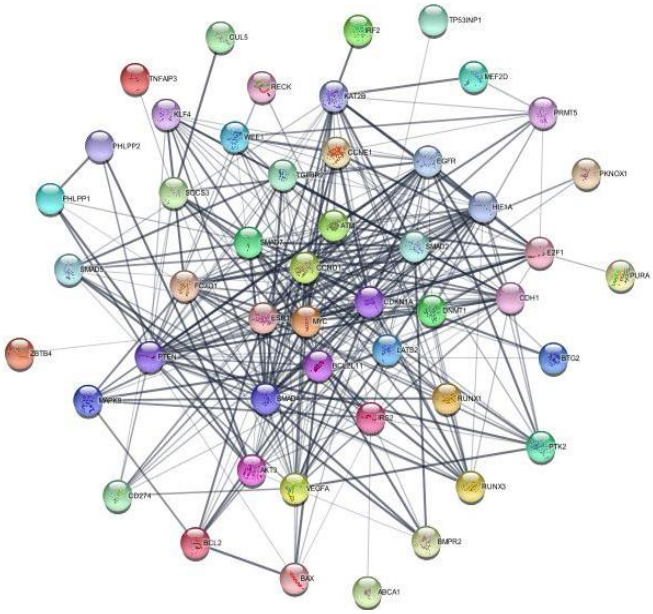


Figure 18: Protein-protein interaction network (PPI) for the potentially upregulated proteins.

4.3. Functional and Pathway Enrichment Analysis

Subsequently, we used the STRING enrichment analysis tool to perform functional enrichment analyses of each protein cluster using data from the Gene Ontology (GO), Kyoto Encyclopaedia of Genes and Genomes (KEGG), and Reactome pathways databases in order to investigate the biological effects of these proteins.

The functional enrichment analysis resulted in a total of 785 functional enriched terms for the potentially upregulated proteins (Supplementary tables 3, 4, 5, 6 and 7) and 1081 terms for the potentially downregulated proteins (Supplementary tables 8, 9, 10, 11 and 12), among the databases considered.

The top 20 enriched terms for each category are represented in Figures 19 and 20, for the potentially downregulated and upregulated proteins, respectively.

Regarding the functional enrichment analysis for the potentially downregulated target genes (Figure 19), in Reactome Pathways database we found that they are mostly involved in activation of the PI3K/AKT and immune response. Regarding KEGG Pathways, we find the terms p53, TNF, FOXO and PI3K/AKT signaling (Figure 19).

Among the functionally enriched terms in the Reactome Pathways in Figure 20 we can observe that the potentially upregulated proteins are involved in cell cycle regulation, transcriptional regulation by FOXO and RUNX3, and modulation of immune response. These genes are also involved in PI3K/AKT pathway and signaling by TGF-β. Regarding KEGG pathways, we can find once again FOXO and PI3K/AKT signaling pathways.

For GO: Molecular Function terms, most of these potentially down and upregulated proteins are DNA-binding proteins or involved in DNA transcription, as shown in Figure 19 and 20, respectively. In the same figures, but regarding GO: Biological Process, we observed that both the potentially down and upregulated proteins were mostly involved in proliferation, regulation of cell cycle, migration, cell death and metabolic process.

Importantly, for both potentially down and upregulated proteins the terms “Prostate cancer” and “MicroRNAs in cancer” and “Pathways in cancer” appeared.

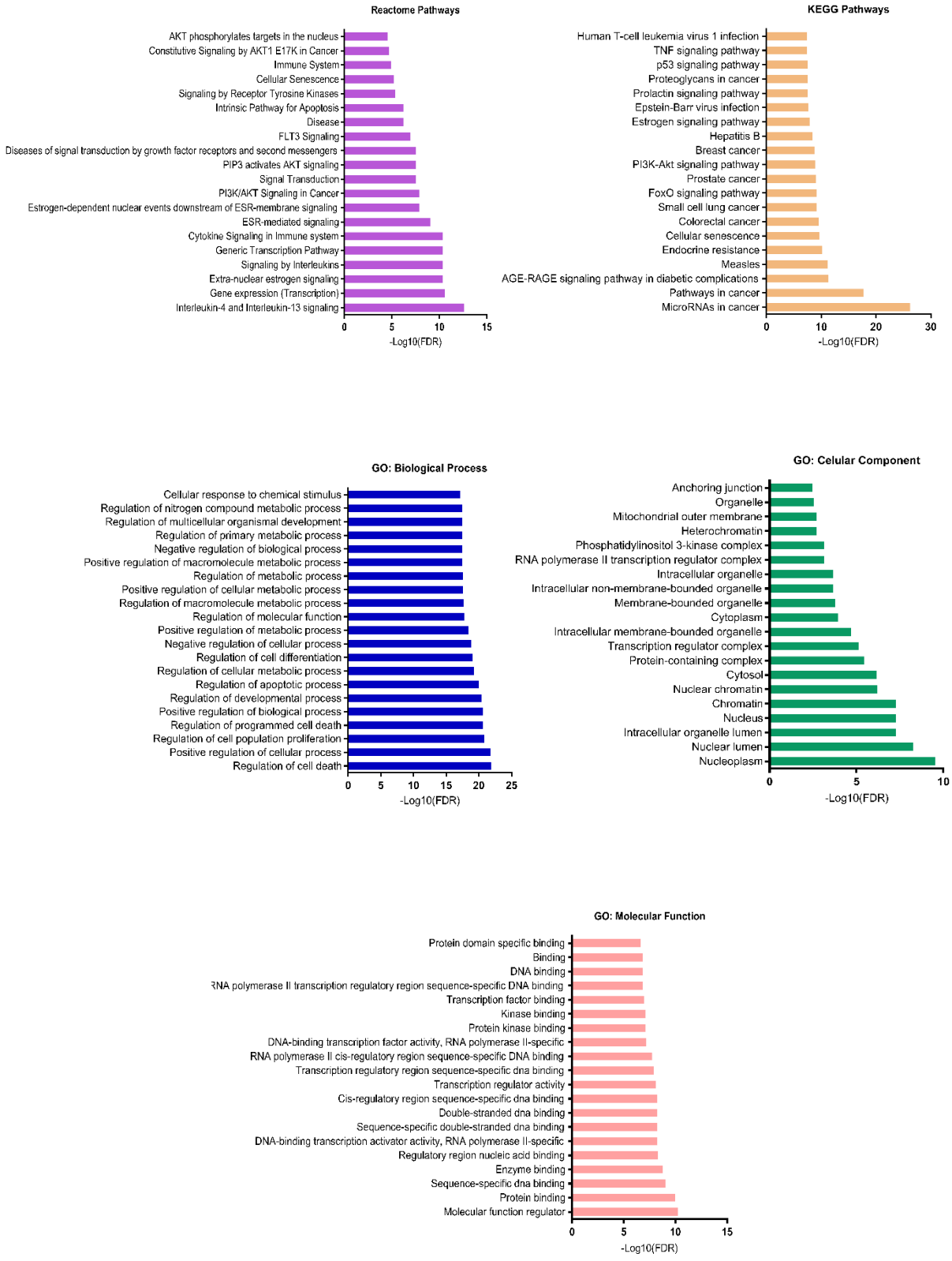


Figure 19: Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome and Gene Ontology (GO) analysis of the 76 selected potentially downregulated target genes.

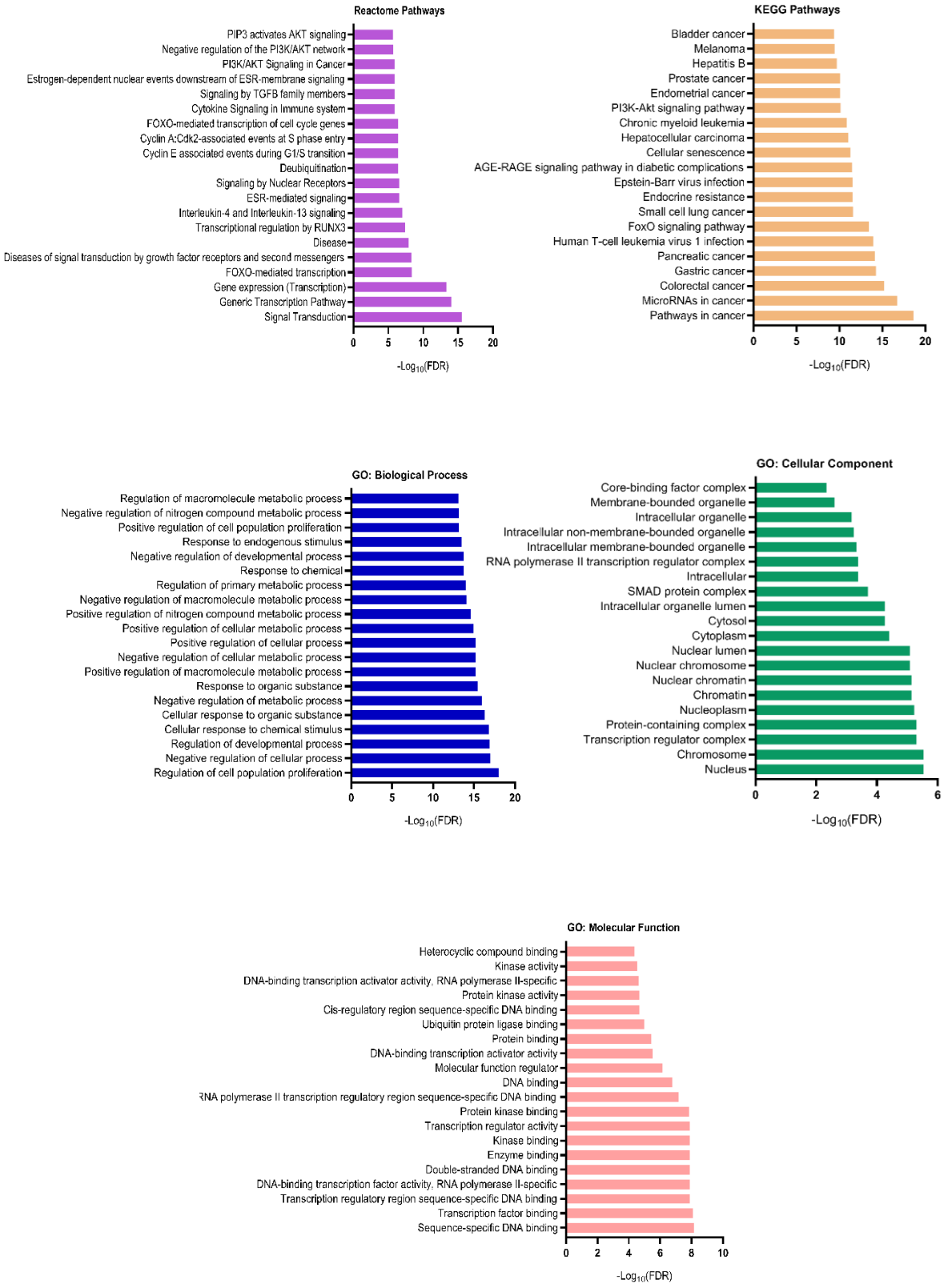


Figure 20: Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome and Gene Ontology (GO) analysis of the 50 selected potentially upregulated target genes.

To assess the potential of previously identified miRNAs as predictive biomarkers of ENZ resistance, as well as their expression dynamics through several stages of the resistance process, we selected the top 5 overexpressed miRNAs that regulated the greatest number of the PPI selected genes to be quantified in EVs secreted by LNCaP and ENZ resistant spheroids. As a result, we selected hsa-miR-221-3p (48 target mRNAs), hsa-miR-222-3p (37 target mRNAs), hsa-miR-21-5p (27 target mRNAs), hsa-miR-200a-3p (18 target mRNAs) and hsa-miR-22-3p (15 target mRNAs) to be analyzed in the *in vitro* model established in the present study.

4.4. Establishment of the *in vitro* 3D model- spheroid

In order to establish a 3D study model of the development process of cell resistance to ENZ, we started by performing a preliminary assay to optimize spheroid assembly conditions and determine the most suitable time points for analysis. LNCaP cells were seeded at three different cell densities of 5 000 cells/sph (C1), 10 000 cells/sph (C2) and 20 000 cells/sph (C3). Time points of 24h, 48h, 72h and 7 days were considered for observations.

The acquired bright-field images of spheroids after different time intervals are displayed in Figure 21-A. The measured spheroid diameters for each density and time point are depicted in Figure 21-B.

During the 7 days of culture, we noticed accentuated changes in the morphology, size, and organization of the spheroids across all three concentrations (C1, C2, and C3). While analyzing Figure 21-A, we observed that different cell densities yielded spheroids with different sizes, and a higher initial seeding densities resulting in a large and more cohesive spheroids, as expected. At the beginning of spheroid culture (24h), C3 spheroids presented a larger diameter than C2 spheroids, which in turn was larger than C1 spheroids. Over time, a progressive increase in spheroid diameter was observed in C1 concentration spheroids (24h vs. 48h: $p=0.0134$; 24h vs. 7 days, 48h vs. 7 days and 72h vs. 7 days: $p<0.0001$). By the end of the test period (7 days), there was a 14% diameter increase in diameter compared to the initial 24h (Figure 21-B). In C2 spheroids, there was no significant variation in diameter over time, whereas in C3 spheroids, there was a gradual but significant decrease in diameter over time (24h vs. 48h: $p=0.0026$; 24h vs. 72h: $p=0.0001$; 24h vs. 7 days, 48h vs. 7 days and 72h vs. 7 days: $p<0.0001$) (Figure 21-B). After 7 days, a 9% reduction in the diameter of the C3 spheroids was registered.

In general, the spheroids present an approximately spherical shape, with C3 concentration spheroids displaying the highest cohesiveness, a more rounded shape, and

well-defined, smoother edges. Moreover, over time, spheroids acquired a more cohesive and compact structure. This was evidenced by the darkening of the spheroid centers over time and was observed across all three concentration conditions studied (Figure 21-A).

The results of the spheroid metabolic activity, estimated by the luminescence intensity, are shown in relation to 24h (fold change) in Figure 21-C. The analysis reveals distinct trends on cell viability among the different spheroid concentrations and time points. For C1 spheroids, cell viability increases over the 7 days of spheroid development, being significantly higher in 72h ($p<0.0334$) and 7 days ($p<0.0075$), comparatively to 24h. In contrast, C2 spheroids do not exhibit significant change in cell viability over time, suggesting that the metabolic activity remains relatively constant during the course of spheroid development. In C3 spheroids initially maintain relatively viability, however, a significant decrease is observed at 7 days compared to 48h ($p=0.0274$). These results highlight the differential impact of time and spheroid density on the metabolic activity of LNCaP cells within the 3D culture system.

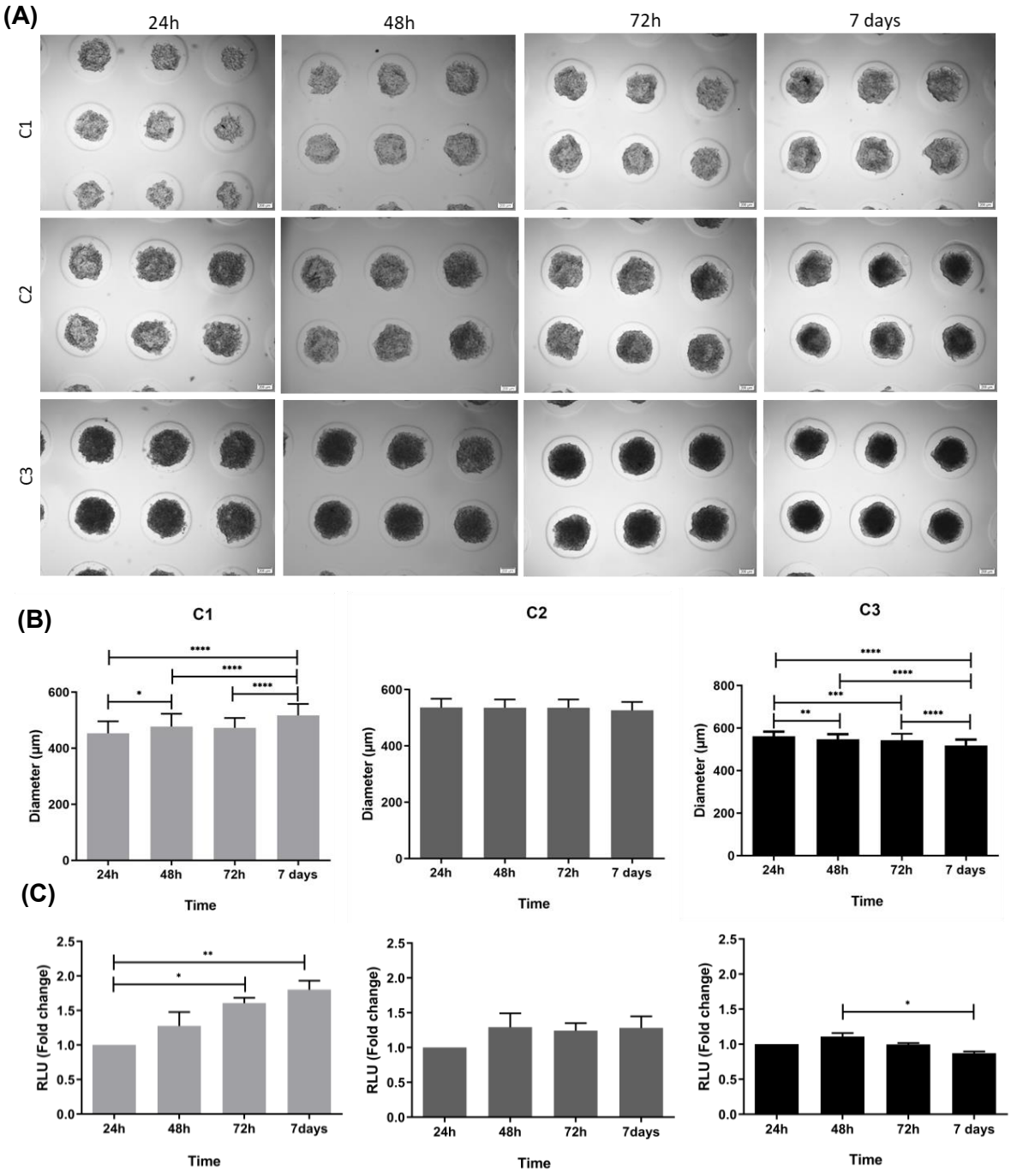


Figure 21: Optimization of 3D LNCaP spheroids with different cellular densities: 5 000cells/sph (C1), 10 000 cell/sph (C2), 20 000 cells/sph (C3) and their evolution through 24h, 48h, 72h and 7 days after cell seeding. (A) Bright-field microscopy images (40x). Scale bar: 200µm. (B) Spheroid diameter measurements using Fiji imaging processing software (*: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001). (C) Metabolic activity assessment of C1, C2 and C1 spheroids, in relation to 24h (fold change).

Based on the outcomes of our spheroid metabolic activity analysis, we have made an informed selection for subsequent investigations. Specifically, the C3 cell concentration was chosen as the preferred condition for generating spheroids with the desired characteristics.

These characteristics include larger spheroid size, enhanced cohesiveness, and a more compact overall structure.

In the interest of studying the evolution of these spheroids over time, we have opted to focus on three distinct time points. The initial time point, at 24 hours, provides a baseline for spheroid formation and early characteristics. The intermediate time point, set at 72 hours, offers insights into the evolving nature of the spheroids as they progress through the 7-day assay. Finally, the final time point at 7 days serves as an endpoint, providing a comprehensive view of the spheroids' maturity and any significant changes in their structure and properties.

This selection of time points and spheroid concentration will allow for a thorough and holistic analysis of the spheroids, offering valuable insights into the behavior of cells within the 3D culture system.

4.5. ENZ-resistant LNCaP spheroid assembly and characterization

The images of the spheroids acquired by bright-field microscopy after 24 hours, 72 hours, and 7 days of culture are shown in Figure 22-A. Figure 22-B graphically represents the spheroid diameters obtained for each of the concentration and time point conditions.

As was observed in the spheroids formed by the LNCaP cells, the spheroids formed by the ENZ-resistant cells also become more compact and denser, as evidenced by the darkening and decrease in spheroid size in the microscopy images. This variation is visible for all the ENZ concentrations studied (Figure 22-A).

The spheroids assembled with resistant cells exhibit a progressive reduction in diameter over time (Figure 22-B). In LNCaP 2.5 μM we observed a significant decrease in all time points studied (24h vs. 72h; 24h vs. 7 days; 72h vs. 7 days: $p<0.0001$), as well as in LNCaP 5 μM (24h vs. 72h; 24h vs. 7days: $p<0.0001$; 72h vs. 7 days: $p=0.0094$), in LNCaP 10 μM (24h vs. 72h: $p=0.0015$; 24h vs. 7 days; 72h vs. 7 days: $p<0.0001$) and in LNCaP 20 μM (24h vs. 72h; 24h vs. 7 days: $p<0.0001$; 72h vs. 7 days: $p=0.0018$). In LNCaPER the decrease in spheroid diameter was significant between 24h and 72h and between 24h and 7 days ($p<0.0001$) but not between 72h and 7 days ($p=0.054$).

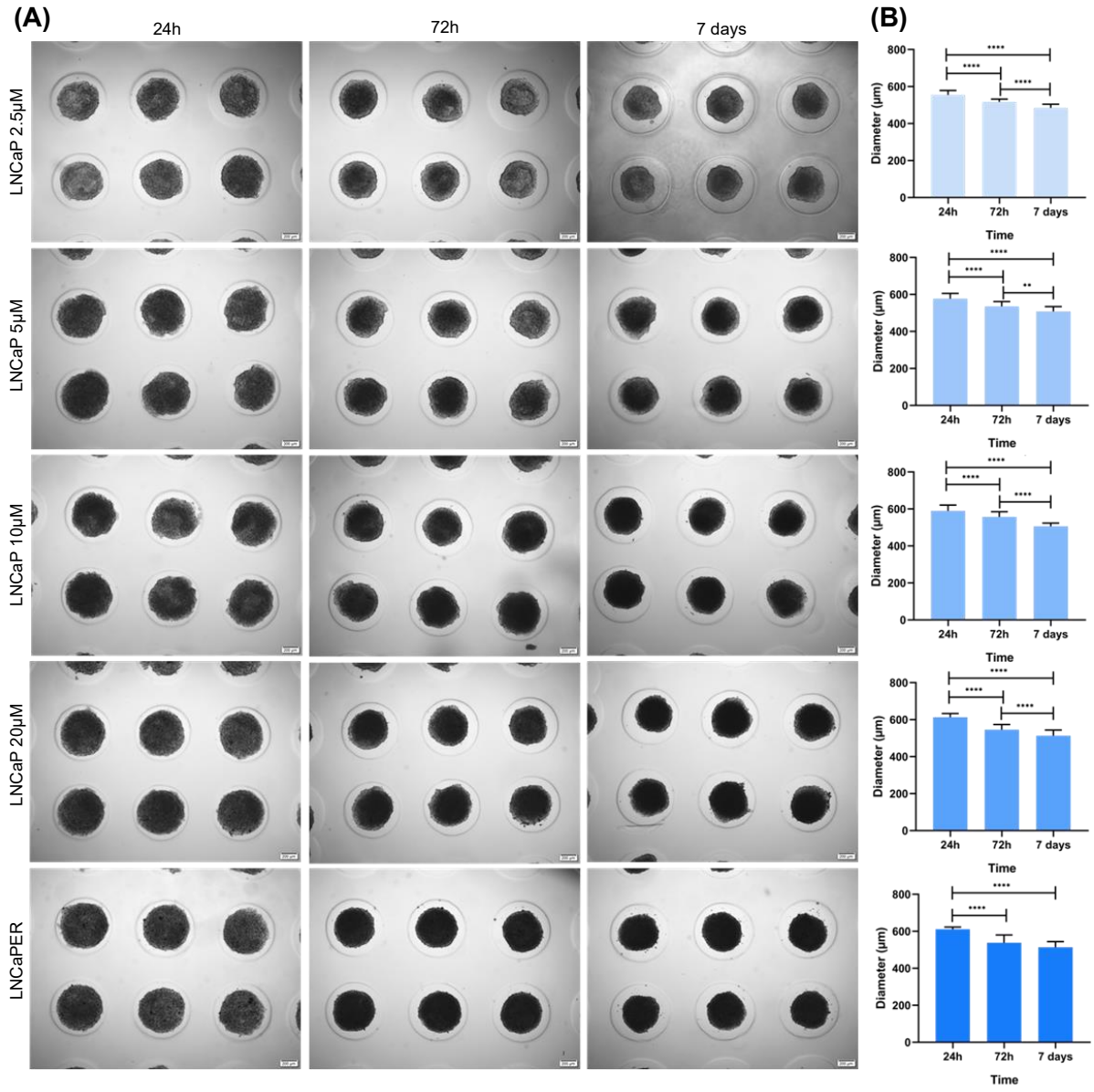


Figure 22: (A) Brightfield microscopy images (40x). Scale bar: 200 µm (B) Spheroid diameter measurements (**: p<0.01; ***: p<0.001; ****: p<0.0001).

Figure 23 shows the metabolic activity of LNCaP and LNCaP cells resistant to the various concentrations used in the spheroid model at 72h and 7 days in relation to the metabolic activity measured at 24h (fold change), estimated by the luminescence values obtained.

The metabolic activity of LNCaP tends to increase in 72h and 7 days relatively to 24h, but this variation is not statistically significant (24h vs. 72h: p=0.0742; 24h vs. 7 days: p=0.217; 72h vs. 7 days: p>0.999). However, metabolic activity of LNCaP 2.5 µM significantly increased at 72 hours (24h vs. 72h: p=0.0185; 24h vs. 7 days: p=0.5116; 72h vs. 7 days: p=0.5116). In LNCaP 5 µM there were no significant changes in metabolic

activity over time (24h vs. 72h: p=0.1060; 24h vs. 7 days: p> 0.999; 72h vs. 7 days: p=0.1520. In LNCaP 10 µM and 20 µM metabolic activities decreased over the course of the test. In fact, at 7 days the metabolic activity of LNCaP 10 µM is significantly lower than the metabolic activity recorded in these spheroids at 24h (24h vs. 72h: p=0.0834; 24h vs. 7 days: p=0.003; 72h vs. 7 days: p=0.0554). In LNCaP 20 µM there is also a significant decrease at 72h and 7 days relatively to 24h (24h vs. 72h: p=0.0346; 24h vs. 7 days: p=0.003; 72h vs. 7 days: p> 0.129). However, no significant variations on metabolic activity were registered in LNCaPER spheroids.

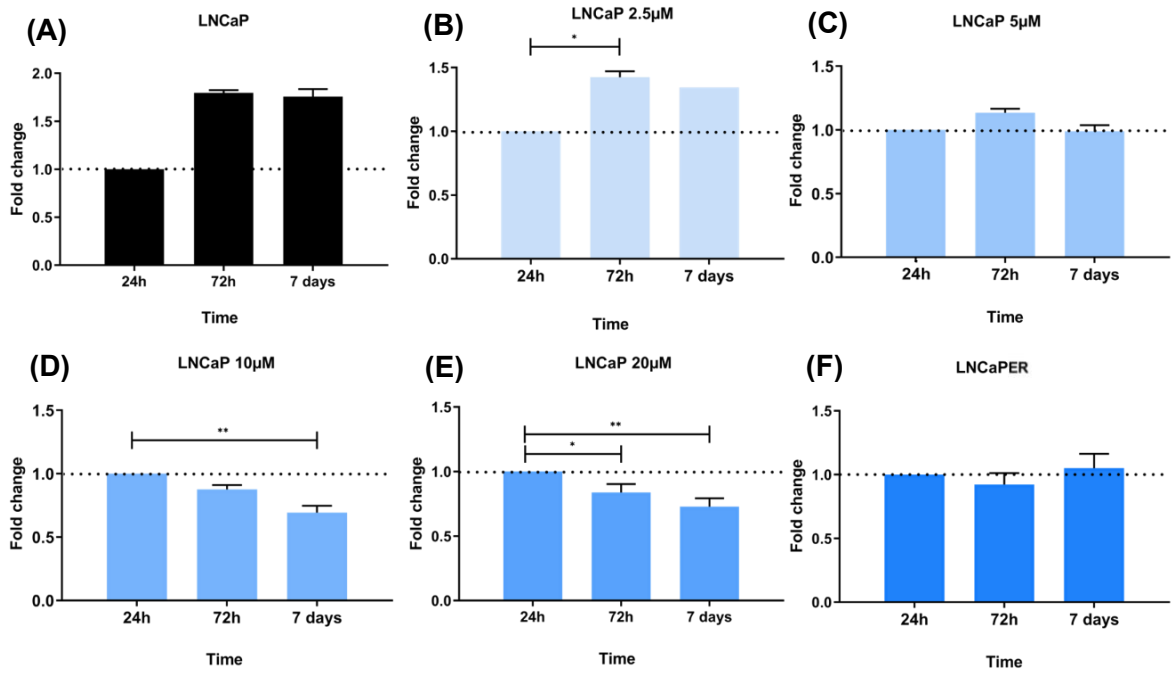


Figure 23: Metabolic activity for (A) LNCaP; (B) LNCaP 2.5 µM; (C) LNCaP 5 µM; (D) LNCaP 10 µM; (E) LNCaP 20 µM and (F) LNCaPER over time in relation to 24h (fold change) (*: p<0.05; **: p<0.01).

Figures 24, 25, 26 and 27 show the CLSM images of immunofluorescence-stained sections from LNCaP and ENZ-resistant spheroids at the time points of 24h, 72h and 7 days after spheroid assemble.

For immunofluorescence staining, specific target molecules were thoughtfully selected to give valuable insights into the structural organization and molecular characteristics of the spheroids. The presence or absence and distribution of these markers shed light on various aspects of spheroids biology. These selected markers include fibronectin and E-cadherin, which provide insides into the organization of the extracellular matrix and the cell adhesion capacity, respectively. Vimentin, a cytoskeleton protein involved in cell mobility and associated with the EMT, was also studied. To assess the

proliferative or necrotic status of cells in the spheroid, Ki-67 and caspase 3 staining were used, respectively. B-catenin staining was also performed to explore the involvement of the Wnt signaling pathway, a relevant pathway in PC.

Figure 24 presents confocal images of DAPI, vimentin and E-cadherin immunostaining. These images reveal that LNCaP spheroids exhibit few or non-vimentin expression. However, it is possible to notice a very slight increase of vimentin expression in the spheroids assembled with resistant cells, but there are no evident changes across ENZ concentrations or time points. Both LNCaP and resistant cells within the spheroid maintain high and consistent E-cadherin production in all the concentrations and time points studied.

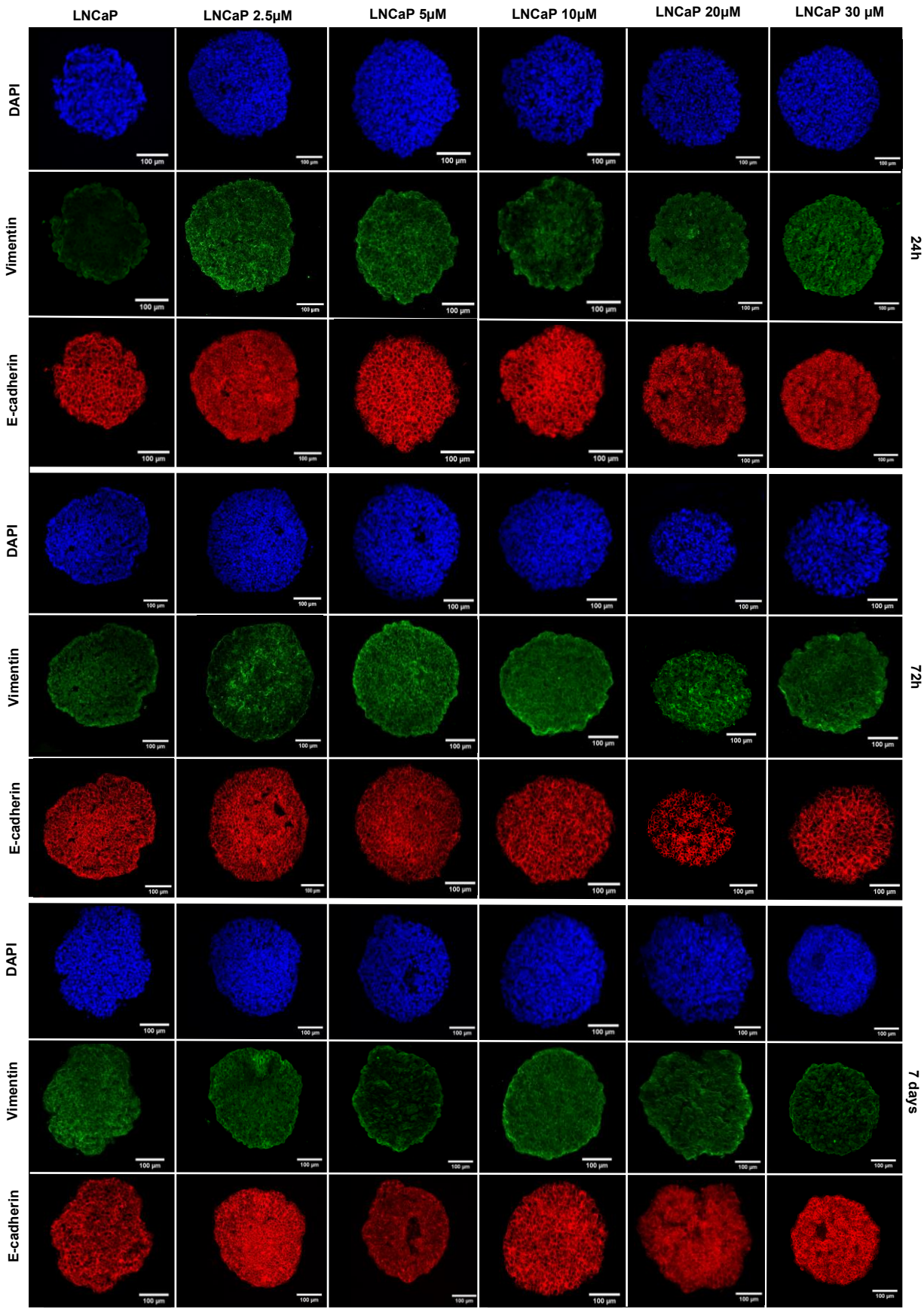


Figure 24: Immunostaining of DNA (DAPI), vimentin and E-cadherin in LNCaP and ENZ-resistant spheroids 24h, 72h and 7 days after spheroid assembly.

Figure 25 presents CLSM images of DAPI and fibronectin immunostaining. These images reveal expression of fibronectin in both LNCaP and ENZ-resistant cells spheroids and in all time points studied. No differences in fibronectin production were observed between ENZ concentrations and time points.

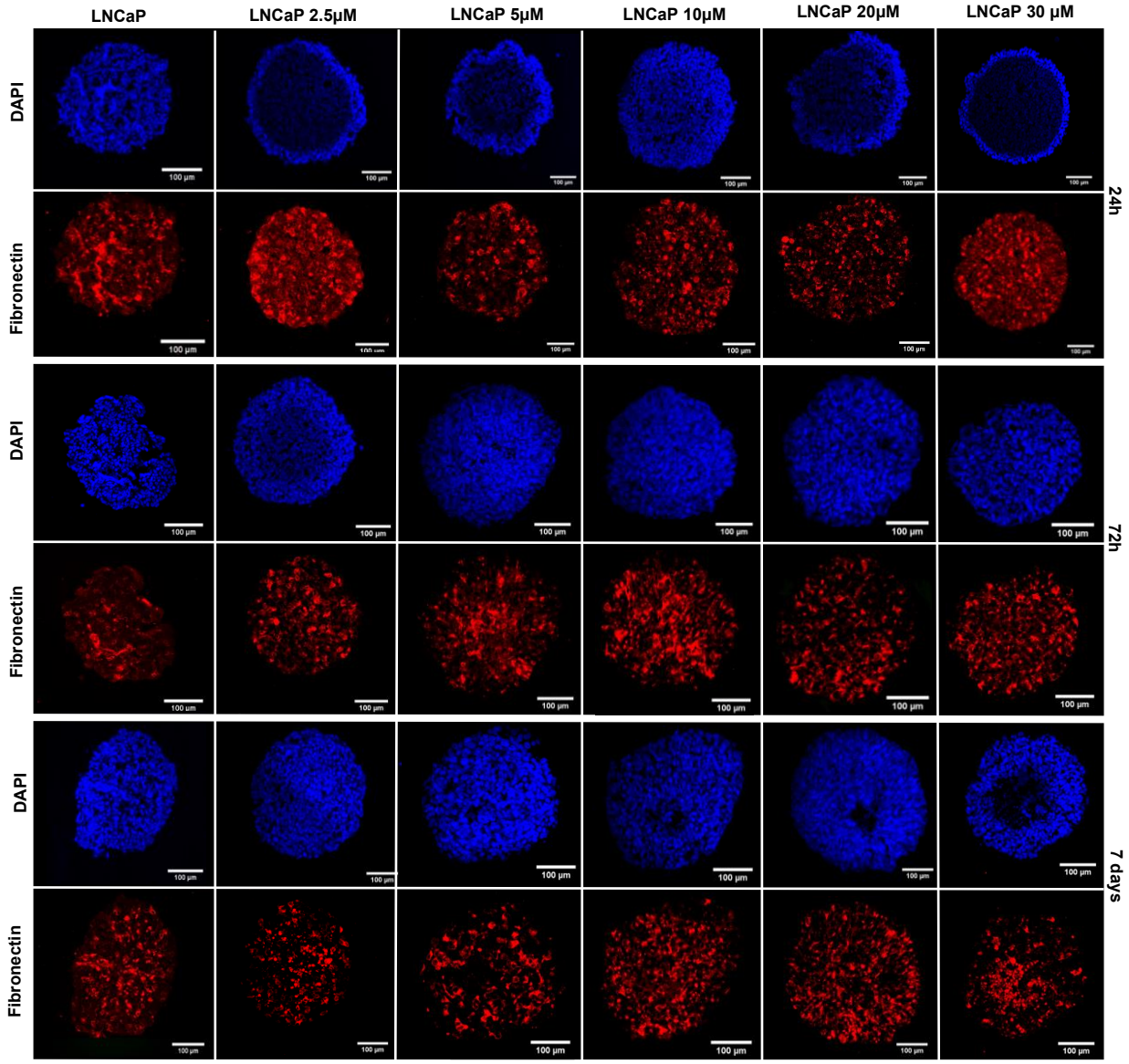


Figure 25: Immunostaining of DNA (DAPI) and fibronectin in LNCaP and ENZ-resistant spheroids 24h, 72h and 7 days after spheroid assembly.

Figure 26 presents CLSM images of DAPI and β-catenin immunostaining. LNCaP and resistant cells spheroids present a high membranal expression of β-catenin in all time points. Similarly, no differences in β-catenin production/cellular localization were observed between time points or ENZ concentrations.

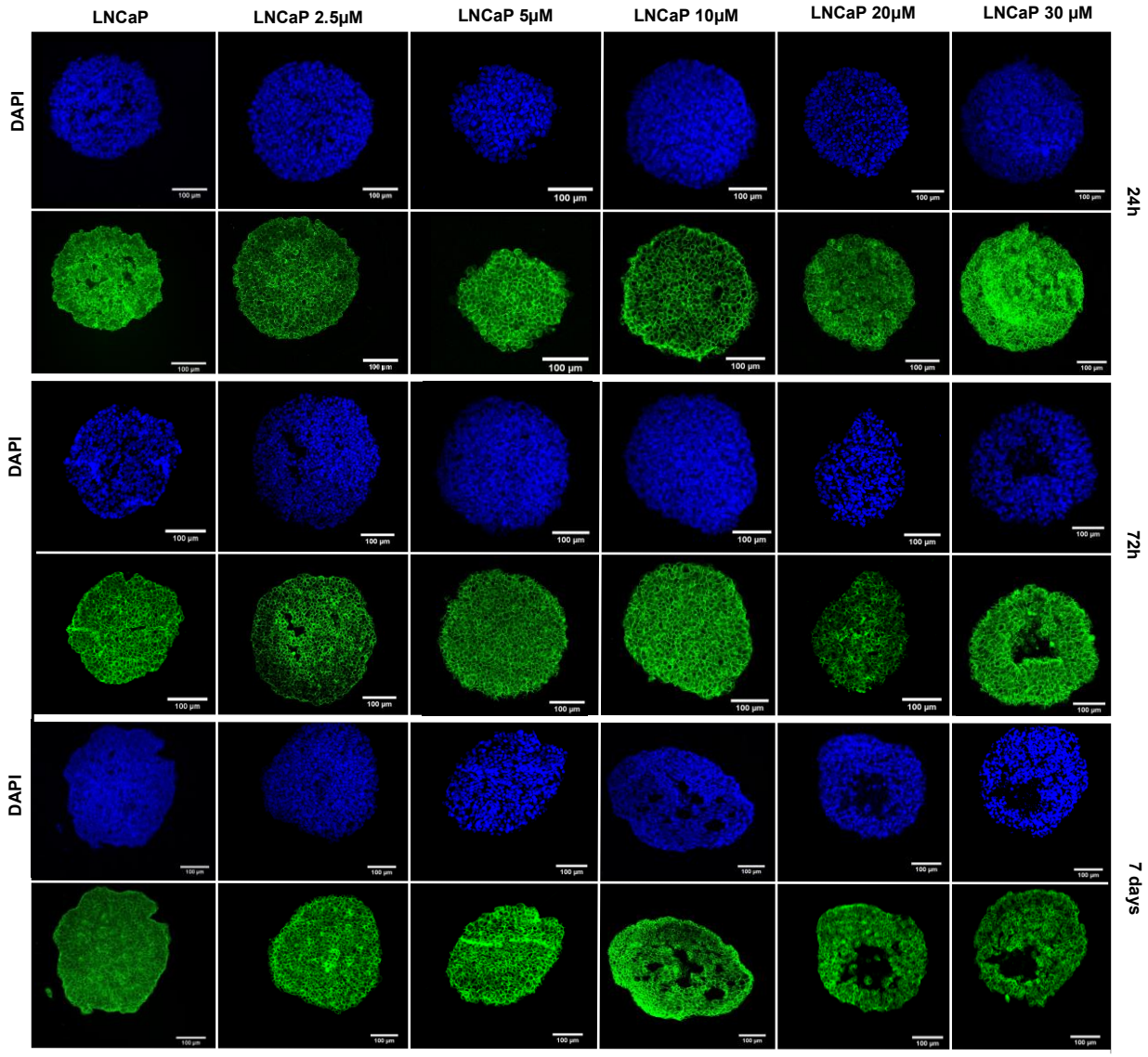


Figure 26: Immunostaining of DNA (DAPI), B-catenin in LNCaP and ENZ-resistant spheroids 24h, 72h and 7 days after spheroid assembly.

Ki-67 and caspase 3 staining results are represented in Figure 27. It was observed that the Ki-67 labelling decreased over time, with a progressive shift towards being more prominent in the spheroid's outer regions. In addition, at the 72h and 7 days after the formation of the spheroids, there is a clear trend of diminishing Ki-67 labelling as the concentration of ENZ increases. In LNCaP 30 μM, a particularly marked decrease in Ki-67 labeling was observed over time, compared to LNCaP and cells treated with the intermediate ENZ concentrations in the spheroid. After 72h and 7 days of culture LNCaP 30 μM spheroids presented a reduced number of proliferative cells.

In contrast to the observations for Ki-67 labelling, there is perceptible increase in caspase 3 labelling over time, mostly in LNCaP 30 μM spheroids. Additionally, Figure 27 shows an alteration in the distribution of caspase 3 staining, from a dispersed pattern throughout the spheroid to a more concentrated presence in the spheroid's central region. These findings shed light on the evolving proliferation and apoptosis dynamics within the spheroids, especially in the context of ENZ resistance.

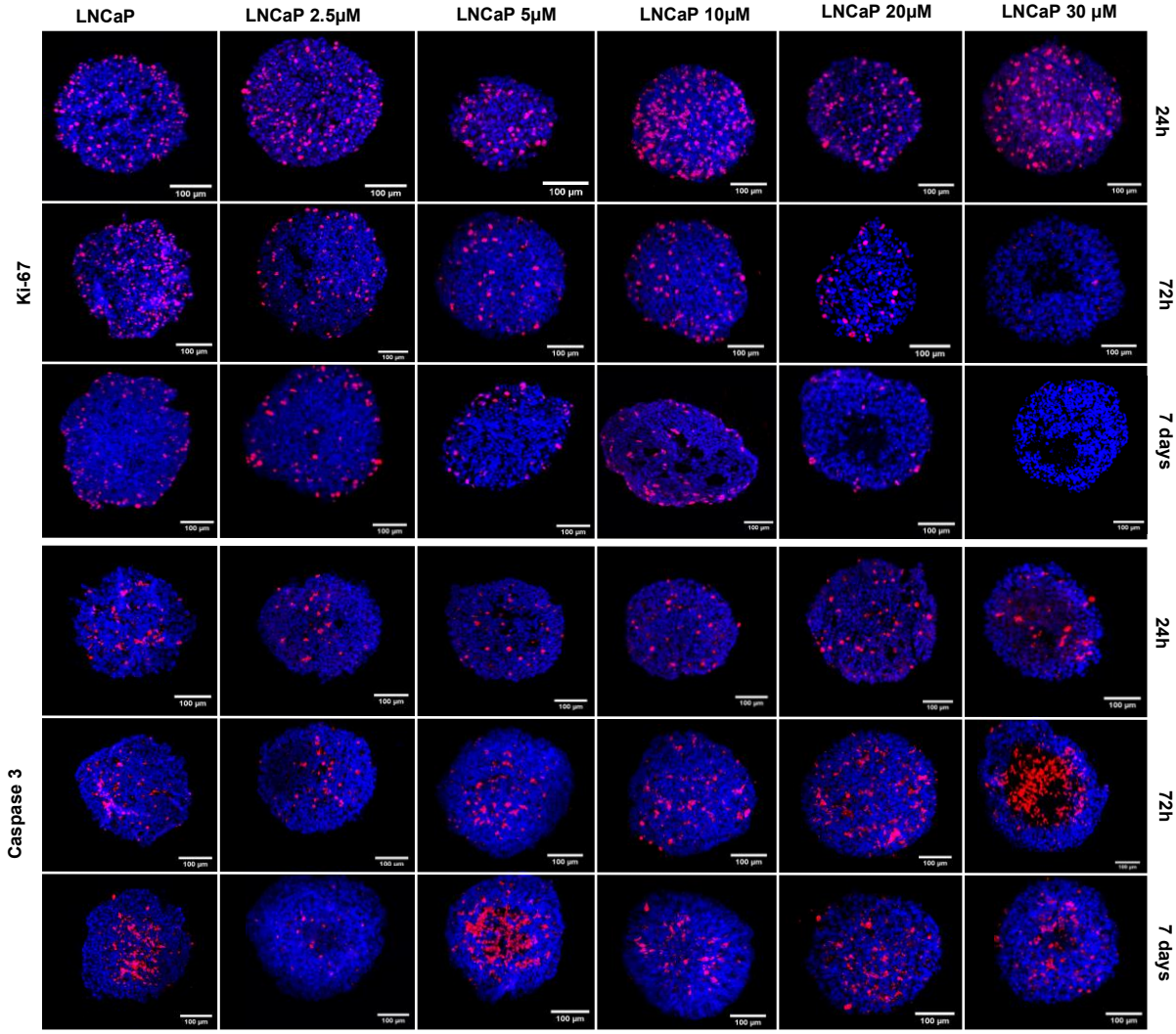


Figure 27: Immunostaining of Ki-67 and caspase 3 merged with DAPI in LNCaP and ENZ-resistant spheroids 24h, 72h and 7 days after spheroid assembly.

4.6. EVs characterization

The NTA analysis indicated that the isolated EVs from both LNCaP and LNCaPER presented a size range between 50 and 200 nm, and a mean of 92.4 nm and 113.2 nm, respectively, which is consistent with the size of exosomes and small microvesicles (Figure 28-A). Moreover, the TEM images revealed cup-shaped membrane- surrounded structures, confirming that these structures in our samples are EVs. The TEM images also showed some variability of sizes (Figure 28-B).

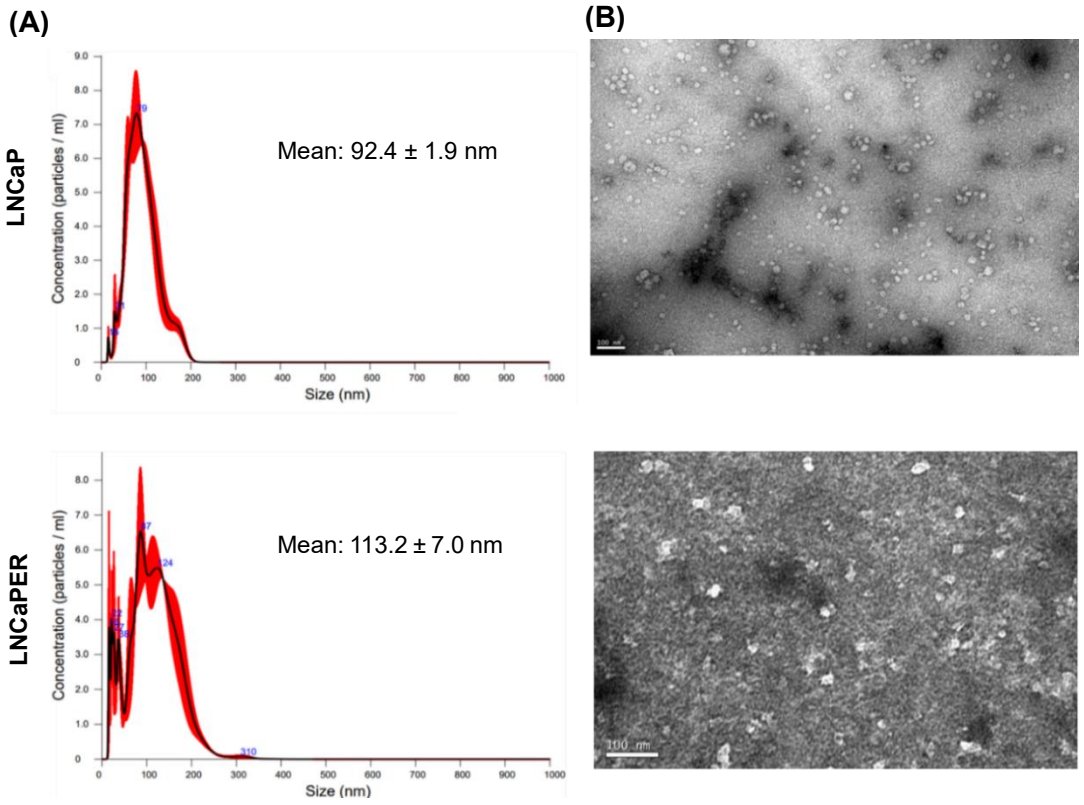


Figure 28: (A) Nanoparticle tracking analysis (NTA) and (B) Transmission electron microscopy (TEM) of EVs derived from LNCaP and LNCaPER spheroids culture medium after 7 days of culture.

4.7. Relative quantification of overexpressed EV-derived miRNAs levels throughout the development of ENZ resistance

Figure 29 represents the variation of EVs derived miRNAs levels of the selected profile at 72h and 7 days after spheroid seeding. Due to the accentuated decrease of proliferative cells observed after Ki-67 staining and increase of caspase 3 labeling in LNCaPER spheroids, which suggest these cells were not resistant to ENZ, these spheroids were not considered for miRNA and EVs-derived miRNAs levels quantifications.

Regarding LNCaP cells, it is observed that only the EV-derived hsa-miR-22-3p is significantly higher at the 7-day timepoint in comparison to the 72h timepoint (6.87 fold-

increase, p =0.026) (Figure 29-A). In LNCaP 2.5 µM condition, we found higher expression levels of EVs-derived-hsa-miR-200a-3p (1.71 fold-increase, p=0.005), -hsa-miR-221-3p (4.79 fold-increase, p<0.001) and -hsa-miR-222-3p (7.01 fold-increase, p<0.001) in day 7 condition compared to the 72 hours condition (Figure 29-B). On the other hand, we observed discreet differences on EVs-derived miRNAs profile between the 72 hours *vs* 7 days conditions for the remain ENZ concentrations tested. However, there was a general tendency for higher EV-derived miRNAs levels in 7-day condition, supporting its selection for further studies.

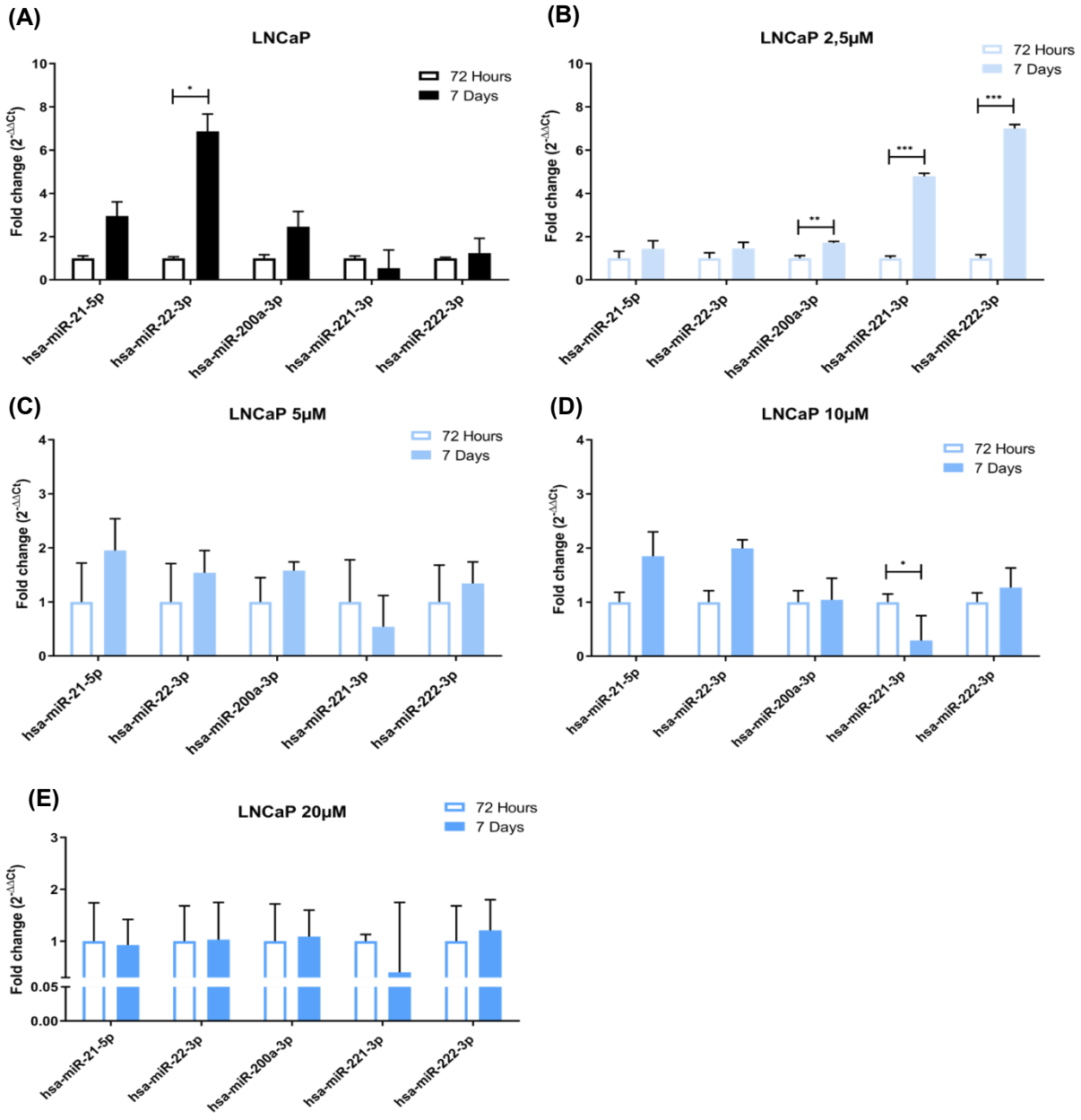


Figure 29:Expression levels of hsa-miR-21-5p, hsa-miR-22-3p, has-miR-200a-3p, has-miR-221 and has-miR-222-3p in EVs derived from (A) LNCaP; (B) LNCaP 2.5 µM; (C) LNCaP 5 µM; (D) LNCaP 10 µM and (E) LNCaP 20 µM, at time points of 72h and 7 days (*: p<0.05; **: p<0.01; ***: p<0.001).

Figure 30 shows the expression levels of the ENZ-resistant miRNA profile defined, as well as the EVs-derived miRNAs at the end of 7 days in the different ENZ concentration tested.

Regarding the intracellular levels, when the cells were exposed to 2.5 μM of ENZ, we observed that there is a tendency for an increase in the expression of the miRNAs profile, compared to LNCaP however, this difference is statistically significant for hsa-miR-221-3p (9.38 fold-increase, p=0.031) and hsa-miR-222-3p (14.72 fold-increase, p=0.022). Moreover, when exposed to higher concentrations of ENZ, the intracellular levels of hsa-miR-21-5p and hsa-miR-200a-3p decreased and remained at a low expression level. Additionally, the intracellular levels of hsa-miR-22-3p presented a statistically significant decrease at concentrations of 5 μM ($2^{-\Delta\Delta Ct}$= 0.67, p= 0.001) and 20 μM ($2^{-\Delta\Delta Ct}$= 0.52, p= 0.012). On the other hand, we found an increase of hsa-miR-221-3p and hsa-miR-222-3p associated with the higher ENZ concentrations administrated compared to LNCaP (LNCaP 5 μM: 10.05 fold-increase, p<0.001, LNCaP 10 μM: 8,63 fold-increase, p=0.001, LNCaP 20 μM:18,63 fold-increase, p<0.001; LNCaP 5 μM: 7,67 fold-increase, LNCaP 10 μM: 15.03 fold-increase, LNCaP 20 μM: 22,78 fold-increase, p<0.001, respectively).

Regarding EV-derived-miRNAs, we found that hsa-miR-22-3p levels increase in LNCaP 20 μM condition compared to LNCaP (11.08 fold-increase, p=0.047). However, despite not being statistically significant, we observed that EV-derived-hsa-miR-21-5p levels, presented a tendency to increase in the concentration of 20 μM (p=0.065).

Regarding EVs-derived-hsa-miR-221-3p and -hsa-miR-222-3p levels, we found a statistical significant increase when cells are treated with 2.5 μM of ENZ (hsa-miR-221-3p: 6.32 fold-increase, p=0.006; hsa-miR-222-3p: 4.11 fold-increase, p=0.003) and 10 μM of ENZ (hsa-miR-221-3p: 4.75 fold-increase, p=0.009; hsa-miR-222-3p: 3.54 fold-increase, p=0.004) compare to LNCaP. However, we observed a more pronounced impact on EVs-derived-miRNAs levels when the cells were treated with 20 μM of ENZ, observing a (37.53 fold-increase of EVs-derived-hsa-miR-221-3p levels (p=0.02) and a 64.45 fold-increase EVs-derived-has-miR-222-3p levels (p=0.004).

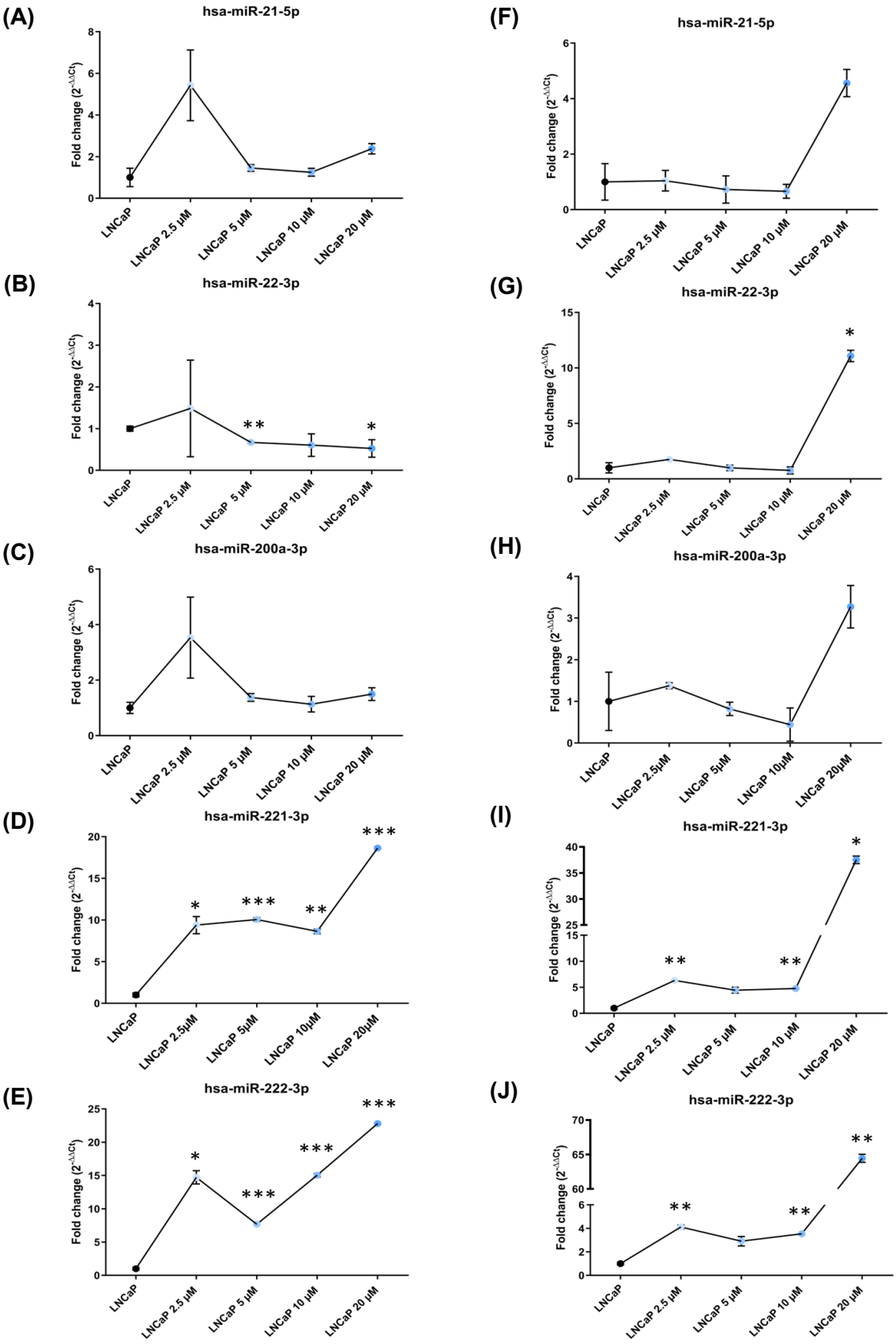


Figure 30: Variation of the (A-E) intracellular and (F-J) EVs levels of hsa-miR-21-5p, hsa-miR-22-3p, hsa-miR-200a-3p, hsa-miR-221-3p and hsa-miR-222-3p ((*: p<0.05; **: p<0.01; ***: p<0.001).

Discussion

5. Discussion

Cancer development and progression is currently a significant health problem worldwide and it deeply relies on the TME, where EVs are important communication mediators [122, 155]. EVs transport bioactive molecules, including miRNAs, which can alter the recipient cells phenotype. Studies have demonstrated that miRNAs play a crucial role in regulating resistance to ENZ in CRPC treatment and are promising predictive biomarker candidates [122, 155]. Therefore, the study of EVs-derived miRNAs can provide insights into the mechanisms underlying resistance and may offer new possibilities for identifying biomarkers to predict ENZ resistance.

The ENZ-resistant cells investigated in the present study exhibited high metabolic capacity and demonstrated robust growth in the presence of ENZ. Moreover, they presented a decrease in *PSA* expression and, in LNCaPER, we observed a decrease of *AR* mRNA expression. These demonstrated that ENZ effectively inhibits the AR pathway and suggests that cells become resistant via AR-independent mechanisms. Yundong H. and colleagues corroborated these observations by reporting transcriptional downregulation of canonical AR target genes, including PSA, in ENZ-resistant LNCaP cells [156]. Similarly, Florian Handle F. and co-workers observed a significant reduction in AR expression in ENZ-resistant LNCaP cells, with no detection of AR-V7 splice variant or *AR* mutations, suggesting a resistant mechanism that does not involve AR activation [157]. In contrast, Hoefer and colleagues developed an ENZ-resistant cell line that employed mechanisms involving AR upregulation and induction of AR-V7, thereby maintaining their reliance on the AR pathway [158]. These findings support the capacity of LNCaP cells to use diverse resistance mechanisms, rather than a predetermined cell type-specific resistance mechanism to escape ENZ treatment. We hypothesize that factors such as the chemical and physical environment pressure and variability in treatment duration and dosages among studies might influence which resistance mechanisms are favored or become predominant.

The establishment of the ENZ-resistant cell line allowed the profiling of miRNAs associated with resistance, enabling the evaluation of their dynamic levels, identifying related pathways, and further assess their potential as resistance prediction biomarkers. In fact, deregulated miRNAs may also be potential therapeutic targets. Replacing their physiological levels using RNA therapies, including miRNA mimics and miRNA inhibitors, may contribute to the reversion of the resistance phenotype and contribute to sensitizing cells for ENZ treatment.

Our analysis revealed significant alterations in miRNA expression patterns in LNCaPER cells, identifying 13 underexpressed and 12 overexpressed miRNAs in

LNCaPER cells, comparatively to ENZ-sensitive cells. Looking for validated target genes of the underexpressed and overexpressed miRNAs, we found that *BCL2L11*, *PTEN* and *TGFBR2* were the genes regulated by the highest number of underexpressed miRNAs, indicating that they might be upregulated during the ENZ acquisition phenotype. However, when we look at the overexpressed miRNAs obtained, we found these genes are targeted by a higher number of overexpressed miRNAs, than underexpressed miRNAs, which suggests that they might be under stronger inhibitory regulation in ENZ resistant LNCaP cells, agreeing with the current literature. *BCL2L11* and *PTEN* genes are tumor suppressor genes and are described as downregulated in PC. In fact, *PTEN* loss is associated with proliferation and cell growth through activation of the PI3K/AKT pathway and BCL2L11 loss cause decreased cell apoptosis [159]. The *TP53* was the most common target of the overexpressed miRNAs. This indicates that this gene is potentially downregulated, which is in line with its known tumor suppressor function. *TP53* is a tumor suppressor gene involved in the regulation of cell cycle progression, apoptosis, promote gene stability and is a crucial gene in the development of PC [160]. These findings shed light on the complex regulatory landscape associated with ENZ resistance.

By a functional enrichment analysis, we found that the potentially upregulated and downregulated proteins are involved in proliferation, apoptosis and cell metabolism regulation. Regarding the pathways in which these proteins are involved, we obtained several terms that were common to potentially up- and downregulated proteins, namely PI3K/AKT, FOXO and TGF-β signaling pathways, which play fundamental role in progression of PC by increasing cell survival, proliferation, growth, and resistance to apoptosis. [161, 162]. In fact, these results agree with previous studies [163]. For example, Shiv V. and colleagues compared the transcriptomic profile of paired ENZ-resistant and parental LNCaP and C4-2B cells and obtained 9409 and 7757 aberrant expressed genes, respectively, in resistant cells compared to parental ENZ sensitive cells [163]. They subsequently performed a pathway enrichment analysis on the differently expressed genes which resulted in enriched terms such as: PI3K/AKT signaling, cytokine-cytokine receptor interaction and pathways in cancer. These analyses also revealed that these genes were mostly associated with altered metabolism of lipids, carbohydrates, fatty acid, and glycoproteins. Consequently, these authors extensively defined the metabolic reprograming as a hallmark of ENZ-resistance in PC [163]. They quantified gene expression levels in a similar study model and identified abnormally regulated pathways essential for proliferation and metabolism [163]. Taken together, this evidence showed the important role of miRNAs in the expression regulation the expression of cancer related genes, which could lead to the

aberrant activation of PI3K/AKT pathway and the metabolism reprogramming, which are essential steps for development of ENZ resistant phenotypes.

Since the 3D models better mimic the *in vivo* conditions, they are particularly valuable for investigating drug resistance mechanisms and genetic expression profiles, which support their application in the present study. Thus, we optimized the best spheroid formation conditions and further established a 3D spheroid model that represents the ENZ resistance development process. It was found that over time the behavior of the spheroids varied between different initial cell concentrations. We observed that spheroids formed with lower cell densities tended to increase in diameter progressively over the 7 days. This may occur because in smaller spheroids cells are less aggregated and have greater access to nutrients and oxygen, and therefore greater capacity for division. Similarly, a study performed by Mittler F. and colleagues reported that smaller LNCaP spheroids, around 400 nm, displayed an increase in area over time[164]. Nonetheless, in the largest spheroids, we observed a significant reduction in diameter over the same period. In fact, Cavo M. and co-workers assembled large spheroids (diameter>1 mm) through distinct spheroid assemble techniques (hanging drop, round-bottom wells and Matrigel embedding) and observed spheroid compaction from the decrease in spheroid diameter accompanied by an increase in darkness and cell density in the first 10 days of culture [165]. According to Smyrek and co-workers, spheroid formation at a non-adhesive surface may be generally divided into three phases: aggregation, compaction and growth. Initially, lose-aggregates are formed, and the subsequent spheroid compaction due to strong cell adhesion through cadherin-cadherin binding, between adjacent cells, contribute to a reduction of the empty spaces between cells, resulting in more cohesive and compact spheroids [166, 167]. The aggregation and compaction phases were clearly observed in our study, while the growth phase was not observed in larger spheroids. These insights underscore the influence of initial cell density on spheroid behavior and provide a foundation for optimizing the 3D spheroid model for our study.

Regarding metabolic activity, our findings show that as the diameter of C1 spheroids increases, so does their metabolic activity, which provides validation for the hypothesis that the increasing spheroid diameter is linked to a higher number of cells. In contrast, the metabolic activity of C3 spheroids registered a decrease. In fact, Sart and co-workers suggested that cell proliferation and viability decrease in large and compact spheroids due to limitations in the diffusion of nutrients, oxygen and metabolites [168]. One noteworthy aspect that emerged from our results is the formation of necrotic cores in the C3 spheroids. The formation of necrotic cores is a desirable characteristic for 3D models, since it proper mimic the cellular heterogeneity and tumor microenvironment seen in solid *in vivo* tumors

[165, 169]. Consequently, we decided to proceed with C3 spheroids, considering their greater robustness and the relevance of necrotic cores for our study's objectives. Nevertheless, we observed variations in the metabolic activity of cells in 3D culture over time, even though they presented similar metabolic rates in 2D culture. This discrepancy might be attributed to distinct physical and structural culture conditions between 2D and 3D models, which can influence signaling pathways and gene expression patterns [139]. Basu H.S. and co-workers observed that ENZ-resistant C4-2 cells are much less glycolytic than its parental LNCaP cells [170]. The authors also found that the increase of ENZ concentration caused a significant decrease in glycolysis in LNCaP cells and an increase in mitochondrial oxidative phosphorylation for ATP synthesis and thus, in their oxygen demands [170]. This observed switch to oxidative phosphorylation can explain the metabolic activity results for ENZ resistant cells in 3D culture. This allows an increase in ATP production, which contributes to cell proliferation and survival in the presence of ENZ under conditions of oxygen availability, as seen in 2D culture. However, such energetic reprogramming may be disadvantageous under conditions of low oxygen availability, as seen in spheroid cores, which may explain the increase of ATP production in LNCaP 2.5 μM after 72h of culture, but a decrease in LNCaP treated with higher concentrations.

In terms of spheroids structural organization characterization, we found that both the LNCaP and ENZ-treated spheroids presented a high expression of E-cadherin, but no differences were visible between ENZ concentrations or time points. E-cadherin, besides being an important mediator of cell-cell adhesion, is also an epithelial marker, thus loss of E-cadherin is a key step to initiate epithelial to mesenchymal transition (EMT), which is associated with CRPC development and ENZ resistance progression [166, 171, 172]. In contrast, we observed low expression of vimentin, which is a critical mesenchymal marker and is reported to be highly expressed during EMT and in ENZ resistant cells [171, 172]. The fact that we observed high expression of E-cadherin and low expression of vimentin in all the spheroids may indicate that, despite several studies demonstrate that EMT is associated with CRPC and promote ENZ resistance, it is not one of the most relevant mechanisms to maintain resistance in the 3D model [172, 173]. However, the immunofluorescence images obtained can only detect marked differences in expression, based on qualitative fluorescence intensity. Thus, a more detailed quantification of protein or gene expression would be necessary to detect small differences between LNCaP and cells resistant to various concentrations of ENZ.

Regarding fibronectin, both LNCaP and ENZ-resistant cells secrete this matrix protein. This observation aligns with previous research that has demonstrated the important role of fibronectin in supporting proliferation and invasion of PC cells. It also underscores

the significant contribution of ECM production by these spheroids [174, 175]. The synthesis of ECM, coupled with the adhesive properties of the cells, may be responsible for the observed aggregation and compaction of spheroids [166].

In this work, we observed a high expression and membranal localization of β-catenin by immunofluorescence labeling on both LNCaP and ENZ treated cells within the 3D model. Notably, no differences between ENZ concentrations and time points were observed. In PC, the loss of the E-cadherin-β-catenin complex at the cell surface has been linked to EMT, and thus disease progression via a loss of cell-cell adhesion and increased metastases [176]. Despite the existence of contradictory IHC studies, regarding β-catenin expression and localization in PC, nuclear β-catenin has been described to increase in PC, since Wnt/β-catenin signaling can influence AR signaling, thus playing a major role in ENZ resistance [177, 178]. Since we didn't observe major differences on β-catenin expression and cellular localization on ENZ-treated spheroid comparatively to LNCaP spheroids, it appears that the Wnt/β-catenin pathway might not be crucial in the development of ENZ resistance within this 3D model.

The higher and central localization of caspase 3 labeling in the assembled LNCaP 30 μM spheroids, compared to the other spheroids, demonstrates that these spheroids may not be mimicking the resistant phenotype. At the time of these spheroids' formation, LNCaP 30 μM cells in 2D culture, seemed to present a phenotype with senescence features that could explain the differences observed in the spheroid. Therefore, these assembled spheroids were not considered for further miRNA quantification.

We found an increase in the intracellular levels of the miRNA profile defined in the cell lines treated with the lowest concentration of ENZ, suggesting that its administration leads to an acute effect on cells. We hypothesize that the initial spike in miRNA expression could be a compensatory mechanism employed by the cells to counteract the inhibitory effects of ENZ (blockage of the AR pathway), this could be an answer to the selective pressure introduced into the cell microenvironment. It is accepted, that both hsa-miR-21 and hsa-miR-22 contribute to PC tumorigenesis by downregulating PTEN expression and modulating PI3K/AKT and MAPK/ERK signaling pathways [179]. Furthermore, hsa-miR-21 also suppresses TGFBR2, thus inhibiting TGF-β-mediated Smad2/3 activation and subsequent promotion of cell growth [180]. These authors described these miRNAs as PC oncomiRs which explain their rapid increase expression after cells treatment with ENZ, suggesting their contribution for an early adaptative response. Indeed, the increased expression of these miRNAs allow hyperactivation of other pathways that induce proliferation and cell growth, including PI3K/AKT and MAPK/ERK signaling pathways [179].

Similarly, we observed a tendency for EVs-derived- hsa-miR-21 and hsa-miR-22 levels increase after cells treatment with higher ENZ concentrations. MiRNAs transported in EVs may play a role in mediating the transfer of resistance from one cell to another. The transference of functional miRNAs to recipient cells can downregulate target genes, such as pro-apoptotic genes, and induce the resistant phenotype on recipient cells, by activating survival and proliferation signaling pathways [173]. In fact, this mechanism was already reported by Samuel and Lee and co-workers, that found that EVs-derived hsa-miR-21 levels are higher in ENZ-resistant PC cells and in sera from patients with ENZ-resistant PC [60, 181].

On the other hand, the observed decrease and stabilization of hsa-miR-21,22 and 200a levels, when cells were submitted to higher ENZ concentrations, could occur due to a negative feedback loops or regulatory mechanisms within the cell, attempting to restore miRNA levels to a homeostatic state. Furthermore, hsa-miR-22 has been reported to have tumor suppressive properties, which might also explain the decrease and stabilization of miRNA levels. Xin and colleagues proved that hsa-miR-22 inhibits the proliferation and invasion of cancer cells as well as promotes cell apoptosis by blocking de novo lipid synthesis [182]. Maina P.K. and co-workers reported consistent results, indicating that in CRPC cells, MYC represses hsa-miR-22, which lead to the increase of PHF8 and KDM3A, transcriptional co-activators, resulting in the positive regulation of proliferation and migration in PC cells [183]. In fact, the existence of controversial results regarding the role of hsa-miR-22 in ENZ resistance may indicate that its expression is dynamic and dependent on several factors. These factors might include the molecular context, the cell population in study, the existence of different genetic mutations, namely in AR, and ENZ treatment and microenvironment conditions.

Regarding hsa-miR-200a, despite the observed initial tendency to increase, its levels decreased at higher ENZ concentrations. This miRNA has been associated with EMT suppression and the development CRPC by impairing BRD4-mediated AR signaling, which might prevent the development of Enz resistance in CRPC [122, 184]. Since this miRNA targets different signaling pathways, including Notch, Wnt, TGF-β and contributes to the maintenance of epithelial characteristics, it is frequently downregulated in PC [184, 185]. Nonetheless, hsa-miR-200a have been reported to be overexpressed in circulating EVs in patients with PC [63, 186], which might indicate that cell secret this miRNA to maintain the low intracellular levels and thus promote/maintain the castration resistant phenotype.

Our results support that hsa-miR-221 and hsa-miR-222 cluster were the ones with the greatest potential as a predictive biomarker of ENZ resistance acquisition since its

expression in the resistant cells significantly increased in a dose-dependent manner. Hsa-miR-221 and hsa-miR-222 are encoded in tandem from a gene cluster on chromosome X, which can explain their similar intracellular expression dynamics [187]. Their intracellular levels significantly increased alongside the increase of ENZ concentration. Their oncogenic role derives from the ability to repress cell cycle inhibitor proteins p27/Kip1 and p57, which are known regulators of the G1/S cell cycle checkpoint [188-190]. They inhibit the cell cycle by binding to cyclin-dependent kinase (CDK) complexes and preventing them from driving the cell cycle forward [188-190]. Zhang and co-workers also identified PTEN as a target gene of hsa-miR-221/222 [191]. Teixeira et al. referred that this cluster, whose overexpression is reported to induce development of CRPC, is a downstream target of the EGFR pathway [189]. The hyperactivation of EGFR can induce several downstream intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) and PI3K/AKT signaling. These pathways can then mediate cell cycle progression and cell survival. In fact, constitutively activated MAPK and PI3K/AKT signaling occur in CRPC cells they have been proposed as the important pathways in promoting PC progression to CRPC and ENZ resistance [118]. Moreover, we found that EVs-derived hsa-miR-221/222 levels increase during the ENZ establishment, suggesting the involvement of these molecules in tumor microenvironment modulation in order to modify cells and transfer the “resistance” among the cell population. In fact, the potential of this cluster as circulating biomarker was already demonstrated. Teixeira A.L. and colleagues, suggest the potential of blood hsa-miR-221/222 as circulating predictive biomarkers of early CRPC development. The authors suggested that this up-regulation could be due to the activation of EGFR signaling pathway, leading to the activation of oncogenic pathways that promote tumor growth, progression, metastasis promotion and castration-resistant development [192].

Taken together, our results support the applicability of Evs-derived hsa-miR-221 and -222 as predictive biomarkers of ENZ-resistance development. It is imperative that future studies are conducted to validate this hypothesis. On the other hand, we also concluded that PI3K/AKT pathway is a central pathway in the development of resistance to ENZ, since important genes involved in its regulation, namely PTEN, are simultaneously regulated by the 5 selected upregulated miRNAs. Additionally, these findings indicate that hsa-miR-221 and -222 can also be future therapeutic targets to revert the resistance phenotype.

Conclusion and Future Perspectives

6. Conclusion and Future Perspectives

ENZ, a nonsteroidal second-generation anti-androgen used for CRPC treatment, has been proven to prolongate the OS of patients, reducing the risk of disease progression. However, the relapsing nature of CRPC results in the development of ENZ resistance in a high number of patients. This scenario is currently a huge concern in the management of PC due to the limited specificity of the clinical predictive biomarkers currently in use and the lack of effective alternative therapeutic approaches. The development of resistance deeply relies on the TME dynamics where EVs mediate communication through the release of their molecular content, including miRNAs. MiRNAs are dysregulated in CRPC and their important contribution for ENZ resistance development has attracted more and more attention. Thus, the analyses of EVs-derived miRNAs in body fluids can provide valuable insights into treatment response and allow the definition of new non-invasive biomarkers.

In this study, we have found that ENZ resistance phenotype is associated with the aberrant expression of several miRNAs which led to alterations mostly in pathways related to proliferation, survival and metabolism, being the PI3K/AKT a key pathway in this process. When evaluating the biomarker potential of overexpressed miRNAs, we observed that the hsa-miR-221/222 cluster expression significantly increased in resistant cells in a dose-dependent manner. Additionally, we observed that EVs-derived hsa-miR-221/222 presented an increase for lower ENZ concentrations, followed by a pronounced increase associated with high doses of ENZ. Thus, we concluded that these two miRNAs can be packaged in the EVs derived from ENZ resistant cell lines, highlighting their role on tumor microenvironment modulation and consequently in the resistance acquisition mechanism, which support their potential application as predictive resistance biomarkers.

In the future, the LNCaPER spheroids should be reassessed in order to obtain a 3D study model representative of the resistance at the highest ENZ concentration studied, 30 μM. Following that, it will be useful to analyze the expression dynamics of other deregulated miRNAs in order to complete the EV-derived miRNAs profile and improve its predictive capacity. Subsequently, the EV-derived miRNAs profile needs to be studied in a clinical setting of patients submitted to ENZ therapeutic approach, in order to monitor the profile dynamic during the treatment.

Finally, it would be interesting to see whether these miRNAs could serve as potential therapeutic targets for reversing ENZ resistance. This could be achieved by modulating their expression using miRNA mimics and/or inhibitors in 3D models.

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7. References

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Supplementary table 1: Selected underexpressed miRNAs and their validated target mRNAs by strong evidence.

miRNA	Log_2(fold change)	$-Log_{10}$(padj)	Validated targets
Hsa-miR-7-5p	-3.038	3.026	*ABCC1, AR, ARRB1, BAX, BCL11, BTG2, CAPZA1, CCNE1, CKAP4, CUL5,DIS3L2, DMPK,EGFR, FOS, GPF5, HELLS, HOXB3, HOXB5, IGF1R, ILF2, ILF3, IRS1, IRS2, KEAP1, KLF4, KMT5A, MSH3, NDUFA4, NOTCH4, PAK1, PAX6, PIK3CD, PIK3CG, PIK3R3, PLP2, PSME3, PTK2, RAF1, RAPGEF3, REL, RELA, RGS5, RNF183, SERPINB5, SETD8,SIRT2, SKP2, SLC7A5, SMARCD1, SNCA, Sp1, SRSF1, TET2, TGFB2, UBE2A, VDAC1, XIAP, XRCC2, YY1, ATXN1*
Hsa-miR-18a-5p	-2.367	1.993	*ATM, BCL2, BCL2L10, CCN2, CDK19, CTGF, DICER1, DNMT1, EPHA7, ESR1, FGF1, HIF1A, HSF2, ING4, IRF2, MEF2D, MT1JP, NOCA3, NEDD9, NEO1, NR1I2, NRR3C1, PHLPP1, PIAS3, PTEN, RUNX1, SDC4, SMAD2, STK4, TBPL1, TGFBR2, TNFAIP3, TNFSF11*
Hsa-miR-1973	-2.028	1.545	-
Hsa-135a-5p	-1.908	1.497	*APC, BCL2L14, BMPR2, CEBPD, DAPK2, E2F1, EGFR, ESRRA, FOXO1, HOXA10, HTR1A, IRS2, JAK2, KLF4, KLF8, MMP11, MTSS1, MYC, NR3C2, PHLPP2, PPM1E, PTK2, PTPRD, RAET1K, RBAK, ROCK1, ROCK2, RUNX2, SIAH1, SL6A4, SMAD1, SMAD5, STAT6, TXNIP, VLDLR*
Hsa-miR-19a-3p	-1.564	1.531	*ABCA1, ADRB1, AKT1, ALOX5, ATXN1, BCL2L11, BMPR2, CCND1, CD22, CUL5, DNMT1, DPYSL2, ERBB4, ESR1, FOXP1, HIPK2, HOXA5, IL10, IMPDH1, KAT2B, KIT, MAP3K5, MECP2, MSMO1, MTUS1, MXD1, MYCN, NPEPL1, NR4A2, PHLPP1, PIK3CA, PKNOX1, PMEPA1, PRMT5, PSAP, PTEN, RAB13, RAB14, RHOB, SIVA1, SMAD4, SOCS1, SOCS3, SUZ12, TF, TGFBR2, TLR2, TLR7, TNF, TNFAIP3, TNFRSF12A, TP53INP1, TUSC2, VPS4B, ZBTB4*
Hsa-miR-15b-5p	-1.455	1.453	*AGO2, AKT3, AXIN2, BAX, BCL2L11, CCND1, CCND3, CCNE1, CHEK1, COL12A1, CRIM1, EIF4A1, FOXO1, FUT2, HNF1A, IFNG, INSR, KDR, MMP9, MTSS1, OIP5, PEBP4, PPM1D, PURA, RAB1A, RECK, SMAD2, SMAD7, SMURF1, SOCS3, TBR1, TGFB1, TMLHE, TRIM14, TRIM29, VAMP1, VEGFA, WEE1, ACSS2*
Hsa-miR-20a-5p+Hsa-miR-20b-5p	-1.341	1.467	*ABL2, AKT3, ANKH, APP, ARHGAP12, ARID4B, ATG16L1, ATG7, BAMBI, BCL2, BCL2L11, BMPR2, BNIP2, BRCA1, CCND1, CCND2, CDKN1A, CRIM1, DAPK3, DNMT1, DUSP2, E2F1, E2F3, EFNB2, EGLN3, EGR2, EPAS1, EPHB4, ERBB3, ESR1, ETV1, FBXO31, FZD6, GJA1, HIFIA, HIPK3, IRF2, ITGB8, KIF26B, KIT, LIMPK1, MAP2K3, MAP3K12, MAPK9, MCL1, MEF2D, MYC, MYLIP, NFKBIB, NRAS, PHLPP2, PDK1, PKNOX1, PPARG, PPP2R2A, PRKG1, PTEN, PRPTO, PURA, RB1, RB1CC1, RBL1, RBL2, REST, RGS5, RUNX1, RUNX3, SIRPA, SMAD4, SMAD7, ATAT3, TCEAL1, TCF4, TGFBR1, TGFBR2, TIMP2, TP53INP1, TRIM32, TSG101, UBEC2C, VEGFA, WEE1, WNT9B, ZFYVE9*
Hsa-miR-93-5p	-1.308	1.550	*ABCA1, ANG, ATG16L1, CDKN1A, CERS2, CXCL8, DAB2, E2F1, FOXA1, IGF2BP1, IL8, ITGB8, KAT2B, LATS2, MAP3K2, MAPK9, MMP3, PDCD4, PHLPP2, PTEN, PTENP1, PURA, RAB11FIP, RHOC,*

			RPS6KA4, SLC2A4, SMAD7, STK11, TGFBR2, TP53INP1, TUSC2, VEGFA, ZBTB4, ZNRF3
Hsa-miR-421	-1.188	1.467	*ATM, CASP3, CSH1, SMAD4, FOXO4, RSU1, EXOC6B, SIRT3, CBX7, RBMXL1, FGD5-AS1, MPP7, TMEM100, CMTM-4, SOCS5, SPINK1, ZNF5G4*
Hsa-miR-940	-0.958	1.352	*DUSP1, ZNF24, CD274, E2F2, PRKAA1, ZDHHC19, RIF1, C-MYC, PTPN12*
Hsa-miR-25-3p	-0.957	1.578	*ATP2A2, BCL2L11, BTG2, CBR3-AS1, CCL26, CDH1, CDKN1C, CPEB1, CYP2B6, DHFR, DSC2, ERBB2, EZH2, FBXW7, HAND2, KAT2B, KLF4, LATS2, MAP2K4, MDM2, MTF1, PRMT5, PTEN, RECK, REV3L, RGS3, RUNX3, SMAD5, TCEAL1, TGFBR2, TNFSF10, TP53, TWIST1, WDR4*
Hsa-miR-570-3p	-0.834	1.511	*EYA3, FUT8, KLF10, CD274*
Hsa-miR-328-3p	-0.702	1.525	*ABCG2, BACE1, CD44, CPT1A, H2AFX, KCNH2, MMP16, NRBP1, PLCE1, PTRJ, SFRP1*

Supplementary table 2: Selected overexpressed miRNAs and their validated target mRNAs by strong evidence.

microRNA	Log2(fold change)	-Log10(padj)	Validated targets
Hsa-miR-22-3p	0.799	1.616	*ACLY, ACVR1C, AKT1, ARPC5, BDNF, BMP6, BMP7, BMPR1B, BSG, BTG1, CCNA2, CD151, CDH5, CDKN1A, CREB1, CSF1R, CXCR2, CYR61, EEF2K, ERRB2, ERBB3, ESR1, H19, HDAC4, HDAC6, HIF1A, HMGB1, HTR2C, IRF5, KDM3A, LGALS1, LGALS9, MALAT1, MAOA, MAPK14, MECOM, MECP2, MMP14, MTA1, MTDH, MTHFR, MYC, MYCBP, NCOA1, NEAT1, NET1, NLRP3, NTRK2, PHF8, PLK1, PPARA, PPMK1, PTEN, PTMS, RAB5B, RCOR1, RGS2, SFRP2, SIRT1, SLC2A1, SNAI1, SP1, TACC1, TCEAL1, TCF7, TET2, TFRC, TIAM1, UBR5, WNT1, ZFP91*
Hsa-miR-30d-5p	1.014	1.453	*ATG12 ATG2B ATG5 BCL9 BECN1 BNIP3L CASP3 EXH2 GNAI2 GPR78 KPNB1 LIMS1 MAP4K4 NOTCH1 PIK3R2 RUNX2 SMAD1 SNAI1 SOCS1 TP53*
Hsa-miR-1269b	1.090	1.497	*PTEN, CDC40*
Hsa-miR-200a-3p	1.092	1.983	*FOG2 AREG ATRX BAP1 CCNE2 CDK6 CTNNB1 DEK DLC1 DLX5 DNMT1 EGFR ELMO2 ERBB2IP ERBIN EZH2 FOXA2 FOXG1A FOXJ1 GDAP1 GEMIN2 GJA1 GRB2 HFE HGF HMGB1 HOXB5 KEAP1 KLF11 KLHL20 MACC1 MALAT1 MAP2K4 MFF MMP2 MNX1 MYB MYH10 PDCD4 PINK1-AS PTEN PTPRD2 RASSF2 RIN2 SHC1 SIP1 SMAD2 SMAD3 SPAG9 SRF STAT4 STMN1 TCF7L1 TFAM TGFB2 THRB TP53 TP73-AS1 TRAPPC2B TRAPPC2P1 UBASH3B VAC14 WASF3 WDR37 YAP1 ZEB1 ZEB2 ZFPM2*
Hsa-miR-30e-5p	1.095	1.497	*ATG12 BECN1 BMI1 CASP3 MBNL1 MBNL3 MTTP MYBL2 NOTCH1 P4HA1 SNAI1 SOCS1 SOCS3 TANK TP53 TRIM38 TUSC3 UBE2I*
Hsa-miR-21-5p	1.519	1.732	*ABCB1, AKT2, ANKRD46, ANP43A, APAF1, BASP1, BCL10, BCL2, BCL6, BMPR2, BTG2, CADM1, CASC2, CASP8, CBX4, CCL20, CCR1, CCR7, CDC25A, CDIP1, CDK2AP1, CDK6, CDKN1B, CEBPB, CLU, COL4A1, COX2, DAXX, DDAH1, DERL1, DNM1L, DOCK4, DOCK5, DOCK7, DUSP10, E2F1, EGFR, EGLN1, EIF4A2, ENO4, ERBB2, FASLG, FBXO11, FMOD, FZD6, GAS5, GDF5, GREM1, HMGB1, HNRNPK, HPGD, ICAM1, ICOSLG, IL12A, IL1B, IL6R, INTU, IRAK1, ISCU, JAG1, JMY, KIF27, LRP6, LRRFIP1, MAP2K3, MARCKS, MBD2, MEF2C, MMP9, MSH2, MSH6,*

			MSLN, MTAP, MYD88, NAV3, NCAPG, NCOA3, NFIA, NFIB, NTF3, OXTR, PACRG, PCBP1, PCGF2, PDCD4, PIAS3, PIK3R1, PLAT, PKOD3, PPARA, PPIF, PSMD9, PTEN, PTPN14, PTX3, RASA1, RASGRP1, RECK, REST, RHO, RHOB, RPS7, RTN4, RUNX2, SATB1, SERPINB5, SETD2, SIRT2, SLC25A15, SMAD7, SMARCA4, SMN1, SOCS1, SOCS6, SOD3, SOX17, SOX2, SOX5, SOX6, SP1, SPRY2, SSSCA1, STAT3, STK36, STRN, STUB1, TAP1, TCF21, TGFB2, TGFBI, TGFBR2, TGFBR3, TGIF1, TIAM1, TICAM2, TIMP3, TLR2, TM9SF3, TNFAIP3, TNFRSF10B, TOPORS
Hsa-miR-30a-5p	1.732	1.578	*FOG2, ABL1, ATF1, ATG5, AVEN, BCL11A, BCL9, BDNF, BECN1, CBX3, CCNE2, CD99, CDH1, DNMT1, DTL, ERG, ESR2, EYA2, FOXD1, FOXL2, HCC, HIF1A, HNF4G, HSPA5, IL21R, ITGB3, KLF9, LOX, MBNL1, MBNL2, MBNL3, MTDH, NCAM1, NEDD4L, NEUROD1, NOTCH1, NT5E, PIK3CD, PRDM1, PRRT2, RAB38, RPA1, RUNX2, SKP2, SMAD1, SNAI1, SOX4, TAB3, TP53, TRAF3IP2, TUBB4B, UBE3C, VIM*
Hsa-miR-221-5p	2.074	1.497	*CDKN1B, CDKN1C, BREM1, IL1B, IL6R, MBD2, RUNX2, SLC25A15, SSSCA1, GCDH*
Hsa-miR-15a-5p	2.146	1.616	*AKT3, APP, BACE1, BCL2, BDNF, BMI1, BRCA1, CADM1, CARM1, CCND1, CCND2, CCNE1, CDC25A, CDCA4, CDKN2B, CHEK1, CHUK, CLCN3, CRKL, CUL3, CXCL10, DMTF1, DRD1, FGF7, FOXO1, HMGA1, HMGA2, HSPA1B, IFNG, IL10RA, KLF4, MN1, MYB, PHF19, PHLPP1, PURA, RECK, REPIN1, RET, SOX5, TMEM184B, TNFAIP1, TP53, TSPYL2, UCP2, VEGFA, WEE1, WNT3A, YAP1, NAT2*
Hsa-miR-221-3p	3.966	1.983	*ADAM1A, ADAMTS6, ANXA1, APAF1, ARF4, ARIH2, ARNT, ASZ1, ATG10, BBC3, BCL2L11, BECN1, BMF, BNIP3, BNIP3L, BRAP, BRWD3, CDKN1B, CDKN1C, CERS2, CORO1A, CREB1, CREBZF, CTCF, CXCL12, DDIT4, DDX58, DICER1, DIRAS3, DKK2, DVL2, ESR1, ETS1, FMR1, FOS, FOXO3, GAS5, GJA1, GRB10, HECTD2, HIF1A, HMGA1, HMGXB4, HOXB5, ICAM1, KIT, MBD2, MDM2, MEOX2, MGMT, MMP2, MX1, MYBL1, NAIP, PAK1, PDGFA, PIK3R1, PTEN, RAB1A, RAD51, RB1, RECK, RUNX1, SELE, SIRT1, SOCS1, SOCS3, SOCS5, SSX2IP, STAT5A, STMN1, TBK1, TCEAL1, TCL6, TICAM1, TIMP3, TEMD7, TNSF10, TP53, TP53INP1, TRPS1, TP53INP1, TRPS1, USP18, WEE1, ZEB2, LASS2, MBD2*
Hsa-miR-196b-5p	4.363	3.026	*AKT1, CD8A, CDKN1B, ETS2, FAZ, FOS, GATA6, HOXA9, HOXB7, HOXB8, HOXC8, IGF2BP1, MEIS1, MTOR, PIK3CG, RDX, RSPO2, TGFBR2, TSPAN12*
Hsa-miR-222-3p	4.472	1.993	*ABCG2, ADAM1A, ARID1A, BBC3, BCL2L11, BMF, CD47, CDKN1B, CDKN1C, CERS2, CORO1A, DICER1, DIRAS3, DIS3L2, DKK2, ESR1, ETS1, FOS, FOXO1, FOXO3, GAS5, GJA1, GNAI2, GNAI3, GRB10, ICAM1, KIT, MGMT, MIAT, MMP1, PDCD10, PLXNC1, PPP2R2A, PRDM1, PTEN, RECK, RUNX2, SELE, SMAD5, SOCS1, SOD2, SSSCA1, SSX2IP, STAT5A, TCEAL1, TIMP2, TIMP3, TMED7, TNFSF10, TP53, TRPS1, VGLL4, ZEB2*

Supplementary table 3: Gene Ontology (GO) Biological Process data enrichment analysis for potentially upregulated proteins. Only the terms with FDR p <0.01 were considered.

Number of genes	Term name	Description	FDR value
32	GO:0042127	Regulation of cell population proliferation	9.43E-19
44	GO:0048523	Negative regulation of cellular process	9.65E-18
36	GO:0050793	Regulation of developmental process	1.17E-17
37	GO:0070887	Cellular response to chemical stimulus	1.45E-17
34	GO:0071310	Cellular response to organic substance	5.03E-17
37	GO:0009892	Negative regulation of metabolic process	1.02E-16
36	GO:0010033	Response to organic substance	3.32E-16
38	GO:0010604	Positive regulation of macromolecule metabolic process	6.28E-16
34	GO:0031324	Negative regulation of cellular metabolic process	6.28E-16
44	GO:0048522	Positive regulation of cellular process	6.28E-16
37	GO:0031325	Positive regulation of cellular metabolic process	1.07E-15
36	GO:0051173	Positive regulation of nitrogen compound metabolic process	2.34E-15
34	GO:0010605	Negative regulation of macromolecule metabolic process	7.52E-15
44	GO:0080090	Regulation of primary metabolic process	9.23E-15
39	GO:0042221	Response to chemical	1.70E-14
23	GO:0051093	Negative regulation of developmental process	1.84E-14
26	GO:0009719	Response to endogenous stimulus	3.27E-14
22	GO:0008284	Positive regulation of cell population proliferation	6.68E-14
31	GO:0051172	Negative regulation of nitrogen compound metabolic process	6.68E-14
44	GO:0060255	Regulation of macromolecule metabolic process	7.89E-14
45	GO:0019222	Regulation of metabolic process	1.31E-13
34	GO:0043412	Macromolecule modification	2.65E-13
33	GO:0006464	Cellular protein modification process	3.58E-13
43	GO:0031323	Regulation of cellular metabolic process	3.58E-13
42	GO:0051171	Regulation of nitrogen compound metabolic process	3.58E-13
27	GO:0045595	Regulation of cell differentiation	9.21E-13
26	GO:0010941	Regulation of cell death	9.31E-13
19	GO:0008285	Negative regulation of cell population proliferation	1.01E-12
33	GO:0048513	Animal organ development	1.01E-12
25	GO:0042981	Regulation of apoptotic process	1.24E-12
39	GO:0044260	Cellular macromolecule metabolic process	1.25E-12
29	GO:0007166	Cell surface receptor signaling pathway	1.50E-12
37	GO:0048731	System development	2.29E-12
35	GO:2000112	Regulation of cellular macromolecule biosynthetic process	3.11E-12
25	GO:0042325	Regulation of phosphorylation	3.15E-12
32	GO:0009966	Regulation of signal transduction	3.58E-12
33	GO:0006355	Regulation of transcription, DNA-templated	4.47E-12
17	GO:0045786	Negative regulation of cell cycle	7.56E-12
24	GO:0045934	Negative regulation of nucleobase-containing compound metabolic process	7.61E-12
27	GO:2000026	Regulation of multicellular organismal development	8.87E-12
32	GO:0051239	Regulation of multicellular organismal process	8.88E-12

23	GO:0051094	Positive regulation of developmental process	1.06E-11
33	GO:0010646	Regulation of cell communication	1.08E-11
42	GO:0051716	Cellular response to stimulus	1.10E-11
38	GO:0007275	Multicellular organism development	1.17E-11
24	GO:1901700	Response to oxygen-containing compound	1.17E-11
33	GO:0023051	Regulation of signaling	1.39E-11
15	GO:0048732	Gland development	1.55E-11
30	GO:0051246	Regulation of protein metabolic process	1.69E-11
27	GO:0006357	Regulation of transcription by RNA polymerase II	1.71E-11
23	GO:0001932	Regulation of protein phosphorylation	2.47E-11
26	GO:0010629	Negative regulation of gene expression	2.57E-11
37	GO:0007165	Signal transduction	3.35E-11
29	GO:0032268	Regulation of cellular protein metabolic process	3.70E-11
34	GO:0019219	Regulation of nucleobase-containing compound metabolic process	3.75E-11
19	GO:0000122	Negative regulation of transcription by RNA polymerase II	3.88E-11
37	GO:0065009	Regulation of molecular function	3.99E-11
25	GO:0031399	Regulation of protein modification process	4.16E-11
10	GO:2000134	Negative regulation of G1/S transition of mitotic cell cycle	4.58E-11
11	GO:2000045	Regulation of G1/S transition of mitotic cell cycle	4.67E-11
49	GO:0050794	Regulation of cellular process	7.28E-11
25	GO:0045935	Positive regulation of nucleobase-containing compound metabolic process	7.51E-11
27	GO:0010628	Positive regulation of gene expression	7.56E-11
34	GO:0048583	Regulation of response to stimulus	8.62E-11
24	GO:0051254	Positive regulation of RNA metabolic process	9.33E-11
19	GO:0006468	Protein phosphorylation	9.56E-11
23	GO:0045893	Positive regulation of transcription, DNA-templated	1.02E-10
24	GO:0051240	Positive regulation of multicellular organismal process	1.02E-10
26	GO:0009653	Anatomical structure morphogenesis	1.04E-10
36	GO:0010468	Regulation of gene expression	1.43E-10
18	GO:0035295	Tube development	1.68E-10
17	GO:0045596	Negative regulation of cell differentiation	1.79E-10
18	GO:0072359	Circulatory system development	2.45E-10
14	GO:0048608	Reproductive structure development	2.55E-10
20	GO:0071495	Cellular response to endogenous stimulus	3.23E-10
24	GO:0010557	Positive regulation of macromolecule biosynthetic process	4.27E-10
22	GO:0010558	Negative regulation of macromolecule biosynthetic process	4.27E-10
16	GO:0035239	Tube morphogenesis	4.53E-10
20	GO:0051726	Regulation of cell cycle	6.32E-10
23	GO:0009888	Tissue development	6.82E-10
22	GO:0031327	Negative regulation of cellular biosynthetic process	8.25E-10
21	GO:0051253	Negative regulation of RNA metabolic process	8.81E-10
20	GO:0016310	Phosphorylation	1.13E-09
24	GO:0031328	Positive regulation of cellular biosynthetic process	1.13E-09
21	GO:2000113	Negative regulation of cellular macromolecule biosynthetic process	1.55E-09

13	GO:0031331	Positive regulation of cellular catabolic process	1.79E-09
17	GO:0033993	Response to lipid	1.79E-09
43	GO:0050896	Response to stimulus	1.92E-09
17	GO:0030334	Regulation of cell migration	1.98E-09
20	GO:1903507	Negative regulation of nucleic acid-templated transcription	2.05E-09
22	GO:0035556	Intracellular signal transduction	2.98E-09
22	GO:0033554	Cellular response to stress	3.42E-09
14	GO:0070848	Response to growth factor	3.42E-09
18	GO:1901701	Cellular response to oxygen-containing compound	4.01E-09
40	GO:0032501	Multicellular organismal process	4.73E-09
19	GO:0045944	Positive regulation of transcription by RNA polymerase II	6.92E-09
13	GO:0042326	Negative regulation of phosphorylation	7.13E-09
18	GO:0051248	Negative regulation of protein metabolic process	7.14E-09
19	GO:0009968	Negative regulation of signal transduction	8.54E-09
30	GO:0030154	Cell differentiation	8.70E-09
17	GO:0009887	Animal organ morphogenesis	9.67E-09
21	GO:0009967	Positive regulation of signal transduction	1.14E-08
18	GO:0009628	Response to abiotic stimulus	1.38E-08
17	GO:0060548	Negative regulation of cell death	1.52E-08
11	GO:0062197	Cellular response to chemical stress	1.64E-08
8	GO:0000079	Regulation of cyclin-dependent protein serine/threonine kinase activity	1.75E-08
23	GO:0006796	Phosphate-containing compound metabolic process	1.87E-08
39	GO:0006807	Nitrogen compound metabolic process	2.01E-08
21	GO:0051247	Positive regulation of protein metabolic process	2.07E-08
17	GO:0009894	Regulation of catabolic process	2.54E-08
11	GO:0045930	Negative regulation of mitotic cell cycle	2.55E-08
24	GO:0007399	Nervous system development	2.68E-08
17	GO:0032269	Negative regulation of cellular protein metabolic process	2.68E-08
16	GO:0043066	Negative regulation of apoptotic process	2.68E-08
11	GO:0001655	Urogenital system development	3.09E-08
12	GO:0001933	Negative regulation of protein phosphorylation	3.18E-08
16	GO:0014070	Response to organic cyclic compound	3.37E-08
13	GO:0007507	Heart development	3.41E-08
19	GO:0022414	Reproductive process	3.47E-08
16	GO:0006915	Apoptotic process	3.67E-08
11	GO:0010948	Negative regulation of cell cycle process	4.41E-08
21	GO:1902531	Regulation of intracellular signal transduction	4.72E-08
34	GO:1901564	Organonitrogen compound metabolic process	5.09E-08
16	GO:0003006	Developmental process involved in reproduction	5.43E-08
20	GO:0048468	Cell development	5.50E-08
40	GO:0044237	Cellular metabolic process	5.64E-08
20	GO:0032270	Positive regulation of cellular protein metabolic process	5.79E-08
20	GO:0048585	Negative regulation of response to stimulus	5.79E-08
11	GO:0050678	Regulation of epithelial cell proliferation	5.83E-08
17	GO:0060429	Epithelium development	5.85E-08

23	GO:0048584	Positive regulation of response to stimulus	5.88E-08
28	GO:0006950	Response to stress	6.07E-08
14	GO:0040008	Regulation of growth	6.07E-08
13	GO:0048729	Tissue morphogenesis	7.19E-08
16	GO:0045597	Positive regulation of cell differentiation	1.01E-07
15	GO:0009725	Response to hormone	1.09E-07
19	GO:0002682	Regulation of immune system process	1.12E-07
39	GO:0044238	Primary metabolic process	1.51E-07
15	GO:0031329	Regulation of cellular catabolic process	1.60E-07
13	GO:0031400	Negative regulation of protein modification process	1.60E-07
8	GO:0048660	Regulation of smooth muscle cell proliferation	1.65E-07
7	GO:0048145	Regulation of fibroblast proliferation	1.67E-07
11	GO:0070482	Response to oxygen levels	1.67E-07
16	GO:0051338	Regulation of transferase activity	1.76E-07
23	GO:0051128	Regulation of cellular component organization	1.82E-07
14	GO:0007420	Brain development	1.90E-07
12	GO:0071363	Cellular response to growth factor stimulus	1.95E-07
7	GO:0009791	Post-embryonic development	2.05E-07
12	GO:0001568	Blood vessel development	2.19E-07
17	GO:0051241	Negative regulation of multicellular organismal process	2.47E-07
12	GO:1902532	Negative regulation of intracellular signal transduction	2.58E-07
16	GO:0022603	Regulation of anatomical structure morphogenesis	3.63E-07
13	GO:0002520	Immune system development	3.72E-07
6	GO:0001541	Ovarian follicle development	4.48E-07
13	GO:0043068	Positive regulation of programmed cell death	4.70E-07
9	GO:0097305	Response to alcohol	4.70E-07
5	GO:0001782	B cell homeostasis	5.48E-07
10	GO:0048638	Regulation of developmental growth	6.41E-07
15	GO:0010243	Response to organonitrogen compound	7.06E-07
15	GO:0007417	Central nervous system development	7.12E-07
15	GO:0009790	Embryo development	8.49E-07
22	GO:0050790	Regulation of catalytic activity	8.79E-07
18	GO:0048699	Generation of neurons	1.01E-06
9	GO:2000027	Regulation of animal organ morphogenesis	1.02E-06
9	GO:0007548	Sex differentiation	1.08E-06
13	GO:0007167	Enzyme linked receptor protein signaling pathway	1.09E-06
5	GO:0045736	Negative regulation of cyclin-dependent protein serine/threonine kinase activity	1.20E-06
8	GO:0022408	Negative regulation of cell-cell adhesion	1.28E-06
18	GO:0034645	Cellular macromolecule biosynthetic process	1.44E-06
13	GO:0080135	Regulation of cellular response to stress	1.44E-06
6	GO:0031571	Mitotic G1 DNA damage checkpoint	1.49E-06
9	GO:0001822	Kidney development	1.50E-06
8	GO:2000377	Regulation of reactive oxygen species metabolic process	1.52E-06
8	GO:0050679	Positive regulation of epithelial cell proliferation	1.76E-06

12	GO:0048534	Hematopoietic or lymphoid organ development	1.87E-06
9	GO:0007162	Negative regulation of cell adhesion	1.91E-06
13	GO:0010564	Regulation of cell cycle process	1.91E-06
9	GO:0016579	Protein deubiquitination	1.95E-06
6	GO:0008630	Intrinsic apoptotic signaling pathway in response to DNA damage	1.96E-06
17	GO:0080134	Regulation of response to stress	1.98E-06
7	GO:0030879	Mammary gland development	2.01E-06
14	GO:0043549	Regulation of kinase activity	2.03E-06
7	GO:0000082	G1/S transition of mitotic cell cycle	2.21E-06
8	GO:0048872	Homeostasis of number of cells	2.60E-06
8	GO:0008406	Gonad development	2.68E-06
18	GO:0042592	Homeostatic process	2.86E-06
11	GO:0030335	Positive regulation of cell migration	3.13E-06
12	GO:0043065	Positive regulation of apoptotic process	3.45E-06
11	GO:0071396	Cellular response to lipid	3.46E-06
14	GO:0022402	Cell cycle process	4.09E-06
12	GO:0007346	Regulation of mitotic cell cycle	4.40E-06
9	GO:0071214	Cellular response to abiotic stimulus	4.43E-06
7	GO:0009411	Response to UV	4.49E-06
6	GO:0001657	Ureteric bud development	5.24E-06
8	GO:0051896	Regulation of protein kinase B signaling	5.24E-06
8	GO:0010632	Regulation of epithelial cell migration	5.40E-06
10	GO:0002009	Morphogenesis of an epithelium	5.83E-06
7	GO:0097193	Intrinsic apoptotic signaling pathway	5.95E-06
4	GO:0048569	Post-embryonic animal organ development	6.12E-06
6	GO:0110110	Positive regulation of animal organ morphogenesis	6.12E-06
9	GO:0048545	Response to steroid hormone	6.17E-06
11	GO:0006351	Transcription, DNA-templated	6.52E-06
11	GO:0030097	Hemopoiesis	6.82E-06
10	GO:0051249	Regulation of lymphocyte activation	6.86E-06
7	GO:0007093	Mitotic cell cycle checkpoint	7.01E-06
12	GO:0030155	Regulation of cell adhesion	7.01E-06
7	GO:0046661	Male sex differentiation	7.01E-06
8	GO:0070663	Regulation of leukocyte proliferation	7.01E-06
7	GO:0071559	Response to transforming growth factor beta	7.01E-06
9	GO:1901342	Regulation of vasculature development	7.21E-06
7	GO:0061138	Morphogenesis of a branching epithelium	7.78E-06
22	GO:0032879	Regulation of localization	8.05E-06
9	GO:0001666	Response to hypoxia	8.23E-06
8	GO:0034599	Cellular response to oxidative stress	8.39E-06
13	GO:0048646	Anatomical structure formation involved in morphogenesis	8.65E-06
8	GO:0045926	Negative regulation of growth	9.09E-06
6	GO:0007179	Transforming growth factor beta receptor signaling pathway	9.72E-06
15	GO:0031401	Positive regulation of protein modification process	1.11E-05
7	GO:0051216	Cartilage development	1.20E-05

9	GO:0051052	Regulation of DNA metabolic process	1.21E-05
4	GO:0060749	Mammary gland alveolus development	1.25E-05
5	GO:0048146	Positive regulation of fibroblast proliferation	1.27E-05
11	GO:1903047	Mitotic cell cycle process	1.33E-05
14	GO:0034097	Response to cytokine	1.47E-05
5	GO:0070228	Regulation of lymphocyte apoptotic process	1.76E-05
6	GO:0071156	Regulation of cell cycle arrest	1.77E-05
5	GO:0010332	Response to gamma radiation	2.05E-05
6	GO:2000278	Regulation of DNA biosynthetic process	2.14E-05
8	GO:0044772	Mitotic cell cycle phase transition	2.15E-05
5	GO:0006977	DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	2.20E-05
13	GO:0044087	Regulation of cellular component biogenesis	2.33E-05
4	GO:1901522	Positive regulation of transcription from RNA polymerase II promoter involved in cellular response to chemical stimulus	2.34E-05
26	GO:0065008	Regulation of biological quality	2.35E-05
12	GO:0045859	Regulation of protein kinase activity	2.43E-05
5	GO:2000514	Regulation of CD4-positive, alpha-beta T cell activation	2.49E-05
7	GO:0007178	Transmembrane receptor protein serine/threonine kinase signaling pathway	2.60E-05
14	GO:0044092	Negative regulation of molecular function	2.65E-05
12	GO:0048666	Neuron development	2.88E-05
8	GO:0051348	Negative regulation of transferase activity	2.90E-05
9	GO:0006366	Transcription by RNA polymerase II	2.92E-05
4	GO:0002053	Positive regulation of mesenchymal cell proliferation	3.00E-05
11	GO:0019221	Cytokine-mediated signaling pathway	3.07E-05
9	GO:0048514	Blood vessel morphogenesis	3.13E-05
10	GO:0010035	Response to inorganic substance	3.14E-05
8	GO:0009416	Response to light stimulus	3.47E-05
13	GO:0071345	Cellular response to cytokine stimulus	3.48E-05
8	GO:0045765	Regulation of angiogenesis	3.54E-05
13	GO:0030182	Neuron differentiation	3.70E-05
6	GO:0048565	Digestive tract development	3.75E-05
9	GO:0022407	Regulation of cell-cell adhesion	4.02E-05
6	GO:0048754	Branching morphogenesis of an epithelial tube	4.21E-05
10	GO:0007423	Sensory organ development	4.54E-05
6	GO:0008584	Male gonad development	4.54E-05
9	GO:0009314	Response to radiation	4.54E-05
7	GO:0071453	Cellular response to oxygen levels	4.68E-05
8	GO:0043010	Camera-type eye development	4.85E-05
10	GO:0032870	Cellular response to hormone stimulus	4.91E-05
10	GO:0048598	Embryonic morphogenesis	5.05E-05
5	GO:0003151	Outflow tract morphogenesis	5.23E-05
4	GO:2000515	Negative regulation of CD4-positive, alpha-beta T cell activation	5.41E-05
5	GO:0031100	Animal organ regeneration	5.88E-05
3	GO:0031077	Post-embryonic camera-type eye development	6.11E-05
10	GO:0071417	Cellular response to organonitrogen compound	6.53E-05

6	GO:0010212	Response to ionizing radiation	7.00E-05
6	GO:0033135	Regulation of peptidyl-serine phosphorylation	7.25E-05
6	GO:0070665	Positive regulation of leukocyte proliferation	7.25E-05
13	GO:0042327	Positive regulation of phosphorylation	7.37E-05
6	GO:0051384	Response to glucocorticoid	7.46E-05
49	GO:0009987	Cellular process	8.35E-05
6	GO:0051250	Negative regulation of lymphocyte activation	8.87E-05
14	GO:0033043	Regulation of organelle organization	8.96E-05
9	GO:1901652	Response to peptide	9.25E-05
5	GO:0002062	Chondrocyte differentiation	9.43E-05
6	GO:0031099	Regeneration	1.00E-04
7	GO:0003007	Heart morphogenesis	1.10E-04
10	GO:0010638	Positive regulation of organelle organization	1.10E-04
7	GO:0031334	Positive regulation of protein-containing complex assembly	1.10E-04
5	GO:0050673	Epithelial cell proliferation	1.10E-04
12	GO:0060284	Regulation of cell development	1.10E-04
6	GO:0006367	Transcription initiation from RNA polymerase II promoter	1.20E-04
11	GO:0006974	Cellular response to DNA damage stimulus	1.20E-04
9	GO:0008283	Cell population proliferation	1.20E-04
5	GO:0010717	Regulation of epithelial to mesenchymal transition	1.20E-04
7	GO:0033673	Negative regulation of kinase activity	1.20E-04
10	GO:0043009	Chordate embryonic development	1.20E-04
12	GO:0044703	Multi-organism reproductive process	1.20E-04
5	GO:0097306	Cellular response to alcohol	1.20E-04
6	GO:0010594	Regulation of endothelial cell migration	1.30E-04
11	GO:0043086	Negative regulation of catalytic activity	1.30E-04
11	GO:0048609	Multicellular organismal reproductive process	1.30E-04
29	GO:0016043	Cellular component organization	1.40E-04
7	GO:0030336	Negative regulation of cell migration	1.40E-04
6	GO:0045766	Positive regulation of angiogenesis	1.50E-04
8	GO:0001701	In utero embryonic development	1.60E-04
6	GO:0021543	Pallium development	1.60E-04
5	GO:0035265	Organ growth	1.60E-04
5	GO:0046620	Regulation of organ growth	1.60E-04
4	GO:0060412	Ventricular septum morphogenesis	1.60E-04
8	GO:0030900	Forebrain development	1.70E-04
8	GO:0045787	Positive regulation of cell cycle	1.70E-04
8	GO:0048589	Developmental growth	1.70E-04
9	GO:0071900	Regulation of protein serine/threonine kinase activity	1.70E-04
6	GO:0018105	Peptidyl-serine phosphorylation	1.80E-04
9	GO:0032990	Cell part morphogenesis	1.80E-04
12	GO:0001934	Positive regulation of protein phosphorylation	1.90E-04
18	GO:0009605	Response to external stimulus	1.90E-04
10	GO:0031175	Neuron projection development	1.90E-04
13	GO:0051130	Positive regulation of cellular component organization	1.90E-04

12	GO:0070647	Protein modification by small protein conjugation or removal	1.90E-04
6	GO:0060173	Limb development	2.00E-04
6	GO:2001235	Positive regulation of apoptotic signaling pathway	2.00E-04
4	GO:0048662	Negative regulation of smooth muscle cell proliferation	2.10E-04
4	GO:0051898	Negative regulation of protein kinase B signaling	2.10E-04
9	GO:0071407	Cellular response to organic cyclic compound	2.10E-04
5	GO:2000379	Positive regulation of reactive oxygen species metabolic process	2.10E-04
7	GO:0051251	Positive regulation of lymphocyte activation	2.20E-04
11	GO:0018193	Peptidyl-amino acid modification	2.40E-04
4	GO:0048546	Digestive tract morphogenesis	2.40E-04
8	GO:2001233	Regulation of apoptotic signaling pathway	2.40E-04
6	GO:0071456	Cellular response to hypoxia	2.50E-04
5	GO:0002262	Myeloid cell homeostasis	2.60E-04
8	GO:0042176	Regulation of protein catabolic process	2.60E-04
7	GO:0090068	Positive regulation of cell cycle process	2.60E-04
3	GO:0034616	Response to laminar fluid shear stress	2.70E-04
6	GO:0030278	Regulation of ossification	3.10E-04
6	GO:0000302	Response to reactive oxygen species	3.20E-04
4	GO:1904705	Regulation of vascular associated smooth muscle cell proliferation	3.20E-04
8	GO:0007389	Pattern specification process	3.30E-04
14	GO:0043085	Positive regulation of catalytic activity	3.30E-04
3	GO:0048070	Regulation of developmental pigmentation	3.30E-04
6	GO:0051054	Positive regulation of DNA metabolic process	3.30E-04
7	GO:0060562	Epithelial tube morphogenesis	3.40E-04
17	GO:0090304	Nucleic acid metabolic process	3.40E-04
5	GO:0007498	Mesoderm development	3.60E-04
10	GO:0030162	Regulation of proteolysis	3.90E-04
5	GO:0010822	Positive regulation of mitochondrion organization	4.20E-04
8	GO:0031667	Response to nutrient levels	4.30E-04
3	GO:0002643	Regulation of tolerance induction	4.40E-04
5	GO:0014066	Regulation of phosphatidylinositol 3-kinase signaling	4.50E-04
6	GO:0043523	Regulation of neuron apoptotic process	4.50E-04
12	GO:0048878	Chemical homeostasis	4.50E-04
6	GO:0060485	Mesenchyme development	4.50E-04
3	GO:0002902	Regulation of B cell apoptotic process	5.00E-04
3	GO:2001185	Regulation of CD8-positive, alpha-beta T cell activation	5.00E-04
11	GO:0002684	Positive regulation of immune system process	5.10E-04
6	GO:0050670	Regulation of lymphocyte proliferation	5.10E-04
7	GO:1901653	Cellular response to peptide	5.20E-04
5	GO:0003206	Cardiac chamber morphogenesis	5.30E-04
5	GO:0050864	Regulation of B cell activation	5.30E-04
5	GO:1903038	Negative regulation of leukocyte cell-cell adhesion	5.30E-04
5	GO:0003231	Cardiac ventricle development	5.40E-04
7	GO:0010506	Regulation of autophagy	6.00E-04
4	GO:0046637	Regulation of alpha-beta T cell differentiation	6.10E-04

5	GO:0034614	Cellular response to reactive oxygen species	6.20E-04
4	GO:0030888	Regulation of B cell proliferation	6.40E-04
5	GO:0050671	Positive regulation of lymphocyte proliferation	6.40E-04
3	GO:0043371	Negative regulation of CD4-positive, alpha-beta T cell differentiation	7.30E-04
6	GO:0006469	Negative regulation of protein kinase activity	7.60E-04
5	GO:0007050	Cell cycle arrest	7.90E-04
8	GO:0048812	Neuron projection morphogenesis	8.00E-04
5	GO:0032355	Response to estradiol	8.20E-04
3	GO:0035162	Embryonic hemopoiesis	8.20E-04
6	GO:0045732	Positive regulation of protein catabolic process	8.20E-04
8	GO:0001501	Skeletal system development	8.40E-04
11	GO:0032101	Regulation of response to external stimulus	8.70E-04
4	GO:2000573	Positive regulation of DNA biosynthetic process	8.70E-04
7	GO:0010038	Response to metal ion	9.30E-04
5	GO:0010634	Positive regulation of epithelial cell migration	9.40E-04
4	GO:0003208	Cardiac ventricle morphogenesis	9.60E-04
7	GO:0030111	Regulation of Wnt signaling pathway	9.70E-04
3	GO:0003181	Atrioventricular valve morphogenesis	1.00E-03
3	GO:0062009	Secondary palate development	1.00E-03
5	GO:0001890	Placenta development	1.10E-03
3	GO:0032924	Activin receptor signaling pathway	1.10E-03
7	GO:0045862	Positive regulation of proteolysis	1.10E-03
4	GO:0046902	Regulation of mitochondrial membrane permeability	1.10E-03
6	GO:0001503	Ossification	1.30E-03
5	GO:0007369	Gastrulation	1.30E-03
8	GO:0009611	Response to wounding	1.30E-03
2	GO:0001922	B-1 B cell homeostasis	1.40E-03
3	GO:0003148	Outflow tract septum morphogenesis	1.40E-03
2	GO:0003250	Regulation of cell proliferation involved in heart valve morphogenesis	1.40E-03
2	GO:0006808	Regulation of nitrogen utilization	1.40E-03
8	GO:0044089	Positive regulation of cellular component biogenesis	1.40E-03
4	GO:0045913	Positive regulation of carbohydrate metabolic process	1.40E-03
6	GO:0045927	Positive regulation of growth	1.40E-03
9	GO:0051347	Positive regulation of transferase activity	1.40E-03
3	GO:0070723	Response to cholesterol	1.40E-03
2	GO:1902263	Apoptotic process involved in embryonic digit morphogenesis	1.40E-03
5	GO:1903364	Positive regulation of cellular protein catabolic process	1.40E-03
3	GO:1904019	Epithelial cell apoptotic process	1.40E-03
17	GO:0002376	Immune system process	1.50E-03
4	GO:0006970	Response to osmotic stress	1.50E-03
3	GO:0033137	Negative regulation of peptidyl-serine phosphorylation	1.50E-03
4	GO:0034644	Cellular response to UV	1.50E-03
4	GO:0043502	Regulation of muscle adaptation	1.50E-03
6	GO:0048511	Rhythmic process	1.50E-03
8	GO:0010720	Positive regulation of cell development	1.60E-03

5	GO:0043409	Negative regulation of MAPK cascade	1.60E-03
3	GO:0048147	Negative regulation of fibroblast proliferation	1.60E-03
9	GO:0000902	Cell morphogenesis	1.70E-03
6	GO:0014706	Striated muscle tissue development	1.70E-03
5	GO:0030307	Positive regulation of cell growth	1.70E-03
5	GO:0030324	Lung development	1.70E-03
6	GO:0042493	Response to drug	1.70E-03
9	GO:0043408	Regulation of MAPK cascade	1.70E-03
5	GO:0071478	Cellular response to radiation	1.70E-03
6	GO:0090287	Regulation of cellular response to growth factor stimulus	1.70E-03
4	GO:0048661	Positive regulation of smooth muscle cell proliferation	1.80E-03
4	GO:2000177	Regulation of neural precursor cell proliferation	1.80E-03
3	GO:0010165	Response to X-ray	1.90E-03
5	GO:0048639	Positive regulation of developmental growth	1.90E-03
10	GO:0051050	Positive regulation of transport	1.90E-03
5	GO:0060759	Regulation of response to cytokine stimulus	1.90E-03
3	GO:0097421	Liver regeneration	1.90E-03
7	GO:0001558	Regulation of cell growth	2.00E-03
4	GO:0030509	BMP signaling pathway	2.00E-03
4	GO:0030510	Regulation of BMP signaling pathway	2.00E-03
2	GO:0043378	Positive regulation of CD8-positive, alpha-beta T cell differentiation	2.00E-03
7	GO:0045785	Positive regulation of cell adhesion	2.00E-03
2	GO:0060574	Intestinal epithelial cell maturation	2.00E-03
2	GO:1902731	Negative regulation of chondrocyte proliferation	2.00E-03
2	GO:1990086	Lens fiber cell apoptotic process	2.00E-03
6	GO:0070372	Regulation of ERK1 and ERK2 cascade	2.10E-03
5	GO:0007565	Female pregnancy	2.20E-03
3	GO:0010039	Response to iron ion	2.20E-03
15	GO:0010467	Gene expression	2.20E-03
4	GO:0043535	Regulation of blood vessel endothelial cell migration	2.20E-03
3	GO:0046621	Negative regulation of organ growth	2.20E-03
10	GO:0051960	Regulation of nervous system development	2.20E-03
19	GO:1901360	Organic cyclic compound metabolic process	2.20E-03
4	GO:0001938	Positive regulation of endothelial cell proliferation	2.30E-03
13	GO:0016070	RNA metabolic process	2.30E-03
3	GO:0030513	Positive regulation of BMP signaling pathway	2.30E-03
4	GO:0031058	Positive regulation of histone modification	2.30E-03
3	GO:0043029	T cell homeostasis	2.30E-03
8	GO:0044057	Regulation of system process	2.30E-03
7	GO:0051090	Regulation of DNA-binding transcription factor activity	2.30E-03
3	GO:0055026	Negative regulation of cardiac muscle tissue development	2.30E-03
3	GO:1904031	Positive regulation of cyclin-dependent protein kinase activity	2.30E-03
5	GO:0071383	Cellular response to steroid hormone stimulus	2.50E-03
3	GO:0097192	Extrinsic apoptotic signaling pathway in absence of ligand	2.50E-03
18	GO:0006725	Cellular aromatic compound metabolic process	2.60E-03

7	GO:0002683	Negative regulation of immune system process	2.70E-03
2	GO:0003186	Tricuspid valve morphogenesis	2.70E-03
4	GO:0022612	Gland morphogenesis	2.70E-03
2	GO:0070242	Thymocyte apoptotic process	2.70E-03
2	GO:0090071	Negative regulation of ribosome biogenesis	2.70E-03
3	GO:1901030	Positive regulation of mitochondrial outer membrane permeabilization involved in apoptotic signaling pathway	2.80E-03
6	GO:0001525	Angiogenesis	2.90E-03
6	GO:1903037	Regulation of leukocyte cell-cell adhesion	2.90E-03
4	GO:0010595	Positive regulation of endothelial cell migration	3.00E-03
3	GO:0010907	Positive regulation of glucose metabolic process	3.00E-03
9	GO:0050801	Ion homeostasis	3.00E-03
3	GO:0090049	Regulation of cell migration involved in sprouting angiogenesis	3.00E-03
5	GO:1901654	Response to ketone	3.10E-03
4	GO:1902106	Negative regulation of leukocyte differentiation	3.10E-03
3	GO:0051205	Protein insertion into membrane	3.20E-03
4	GO:0048640	Negative regulation of developmental growth	3.30E-03
2	GO:0007182	Common-partner SMAD protein phosphorylation	3.40E-03
2	GO:0061419	Positive regulation of transcription from RNA polymerase II promoter in response to hypoxia	3.40E-03
2	GO:0070141	Response to UV-A	3.40E-03
3	GO:0071392	Cellular response to estradiol stimulus	3.40E-03
2	GO:1903626	Positive regulation of DNA catabolic process	3.40E-03
4	GO:0021761	Limbic system development	3.60E-03
3	GO:1903672	Positive regulation of sprouting angiogenesis	3.60E-03
7	GO:0050769	Positive regulation of neurogenesis	3.80E-03
3	GO:2000826	Regulation of heart morphogenesis	3.80E-03
9	GO:0050767	Regulation of neurogenesis	3.90E-03
6	GO:0002521	Leukocyte differentiation	4.10E-03
3	GO:0030890	Positive regulation of B cell proliferation	4.10E-03
3	GO:2000648	Positive regulation of stem cell proliferation	4.10E-03
5	GO:0009952	Anterior/posterior pattern specification	4.20E-03
3	GO:0001658	Branching involved in ureteric bud morphogenesis	4.30E-03
2	GO:0001844	Protein insertion into mitochondrial membrane involved in apoptotic signaling pathway	4.30E-03
3	GO:0003197	Endocardial cushion development	4.30E-03
2	GO:0007183	SMAD protein complex assembly	4.30E-03
5	GO:0043281	Regulation of cysteine-type endopeptidase activity involved in apoptotic process	4.30E-03
2	GO:0048842	Positive regulation of axon extension involved in axon guidance	4.30E-03
4	GO:2000060	Positive regulation of ubiquitin-dependent protein catabolic process	4.30E-03
10	GO:1902533	Positive regulation of intracellular signal transduction	4.40E-03
7	GO:1903706	Regulation of hemopoiesis	4.40E-03
5	GO:0009636	Response to toxic substance	4.50E-03
5	GO:0030099	Myeloid cell differentiation	4.70E-03
3	GO:0007595	Lactation	4.80E-03
4	GO:0017015	Regulation of transforming growth factor beta receptor signaling pathway	4.80E-03
4	GO:0042542	Response to hydrogen peroxide	4.80E-03

3	GO:0046638	Positive regulation of alpha-beta T cell differentiation	4.80E-03
3	GO:0048538	Thymus development	4.80E-03
6	GO:0050727	Regulation of inflammatory response	4.80E-03
3	GO:0050999	Regulation of nitric-oxide synthase activity	4.80E-03
3	GO:0051402	Neuron apoptotic process	4.80E-03
4	GO:0001952	Regulation of cell-matrix adhesion	4.90E-03
6	GO:0051222	Positive regulation of protein transport	4.90E-03
3	GO:0010718	Positive regulation of epithelial to mesenchymal transition	5.00E-03
2	GO:0001783	B cell apoptotic process	5.10E-03
2	GO:0032909	Regulation of transforming growth factor beta2 production	5.10E-03
2	GO:0048563	Post-embryonic animal organ morphogenesis	5.10E-03
2	GO:1903896	Positive regulation of IRE1-mediated unfolded protein response	5.10E-03
26	GO:0051179	Localization	5.20E-03
5	GO:0032872	Regulation of stress-activated MAPK cascade	5.30E-03
3	GO:0072132	Mesenchyme morphogenesis	5.50E-03
12	GO:0044271	Cellular nitrogen compound biosynthetic process	5.60E-03
5	GO:0090257	Regulation of muscle system process	5.60E-03
7	GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	5.70E-03
4	GO:0030326	Embryonic limb morphogenesis	5.90E-03
4	GO:0010508	Positive regulation of autophagy	6.00E-03
2	GO:0002645	Positive regulation of tolerance induction	6.10E-03
2	GO:0048087	Positive regulation of developmental pigmentation	6.10E-03
3	GO:0072332	Intrinsic apoptotic signaling pathway by p53 class mediator	6.10E-03
5	GO:0021700	Developmental maturation	6.20E-03
6	GO:0051098	Regulation of binding	6.30E-03
9	GO:0050776	Regulation of immune response	6.40E-03
4	GO:0046887	Positive regulation of hormone secretion	6.60E-03
4	GO:0016202	Regulation of striated muscle tissue development	6.80E-03
6	GO:0060249	Anatomical structure homeostasis	6.90E-03
5	GO:0090092	Regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	6.90E-03
3	GO:0010611	Regulation of cardiac muscle hypertrophy	7.00E-03
2	GO:0002664	Regulation of T cell tolerance induction	7.10E-03
19	GO:0006996	Organelle organization	7.10E-03
6	GO:0007409	Axonogenesis	7.10E-03
2	GO:0010749	Regulation of nitric oxide mediated signal transduction	7.10E-03
2	GO:0014745	Negative regulation of muscle adaptation	7.10E-03
2	GO:0015734	Taurine transport	7.10E-03
4	GO:0019827	Stem cell population maintenance	7.10E-03
2	GO:0042118	Endothelial cell activation	7.10E-03
2	GO:0060068	Vagina development	7.10E-03
5	GO:0045165	Cell fate commitment	7.20E-03
4	GO:0009749	Response to glucose	7.50E-03
3	GO:0043536	Positive regulation of blood vessel endothelial cell migration	7.50E-03
3	GO:0008637	Apoptotic mitochondrial changes	7.90E-03

6	GO:0043434	Response to peptide hormone	7.90E-03
3	GO:0043551	Regulation of phosphatidylinositol 3-kinase activity	7.90E-03
3	GO:0061180	Mammary gland epithelium development	7.90E-03
2	GO:2000561	Regulation of CD4-positive, alpha-beta T cell proliferation	8.00E-03
3	GO:0043525	Positive regulation of neuron apoptotic process	8.20E-03
3	GO:0060395	SMAD protein signal transduction	8.50E-03
3	GO:0071385	Cellular response to glucocorticoid stimulus	8.50E-03
4	GO:0062013	Positive regulation of small molecule metabolic process	8.60E-03
3	GO:2001244	Positive regulation of intrinsic apoptotic signaling pathway	8.90E-03
8	GO:0055082	Cellular chemical homeostasis	9.20E-03
2	GO:0033598	Mammary gland epithelial cell proliferation	9.30E-03
3	GO:0042733	Embryonic digit morphogenesis	9.30E-03
3	GO:0048844	Artery morphogenesis	9.30E-03
2	GO:0060736	Prostate gland growth	9.30E-03
4	GO:0043524	Negative regulation of neuron apoptotic process	9.40E-03
5	GO:0071375	Cellular response to peptide hormone stimulus	9.90E-03

Supplementary table 4: Gene Ontology (GO) Cellular Component data enrichment analysis for potentially upregulated proteins. Only the terms with FDR p <0.01 were considered.

Number of genes	Term name	Description	FDR value
39	GO:0005634	Nucleus	2,92E-06
20	GO:0005694	Chromosome	2,92E-06
11	GO:0005667	Transcription regulator complex	4,98E-06
32	GO:0032991	Protein-containing complex	4,98E-06
28	GO:0005654	Nucleoplasm	5,98E-06
16	GO:0000785	Chromatin	7,26E-06
15	GO:0000790	Nuclear chromatin	7,26E-06
16	GO:0000228	Nuclear chromosome	8,22E-06
30	GO:0031981	Nuclear lumen	8,22E-06
45	GO:0005737	Cytoplasm	3,90E-05
30	GO:0005829	Cytosol	5,43E-05
32	GO:0070013	Intracellular organelle lumen	5,43E-05
3	GO:0071141	SMAD protein complex	2,00E-04
48	GO:0005622	Intracellular	4,10E-04
6	GO:0090575	RNA polymerase II transcription regulator complex	4,10E-04
42	GO:0043231	Intracellular membrane-bounded organelle	4,80E-04
27	GO:0043232	Intracellular non-membrane-bounded organelle	5,80E-04
45	GO:0043229	Intracellular organelle	6,70E-04
44	GO:0043227	Membrane-bounded organelle	2,50E-03
2	GO:0016513	Core-binding factor complex	4,50E-03
2	GO:0032444	Activin responsive factor complex	4,50E-03

Supplementary table 5: Gene Ontology (GO) Molecular Function data enrichment analysis for potentially upregulated proteins. Only the terms with FDR p <0.01 were considered.

Number of genes	Term name	Description	FDR value
21	GO:0043565	Sequence-specific DNA binding	6,77E-09
16	GO:0008134	Transcription factor binding	8,14E-09
18	GO:0000976	Transcription regulatory region sequence-specific DNA binding	1,34E-08
18	GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific	1,34E-08
19	GO:0003690	Double-stranded DNA binding	1,34E-08
25	GO:0019899	Enzyme binding	1,34E-08
16	GO:0019900	Kinase binding	1,34E-08
22	GO:0140110	Transcription regulator activity	1,34E-08
15	GO:0019901	Protein kinase binding	1,43E-08
16	GO:0000977	RNA polymerase II transcription regulatory region sequence-specific DNA binding	6,66E-08
24	GO:0003677	DNA binding	1,69E-07
27	GO:0098772	Molecular function regulator	7,14E-07
11	GO:0001216	DNA-binding transcription activator activity	2,96E-06
37	GO:0005515	Protein binding	3,72E-06
9	GO:0031625	Ubiquitin protein ligase binding	1,01E-05
12	GO:0000987	Cis-regulatory region sequence-specific DNA binding	2,11E-05
11	GO:0004672	Protein kinase activity	2,19E-05
10	GO:0001228	DNA-binding transcription activator activity, RNA polymerase II-specific	2,38E-05
12	GO:0016301	Kinase activity	2,90E-05
32	GO:1901363	Heterocyclic compound binding	4,24E-05
32	GO:0097159	Organic cyclic compound binding	5,85E-05
11	GO:0000978	RNA polymerase II cis-regulatory region sequence-specific DNA binding	8,88E-05
46	GO:0005488	Binding	1,40E-04
5	GO:0046332	SMAD binding	1,80E-04
5	GO:0008013	Beta-catenin binding	3,30E-04
3	GO:0034713	Type I transforming growth factor beta receptor binding	3,30E-04
8	GO:0140297	DNA-binding transcription factor binding	3,60E-04
3	GO:0070411	I-SMAD binding	5,00E-04
13	GO:0044877	Protein-containing complex binding	6,10E-04
4	GO:0001221	Transcription cofactor binding	6,90E-04
4	GO:0016538	Cyclin-dependent protein serine/threonine kinase regulator activity	7,10E-04
17	GO:0140096	Catalytic activity, acting on a protein	7,10E-04
6	GO:0019902	Phosphatase binding	8,20E-04
9	GO:0003682	Chromatin binding	9,00E-04
17	GO:0016740	Transferase activity	9,00E-04
5	GO:0042826	Histone deacetylase binding	9,00E-04
6	GO:0019207	Kinase regulator activity	1,30E-03
5	GO:0019903	Protein phosphatase binding	2,90E-03
11	GO:0046983	Protein dimerization activity	3,00E-03

6	GO:0001227	DNA-binding transcription repressor activity, RNA polymerase II-specific	3,40E-03
3	GO:0001223	Transcription coactivator binding	3,70E-03
3	GO:0008327	methyl-CpG binding	4,40E-03
7	GO:0004674	Protein serine/threonine kinase activity	6,80E-03
5	GO:0019887	Protein kinase regulator activity	7,20E-03
15	GO:0000166	Nucleotide binding	8,70E-03
14	GO:0042802	Identical protein binding	9,60E-03
2	GO:0051434	BH3 domain binding	9,60E-03

Supplementary table 6: KEGG pathway enrichment analysis data for potentially upregulated proteins. Only the terms with FDR p <0.01 were considered.

Number of genes	Term name	Description	FDR value
22	hsa05200	Pathways in cancer	2.50E-19
15	hsa05206	MicroRNAs in cancer	2,00E-17
12	hsa05210	Colorectal cancer	6,34E-16
13	hsa05226	Gastric cancer	5,75E-15
11	hsa05212	Pancreatic cancer	7,73E-15
14	hsa05166	Human T-cell leukemia virus 1 infection	1,21E-14
12	hsa04068	FoxO signaling pathway	3,50E-14
10	hsa05222	Small cell lung cancer	2,68E-12
10	hsa01522	Endocrine resistance	3,02E-12
12	hsa05169	Epstein-Barr virus infection	3,02E-12
10	hsa04933	AGE-RAGE signaling pathway in diabetic complications	3,51E-12
11	hsa04218	Cellular senescence	5,03E-12
11	hsa05225	Hepatocellular carcinoma	9,04E-12
9	hsa05220	Chronic myeloid leukemia	1,34E-11
13	hsa04151	PI3K-Akt signaling pathway	7,78E-11
8	hsa05213	Endometrial cancer	7,92E-11
9	hsa05215	Prostate cancer	8,65E-11
10	hsa05161	Hepatitis B	1,98E-10
8	hsa05218	Melanoma	3,73E-10
7	hsa05219	Bladder cancer	4,44E-10
9	hsa04110	Cell cycle	4,54E-10
12	hsa05165	Human papillomavirus infection	4,54E-10
10	hsa05205	Proteoglycans in cancer	1,10E-09
9	hsa05224	Breast cancer	1,97E-09
9	hsa04390	Hippo signaling pathway	2,98E-09
7	hsa04115	p53 signaling pathway	1,27E-08
7	hsa05214	Glioma	1,27E-08
9	hsa05167	Kaposi sarcoma-associated herpesvirus infection	1,44E-08
7	hsa01521	EGFR tyrosine kinase inhibitor resistance	1,92E-08
9	hsa05163	Human cytomegalovirus infection	4,89E-08

7	hsa04350	TGF-beta signaling pathway	4,90E-08
8	hsa05160	Hepatitis C	7,12E-08
8	hsa05202	Transcriptional misregulation in cancer	1,37E-07
7	hsa04919	Thyroid hormone signaling pathway	2.59E-07
6	hsa05223	Non-small cell lung cancer	2,77E-07
8	hsa04510	Focal adhesion	3,76E-07
8	hsa05170	Human immunodeficiency virus 1 infection	4,57E-07
5	hsa05216	Thyroid cancer	4,88E-07
7	hsa05162	Measles	5,95E-07
8	hsa05131	Shigellosis	6,92E-07
6	hsa04012	ErbB signaling pathway	7,21E-07
7	hsa04630	JAK-STAT signaling pathway	1,45E-06
6	hsa04659	Th17 cell differentiation	2,07E-06
6	hsa04066	HIF-1 signaling pathway	2,65E-06
6	hsa04931	Insulin resistance	2,73E-06
6	hsa04926	Relaxin signaling pathway	7,29E-06
5	hsa01524	Platinum drug resistance	7,78E-06
6	hsa04140	Autophagy - animal	7,78E-06
6	hsa04210	Apoptosis	7,78E-06
6	hsa04371	Apelin signaling pathway	7,78E-06
5	hsa04917	Prolactin signaling pathway	7,78E-06
5	hsa05230	Central carbon metabolism in cancer	7,78E-06
6	hsa05418	Fluid shear stress and atherosclerosis	7,78E-06
4	hsa04215	Apoptosis - multiple species	9,38E-06
6	hsa04550	Signaling pathways regulating pluripotency of stem cells	1,01E-05
6	hsa04932	Non-alcoholic fatty liver disease	1,35E-05
5	hsa05235	PD-L1 expression and PD-1 checkpoint pathway in cancer	2,05E-05
5	hsa04071	Sphingolipid signaling pathway	7,31E-05
5	hsa04935	Growth hormone synthesis, secretion and action	7,78E-05
5	hsa04910	Insulin signaling pathway	1,30E-04
4	hsa05211	Renal cell carcinoma	1,50E-04
4	hsa05221	Acute myeloid leukemia	1,50E-04
4	hsa04520	Adherens junction	1,60E-04
4	hsa04920	Adipocytokine signaling pathway	1,70E-04
5	hsa04934	Cushing syndrome	2,40E-04
4	hsa04211	Longevity regulating pathway	4,00E-04
6	hsa04010	MAPK signaling pathway	4,40E-04
5	hsa05203	Viral carcinogenesis	5,00E-04
4	hsa05231	Choline metabolism in cancer	5,50E-04
4	hsa05142	Chagas disease	6,10E-04
4	hsa04928	Parathyroid hormone synthesis, secretion and action	6,90E-04
5	hsa05132	Salmonella infection	8,90E-04
4	hsa04668	TNF signaling pathway	9,20E-04
4	hsa04722	Neurotrophin signaling pathway	9,70E-04
4	hsa04152	AMPK signaling pathway	1,10E-03

3	hsa04930	Type II diabetes mellitus	1,10E-03
4	hsa04380	Osteoclast differentiation	1,20E-03
4	hsa04915	Estrogen signaling pathway	1,60E-03
3	hsa04370	VEGF signaling pathway	1,90E-03
3	hsa04213	Longevity regulating pathway - multiple species	2,30E-03
4	hsa04217	Necroptosis	2,40E-03
3	hsa04137	Mitophagy - animal	2.50E-03
4	hsa04310	Wnt signaling pathway	2,60E-03
4	hsa05152	Tuberculosis	3,60E-03
4	hsa04015	Rap1 signaling pathway	6,80E-03
3	hsa04064	NF-kappa B signaling pathway	8,70E-03
3	hsa05145	Toxoplasmosis	9,60E-03
4	hsa04014	Ras signaling pathway	9,90E-03

Supplementary table 7: Reactome pathway enrichment analysis for potentially upregulated proteins. Only the terms with FDR p <0.01 were considered.

Number of genes	Term name	Description	FDR value
35	HSA-162582	Signal Transduction	2,99E-16
25	HSA-212436	Generic Transcription Pathway	9,27E-15
26	HSA-74160	Gene expression (Transcription)	4,20E-14
8	HSA-9614085	FOXO-mediated transcription	4,30E-09
13	HSA-5663202	Diseases of signal transduction by growth factor receptors and second messengers	4,96E-09
21	HSA-1643685	Disease	1,10E-08
8	HSA-8878159	Transcriptional regulation by RUNX3	3,83E-08
8	HSA-6785807	Interleukin-4 and Interleukin-13 signaling	9,72E-08
9	HSA-8939211	ESR-mediated signaling	2,75E-07
10	HSA-9006931	Signaling by Nuclear Receptors	2,75E-07
10	HSA-5688426	Deubiquitination	3,64E-07
7	HSA-69202	Cyclin E associated events during G1/S transition	3,64E-07
7	HSA-69656	Cyclin A:Cdk2-associated events at S phase entry	3,64E-07
5	HSA-9617828	FOXO-mediated transcription of cell cycle genes	3,64E-07
13	HSA-1280215	Cytokine Signaling in Immune system	1,21E-06
7	HSA-9006936	Signaling by TGFB family members	1,21E-06
5	HSA-9634638	Estrogen-dependent nuclear events downstream of ESR-membrane signaling	1,21E-06
7	HSA-2219528	PI3K/AKT Signaling in Cancer	1,32E-06
7	HSA-199418	Negative regulation of the PI3K/AKT network	1,91E-06
9	HSA-1257604	PIP3 activates AKT signaling	2,24E-06
10	HSA-3700989	Transcriptional Regulation by TP53	2,30E-06
7	HSA-8878166	Transcriptional regulation by RUNX2	2,34E-06
15	HSA-1266738	Developmental Biology	3,74E-06
6	HSA-9009391	Extra-nuclear estrogen signaling	3,86E-06

5	HSA-8864260	Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors	3,91E-06
19	HSA-168256	Immune System	7,57E-06
10	HSA-449147	Signaling by Interleukins	9,45E-06
4	HSA-9661069	Defective binding of RB1 mutants to E2F1,(E2F2, E2F3)	1,31E-05
5	HSA-6791312	TP53 Regulates Transcription of Cell Cycle Genes	1,41E-05
5	HSA-109606	Intrinsic Pathway for Apoptosis	1,65E-05
7	HSA-109581	Apoptosis	1,78E-05
5	HSA-8848021	Signaling by PTK6	1,87E-05
6	HSA-9018519	Estrogen-dependent gene expression	3,51E-05
7	HSA-157118	Signaling by NOTCH	4,21E-05
3	HSA-8952158	RUNX3 regulates BCL2L11 (BIM) transcription	4,21E-05
4	HSA-201451	Signaling by BMP	5,08E-05
4	HSA-114452	Activation of BH3-only proteins	6,43E-05
5	HSA-170834	Signaling by TGF-beta Receptor Complex	6,43E-05
3	HSA-3304349	Loss of Function of SMAD2/3 in Cancer	8,02E-05
3	HSA-8941855	RUNX3 regulates CDKN1A transcription	8,02E-05
4	HSA-2173789	TGF-beta receptor signaling activates SMADs	8,46E-05
4	HSA-2173796	SMAD2/SMAD3:SMAD4 heterotrimer regulates transcription	8,46E-05
3	HSA-8951430	RUNX3 regulates WNT signaling	1,00E-04
3	HSA-8951936	RUNX3 regulates p14-ARF	1,30E-04
9	HSA-9006934	Signaling by Receptor Tyrosine Kinases	1,60E-04
14	HSA-597592	Post-translational protein modification	2,10E-04
3	HSA-8866910	TFAP2 (AP-2) family regulates transcription of growth factors and their receptors	2,40E-04
4	HSA-69231	Cyclin D associated events in G1	2,90E-04
3	HSA-6804116	TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest	3,40E-04
6	HSA-5689880	Ub-specific processing proteases	4,50E-04
16	HSA-392499	Metabolism of proteins	5,30E-04
4	HSA-9616222	Transcriptional regulation of granulopoiesis	6,10E-04
4	HSA-1912408	Pre-NOTCH Transcription and Translation	7,20E-04
3	HSA-6803204	TP53 Regulates Transcription of Genes Involved in Cytochrome C Release	7,20E-04
3	HSA-452723	Transcriptional regulation of pluripotent stem cells	1,30E-03
3	HSA-2173788	Downregulation of TGF-beta receptor signaling	1,60E-03
3	HSA-5674400	Constitutive Signaling by AKT1 E17K in Cancer	1,60E-03
3	HSA-1538133	G0 and Early G1	1,70E-03
5	HSA-2559583	Cellular Senescence	1,80E-03
2	HSA-3311021	SMAD4 MH2 Domain Mutants in Cancer	1,80E-03
2	HSA-3315487	SMAD2/3 MH2 Domain Mutants in Cancer	1,80E-03
3	HSA-8863795	Downregulation of ERBB2 signaling	1,90E-03
3	HSA-9615017	FOXO-mediated transcription of oxidative stress, metabolic and neuronal genes	2,10E-03
2	HSA-211163	AKT-mediated inactivation of FOXO1A	2.50E-03
2	HSA-8866911	TFAP2 (AP-2) family regulates transcription of cell cycle factors	3,50E-03
3	HSA-5689896	Ovarian tumor domain proteases	3,90E-03
2	HSA-3304356	SMAD2/3 Phosphorylation Motif Mutants in Cancer	4,50E-03
2	HSA-3656532	TGFBR1 KD Mutants in Cancer	4,50E-03

2	HSA-8849470	PTK6 Regulates Cell Cycle	4,50E-03
2	HSA-8857538	PTK6 promotes HIF1A stabilization	4,50E-03
5	HSA-8878171	Transcriptional regulation by RUNX1	4,50E-03
2	HSA-8931987	RUNX1 regulates estrogen receptor mediated transcription	4,50E-03
2	HSA-8939256	RUNX1 regulates transcription of genes involved in WNT signaling	4,50E-03
3	HSA-186712	Regulation of beta-cell development	4,70E-03
8	HSA-1640170	Cell Cycle	4,80E-03
2	HSA-111453	BH3-only proteins associate with and inactivate anti-apoptotic BCL-2 members	6,30E-03
3	HSA-2122947	NOTCH1 Intracellular Domain Regulates Transcription	6,30E-03
2	HSA-4411364	Binding of TCF/LEF: CTNNB1 to target gene promoters	6,30E-03
2	HSA-8939242	RUNX1 regulates transcription of genes involved in differentiation of keratinocytes	6,30E-03
2	HSA-198693	AKT phosphorylates targets in the nucleus	8,70E-03
2	HSA-9617629	Regulation of FOXO transcriptional activity by acetylation	8,70E-03
7	HSA-2262752	Cellular responses to stress	8,80E-03

Supplementary table 8: Gene Ontology (GO) Biological Process enrichment analysis for potentially downregulated proteins. Only the terms with FDR p<0.01 were considered.

Number of genes	Term name	Description	FDR value
46	GO:0010941	Regulation of cell death	1.16E-22
71	GO:0048522	Positive regulation of cellular process	1.49E-22
44	GO:0042127	Regulation of cell population proliferation	1.52E-21
43	GO:0043067	Regulation of programmed cell death	2.23E-21
72	GO:0048518	Positive regulation of biological process	2.23E-21
52	GO:0050793	Regulation of developmental process	3.46E-21
42	GO:0042981	Regulation of apoptotic process	9.24E-21
71	GO:0031323	Regulation of cellular metabolic process	5.37E-20
44	GO:0045595	Regulation of cell differentiation	9.48E-20
64	GO:0048523	Negative regulation of cellular process	1.51E-19
58	GO:0009893	Positive regulation of metabolic process	4.04E-19
63	GO:0065009	Regulation of molecular function	1.79E-18
70	GO:0060255	Regulation of macromolecule metabolic process	1.91E-18
54	GO:0031325	Positive regulation of cellular metabolic process	2.64E-18
72	GO:0019222	Regulation of metabolic process	2.84E-18
55	GO:0010604	Positive regulation of macromolecule metabolic process	3.27E-18
65	GO:0048519	Negative regulation of biological process	3.27E-18
68	GO:0080090	Regulation of primary metabolic process	3.27E-18
44	GO:2000026	Regulation of multicellular organismal development	3.59E-18
67	GO:0051171	Regulation of nitrogen compound metabolic process	3.59E-18
50	GO:0070887	Cellular response to chemical stimulus	6.81E-18
52	GO:0051239	Regulation of multicellular organismal process	8.49E-18
62	GO:0007275	Multicellular organism development	2.65E-17

37	GO:0009719	Response to endogenous stimulus	3.04E-17
45	GO:0071310	Cellular response to organic substance	3.79E-17
38	GO:1901700	Response to oxygen-containing compound	4.17E-17
34	GO:0071495	Cellular response to endogenous stimulus	4.44E-17
51	GO:0051173	Positive regulation of nitrogen compound metabolic process	6.22E-17
40	GO:0010647	Positive regulation of cell communication	8.37E-17
40	GO:0023056	Positive regulation of signaling	9.43E-17
49	GO:0010033	Response to organic substance	1.39E-16
56	GO:0048583	Regulation of response to stimulus	1.42E-16
39	GO:0051240	Positive regulation of multicellular organismal process	2.29E-16
43	GO:0048584	Positive regulation of response to stimulus	2.86E-16
31	GO:0060548	Negative regulation of cell death	3.10E-16
30	GO:0008284	Positive regulation of cell population proliferation	3.34E-16
22	GO:0070482	Response to oxygen levels	3.77E-16
31	GO:0051270	Regulation of cellular component movement	3.77E-16
57	GO:0048731	System development	5.62E-16
21	GO:0001666	Response to hypoxia	7.90E-16
64	GO:0032502	Developmental process	9.50E-16
32	GO:0009628	Response to abiotic stimulus	1.20E-15
45	GO:0031324	Negative regulation of cellular metabolic process	1.27E-15
37	GO:0009967	Positive regulation of signal transduction	1.28E-15
41	GO:0009653	Anatomical structure morphogenesis	2.75E-15
48	GO:0009892	Negative regulation of metabolic process	3.05E-15
34	GO:0051094	Positive regulation of developmental process	3.26E-15
29	GO:2000145	Regulation of cell motility	3.42E-15
52	GO:0048869	Cellular developmental process	3.42E-15
46	GO:0010605	Negative regulation of macromolecule metabolic process	4.86E-15
51	GO:0030154	Cell differentiation	1.11E-14
28	GO:0043069	Negative regulation of programmed cell death	1.80E-14
40	GO:0006357	Regulation of transcription by rna polymerase ii	1.91E-14
41	GO:0007166	Cell surface receptor signaling pathway	2.79E-14
44	GO:0032879	Regulation of localization	3.23E-14
65	GO:0051716	Cellular response to stimulus	3.25E-14
27	GO:0033993	Response to lipid	4.14E-14
47	GO:0048513	Animal organ development	4.14E-14
23	GO:0051272	Positive regulation of cellular component movement	4.77E-14
27	GO:0030334	Regulation of cell migration	4.84E-14
29	GO:0012501	Programmed cell death	6.94E-14
25	GO:0010942	Positive regulation of cell death	7.08E-14
27	GO:0043066	Negative regulation of apoptotic process	9.92E-14
80	GO:0050794	Regulation of cellular process	1.00E-13
28	GO:0051093	Negative regulation of developmental process	1.04E-13
41	GO:0051172	Negative regulation of nitrogen compound metabolic process	1.06E-13
22	GO:0030335	Positive regulation of cell migration	1.23E-13
28	GO:0045597	Positive regulation of cell differentiation	1.28E-13

27	GO:0006915	Apoptotic process	1.74E-13
48	GO:0010646	Regulation of cell communication	2.32E-13
26	GO:0009725	Response to hormone	2.60E-13
20	GO:0048608	Reproductive structure development	3.14E-13
48	GO:0023051	Regulation of signaling	3.43E-13
45	GO:0009966	Regulation of signal transduction	4.06E-13
28	GO:1901701	Cellular response to oxygen-containing compound	4.95E-13
55	GO:0010468	Regulation of gene expression	5.70E-13
39	GO:0009605	Response to external stimulus	6.88E-13
24	GO:0045596	Negative regulation of cell differentiation	7.52E-13
65	GO:0032501	Multicellular organismal process	8.52E-13
50	GO:0019219	Regulation of nucleobase-containing compound metabolic process	9.18E-13
23	GO:0043065	Positive regulation of apoptotic process	9.61E-13
39	GO:0010628	Positive regulation of gene expression	9.61E-13
52	GO:0042221	Response to chemical	9.61E-13
33	GO:0042325	Regulation of phosphorylation	1.34E-12
34	GO:0009888	Tissue development	1.82E-12
25	GO:0035295	Tube development	2.08E-12
19	GO:2001233	Regulation of apoptotic signaling pathway	2.17E-12
31	GO:0080134	Regulation of response to stress	2.26E-12
25	GO:0072359	Circulatory system development	3.41E-12
29	GO:0045944	Positive regulation of transcription by rna polymerase ii	3.58E-12
54	GO:0007165	Signal transduction	4.72E-12
23	GO:0007167	Enzyme linked receptor protein signaling pathway	5.02E-12
30	GO:0022414	Reproductive process	7.82E-12
32	GO:0009890	Negative regulation of biosynthetic process	7.87E-12
45	GO:0006355	Regulation of transcription, dna-templated	7.87E-12
56	GO:0007154	Cell communication	7.87E-12
48	GO:2000112	Regulation of cellular macromolecule biosynthetic process	7.95E-12
23	GO:0080135	Regulation of cellular response to stress	7.99E-12
47	GO:0051252	Regulation of rna metabolic process	8.56E-12
32	GO:0048585	Negative regulation of response to stimulus	9.17E-12
31	GO:0010558	Negative regulation of macromolecule biosynthetic process	1.10E-11
38	GO:0051128	Regulation of cellular component organization	1.12E-11
68	GO:0050896	Response to stimulus	1.16E-11
22	GO:0040008	Regulation of growth	1.23E-11
29	GO:1903507	Negative regulation of nucleic acid-templated transcription	1.23E-11
32	GO:1903508	Positive regulation of nucleic acid-templated transcription	1.48E-11
30	GO:2000113	Negative regulation of cellular macromolecule biosynthetic process	2.24E-11
31	GO:0045893	Positive regulation of transcription, dna-templated	2.39E-11
31	GO:0031327	Negative regulation of cellular biosynthetic process	2.58E-11
26	GO:1901698	Response to nitrogen compound	3.00E-11
28	GO:0045892	Negative regulation of transcription, dna-templated	3.03E-11
49	GO:0009889	Regulation of biosynthetic process	3.16E-11
37	GO:0007399	Nervous system development	3.73E-11

25	GO:0010243	Response to organonitrogen compound	3.83E-11
19	GO:0071363	Cellular response to growth factor stimulus	3.98E-11
20	GO:0032870	Cellular response to hormone stimulus	4.32E-11
37	GO:0050790	Regulation of catalytic activity	4.39E-11
33	GO:0031399	Regulation of protein modification process	4.50E-11
25	GO:0009790	Embryo development	5.11E-11
40	GO:0051246	Regulation of protein metabolic process	5.33E-11
30	GO:0045934	Negative regulation of nucleobase-containing compound metabolic process	5.34E-11
34	GO:0010629	Negative regulation of gene expression	5.69E-11
16	GO:1901214	Regulation of neuron death	6.54E-11
30	GO:0048699	Generation of neurons	7.49E-11
44	GO:0006950	Response to stress	8.12E-11
27	GO:0051241	Negative regulation of multicellular organismal process	8.52E-11
33	GO:0045935	Positive regulation of nucleobase-containing compound metabolic process	9.35E-11
29	GO:0001932	Regulation of protein phosphorylation	1.03E-10
25	GO:1902533	Positive regulation of intracellular signal transduction	1.05E-10
15	GO:0007568	Aging	1.14E-10
12	GO:0048660	Regulation of smooth muscle cell proliferation	1.26E-10
27	GO:0009968	Negative regulation of signal transduction	1.69E-10
28	GO:0010648	Negative regulation of cell communication	1.76E-10
28	GO:0023057	Negative regulation of signaling	1.84E-10
18	GO:1901652	Response to peptide	1.98E-10
30	GO:0048468	Cell development	2.34E-10
13	GO:2000377	Regulation of reactive oxygen species metabolic process	2.42E-10
33	GO:0031328	Positive regulation of cellular biosynthetic process	2.53E-10
38	GO:0032268	Regulation of cellular protein metabolic process	2.70E-10
23	GO:0014070	Response to organic cyclic compound	3.55E-10
20	GO:0035239	Tube morphogenesis	4.28E-10
31	GO:1902531	Regulation of intracellular signal transduction	5.13E-10
24	GO:0051338	Regulation of transferase activity	6.22E-10
30	GO:0051247	Positive regulation of protein metabolic process	7.87E-10
18	GO:0007507	Heart development	8.03E-10
28	GO:0002682	Regulation of immune system process	1.35E-09
15	GO:1901342	Regulation of vasculature development	1.61E-09
15	GO:0050678	Regulation of epithelial cell proliferation	1.81E-09
24	GO:0034097	Response to cytokine	2.08E-09
21	GO:0045859	Regulation of protein kinase activity	2.21E-09
14	GO:0097190	Apoptotic signaling pathway	2.44E-09
19	GO:1901699	Cellular response to nitrogen compound	2.64E-09
22	GO:0043549	Regulation of kinase activity	2.82E-09
18	GO:0048598	Embryonic morphogenesis	3.18E-09
14	GO:0062197	Cellular response to chemical stress	3.24E-09
22	GO:0003006	Developmental process involved in reproduction	4.77E-09
29	GO:0033554	Cellular response to stress	4.77E-09

18	GO:0071417	Cellular response to organonitrogen compound	5.23E-09
19	GO:0019221	Cytokine-mediated signaling pathway	5.81E-09
14	GO:0071496	Cellular response to external stimulus	6.18E-09
22	GO:0009887	Animal organ morphogenesis	7.15E-09
16	GO:0048568	Embryonic organ development	7.23E-09
25	GO:0009607	Response to biotic stimulus	7.75E-09
30	GO:0044419	Interspecies interaction between organisms	8.55E-09
19	GO:0008285	Negative regulation of cell population proliferation	8.67E-09
8	GO:1902893	Regulation of pri-mirna transcription by rna polymerase ii	1.13E-08
21	GO:0000122	Negative regulation of transcription by rna polymerase ii	1.16E-08
14	GO:0048545	Response to steroid hormone	1.26E-08
23	GO:0060429	Epithelium development	1.41E-08
13	GO:0048511	Rhythmic process	1.50E-08
14	GO:0048638	Regulation of developmental growth	1.50E-08
12	GO:0071453	Cellular response to oxygen levels	1.57E-08
18	GO:0043009	Chordate embryonic development	1.74E-08
14	GO:0010506	Regulation of autophagy	1.92E-08
16	GO:0009991	Response to extracellular stimulus	1.96E-08
9	GO:0048661	Positive regulation of smooth muscle cell proliferation	2.06E-08
22	GO:0009894	Regulation of catabolic process	2.46E-08
11	GO:2001235	Positive regulation of apoptotic signaling pathway	3.44E-08
15	GO:0009314	Response to radiation	3.82E-08
11	GO:0007565	Female pregnancy	4.02E-08
9	GO:0046620	Regulation of organ growth	4.09E-08
9	GO:0000079	Regulation of cyclin-dependent protein serine/threonine kinase activity	4.43E-08
21	GO:0044087	Regulation of cellular component biogenesis	4.56E-08
13	GO:0009416	Response to light stimulus	4.95E-08
11	GO:0071456	Cellular response to hypoxia	5.43E-08
11	GO:1904018	Positive regulation of vasculature development	5.43E-08
12	GO:0034599	Cellular response to oxidative stress	5.46E-08
22	GO:0042327	Positive regulation of phosphorylation	6.00E-08
12	GO:0045926	Negative regulation of growth	6.15E-08
22	GO:0022603	Regulation of anatomical structure morphogenesis	6.15E-08
15	GO:0031667	Response to nutrient levels	6.18E-08
7	GO:1902895	Positive regulation of pri-mirna transcription by rna polymerase ii	6.47E-08
9	GO:2000379	Positive regulation of reactive oxygen species metabolic process	6.86E-08
13	GO:1903037	Regulation of leukocyte cell-cell adhesion	7.70E-08
18	GO:0030155	Regulation of cell adhesion	8.12E-08
23	GO:0051726	Regulation of cell cycle	8.81E-08
21	GO:0071345	Cellular response to cytokine stimulus	8.93E-08
28	GO:0044093	Positive regulation of molecular function	8.93E-08
14	GO:0048589	Developmental growth	9.04E-08
15	GO:0009896	Positive regulation of catabolic process	9.38E-08
14	GO:0006979	Response to oxidative stress	1.02E-07
14	GO:0043434	Response to peptide hormone	1.05E-07

23	GO:0031401	Positive regulation of protein modification process	1.19E-07
25	GO:0043085	Positive regulation of catalytic activity	1.20E-07
13	GO:1901653	Cellular response to peptide	1.29E-07
16	GO:0045936	Negative regulation of phosphate metabolic process	1.36E-07
11	GO:0043523	Regulation of neuron apoptotic process	1.52E-07
26	GO:0032270	Positive regulation of cellular protein metabolic process	1.57E-07
20	GO:0002684	Positive regulation of immune system process	1.68E-07
10	GO:2001242	Regulation of intrinsic apoptotic signaling pathway	1.89E-07
15	GO:1903706	Regulation of hemopoiesis	1.89E-07
8	GO:0055024	Regulation of cardiac muscle tissue development	2.21E-07
15	GO:0001568	Blood vessel development	2.26E-07
14	GO:0022407	Regulation of cell-cell adhesion	2.41E-07
19	GO:0031329	Regulation of cellular catabolic process	2.72E-07
11	GO:0010632	Regulation of epithelial cell migration	2.87E-07
7	GO:1904705	Regulation of vascular associated smooth muscle cell proliferation	2.95E-07
11	GO:1903039	Positive regulation of leukocyte cell-cell adhesion	2.97E-07
19	GO:0048646	Anatomical structure formation involved in morphogenesis	3.10E-07
18	GO:0060322	Head development	3.37E-07
9	GO:0010508	Positive regulation of autophagy	3.56E-07
15	GO:0071900	Regulation of protein serine/threonine kinase activity	3.73E-07
26	GO:0035556	Intracellular signal transduction	3.78E-07
15	GO:0071396	Cellular response to lipid	4.38E-07
6	GO:0060251	Regulation of glial cell proliferation	4.42E-07
7	GO:0043536	Positive regulation of blood vessel endothelial cell migration	4.54E-07
18	GO:0048609	Multicellular organismal reproductive process	4.69E-07
9	GO:0016202	Regulation of striated muscle tissue development	4.73E-07
7	GO:0008637	Apoptotic mitochondrial changes	5.02E-07
20	GO:0001934	Positive regulation of protein phosphorylation	5.02E-07
20	GO:0030182	Neuron differentiation	5.02E-07
15	GO:0010035	Response to inorganic substance	5.42E-07
7	GO:0043525	Positive regulation of neuron apoptotic process	5.53E-07
8	GO:0043535	Regulation of blood vessel endothelial cell migration	5.55E-07
22	GO:0051707	Response to other organism	5.83E-07
17	GO:0000902	Cell morphogenesis	6.13E-07
12	GO:0071214	Cellular response to abiotic stimulus	6.21E-07
18	GO:0048666	Neuron development	6.39E-07
7	GO:2001244	Positive regulation of intrinsic apoptotic signaling pathway	6.58E-07
7	GO:0055021	Regulation of cardiac muscle tissue growth	7.29E-07
26	GO:0051049	Regulation of transport	7.32E-07
9	GO:0032355	Response to estradiol	7.55E-07
8	GO:1901216	Positive regulation of neuron death	7.74E-07
9	GO:0009411	Response to uv	8.41E-07
17	GO:0007420	Brain development	8.57E-07
13	GO:0031331	Positive regulation of cellular catabolic process	8.64E-07
15	GO:0048729	Tissue morphogenesis	8.67E-07

8	GO:0097191	Extrinsic apoptotic signaling pathway	8.81E-07
10	GO:0000302	Response to reactive oxygen species	8.83E-07
10	GO:1903708	Positive regulation of hemopoiesis	9.21E-07
19	GO:0060284	Regulation of cell development	9.44E-07
7	GO:0046626	Regulation of insulin receptor signaling pathway	9.49E-07
9	GO:0010634	Positive regulation of epithelial cell migration	9.65E-07
15	GO:0045786	Negative regulation of cell cycle	1.06E-06
9	GO:0071560	Cellular response to transforming growth factor beta stimulus	1.07E-06
7	GO:0042698	Ovulation cycle	1.14E-06
10	GO:0008406	Gonad development	1.18E-06
19	GO:0044703	Multi-organism reproductive process	1.18E-06
13	GO:0048732	Gland development	1.19E-06
14	GO:0008283	Cell population proliferation	1.26E-06
14	GO:0048812	Neuron projection morphogenesis	1.32E-06
21	GO:0051130	Positive regulation of cellular component organization	1.40E-06
7	GO:0001776	Leukocyte homeostasis	1.46E-06
16	GO:0031175	Neuron projection development	1.47E-06
9	GO:0048863	Stem cell differentiation	1.49E-06
19	GO:0007417	Central nervous system development	1.49E-06
10	GO:0060485	Mesenchyme development	1.52E-06
13	GO:0045785	Positive regulation of cell adhesion	1.62E-06
14	GO:1902532	Negative regulation of intracellular signal transduction	1.73E-06
15	GO:0031400	Negative regulation of protein modification process	1.94E-06
8	GO:0043200	Response to amino acid	2.08E-06
9	GO:0031960	Response to corticosteroid	2.16E-06
14	GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	2.16E-06
13	GO:0002009	Morphogenesis of an epithelium	2.18E-06
21	GO:0040011	Locomotion	2.35E-06
6	GO:0060043	Regulation of cardiac muscle cell proliferation	2.44E-06
47	GO:0016043	Cellular component organization	2.53E-06
15	GO:0048534	Hematopoietic or lymphoid organ development	2.69E-06
11	GO:0051348	Negative regulation of transferase activity	2.81E-06
13	GO:0042326	Negative regulation of phosphorylation	3.04E-06
12	GO:0030111	Regulation of wnt signaling pathway	3.06E-06
10	GO:0032868	Response to insulin	3.09E-06
14	GO:0071407	Cellular response to organic cyclic compound	3.19E-06
10	GO:2001234	Negative regulation of apoptotic signaling pathway	3.20E-06
16	GO:0043408	Regulation of mapk cascade	3.20E-06
17	GO:0050767	Regulation of neurogenesis	3.27E-06
20	GO:0044092	Negative regulation of molecular function	3.40E-06
5	GO:0035794	Positive regulation of mitochondrial membrane permeability	3.62E-06
10	GO:0006469	Negative regulation of protein kinase activity	3.65E-06
10	GO:0031668	Cellular response to extracellular stimulus	3.65E-06
11	GO:0045765	Regulation of angiogenesis	3.65E-06
18	GO:0051960	Regulation of nervous system development	3.65E-06

20	GO:0030030	Cell projection organization	3.67E-06
6	GO:0010718	Positive regulation of epithelial to mesenchymal transition	3.73E-06
21	GO:0006952	Defense response	3.96E-06
12	GO:0001701	In utero embryonic development	4.10E-06
7	GO:0048145	Regulation of fibroblast proliferation	4.21E-06
5	GO:1901522	Positive regulation of transcription from rna polymerase ii promoter involved in cellular response to chemical stimulus	4.26E-06
5	GO:1902108	Regulation of mitochondrial membrane permeability involved in apoptotic process	4.26E-06
8	GO:0048565	Digestive tract development	4.41E-06
21	GO:0033043	Regulation of organelle organization	4.41E-06
39	GO:0065008	Regulation of biological quality	4.41E-06
11	GO:0032496	Response to lipopolysaccharide	4.80E-06
6	GO:1990090	Cellular response to nerve growth factor stimulus	5.04E-06
8	GO:0034614	Cellular response to reactive oxygen species	5.71E-06
7	GO:2000177	Regulation of neural precursor cell proliferation	5.92E-06
14	GO:0030097	Hemopoiesis	6.02E-06
8	GO:0071901	Negative regulation of protein serine/threonine kinase activity	6.66E-06
7	GO:0010717	Regulation of epithelial to mesenchymal transition	6.78E-06
6	GO:0002260	Lymphocyte homeostasis	6.79E-06
9	GO:0050679	Positive regulation of epithelial cell proliferation	6.83E-06
7	GO:0008585	Female gonad development	7.16E-06
9	GO:0007178	Transmembrane receptor protein serine/threonine kinase signaling pathway	7.68E-06
12	GO:0048514	Blood vessel morphogenesis	7.97E-06
12	GO:0001933	Negative regulation of protein phosphorylation	8.14E-06
19	GO:0120036	Plasma membrane bounded cell projection organization	8.66E-06
13	GO:0001501	Skeletal system development	8.69E-06
17	GO:0016477	Cell migration	8.76E-06
14	GO:0044057	Regulation of system process	8.98E-06
22	GO:0006928	Movement of cell or subcellular component	9.33E-06
18	GO:0032101	Regulation of response to external stimulus	9.42E-06
7	GO:0035265	Organ growth	9.73E-06
10	GO:0045927	Positive regulation of growth	9.77E-06
18	GO:0048870	Cell motility	1.00E-05
9	GO:0031669	Cellular response to nutrient levels	1.06E-05
9	GO:0048872	Homeostasis of number of cells	1.06E-05
7	GO:0007179	Transforming growth factor beta receptor signaling pathway	1.09E-05
8	GO:0035148	Tube formation	1.09E-05
8	GO:0051384	Response to glucocorticoid	1.09E-05
16	GO:0043086	Negative regulation of catalytic activity	1.13E-05
13	GO:0006954	Inflammatory response	1.18E-05
17	GO:0051050	Positive regulation of transport	1.27E-05
8	GO:0097193	Intrinsic apoptotic signaling pathway	1.30E-05
43	GO:0044260	Cellular macromolecule metabolic process	1.33E-05
9	GO:0009612	Response to mechanical stimulus	1.41E-05
23	GO:0042592	Homeostatic process	1.41E-05

8	GO:0001890	Placenta development	1.48E-05
8	GO:0009267	Cellular response to starvation	1.48E-05
8	GO:0051147	Regulation of muscle cell differentiation	1.48E-05
8	GO:2000045	Regulation of g1/s transition of mitotic cell cycle	1.61E-05
12	GO:0051051	Negative regulation of transport	1.64E-05
10	GO:1902105	Regulation of leukocyte differentiation	1.65E-05
9	GO:0071216	Cellular response to biotic stimulus	1.79E-05
11	GO:0010038	Response to metal ion	1.93E-05
6	GO:0007569	Cell aging	2.00E-05
8	GO:2001236	Regulation of extrinsic apoptotic signaling pathway	2.00E-05
5	GO:0043029	T cell homeostasis	2.04E-05
81	GO:0009987	Cellular process	2.24E-05
5	GO:0097192	Extrinsic apoptotic signaling pathway in absence of ligand	2.30E-05
6	GO:0072091	Regulation of stem cell proliferation	2.31E-05
9	GO:0032872	Regulation of stress-activated mapk cascade	2.40E-05
18	GO:0051248	Negative regulation of protein metabolic process	2.65E-05
28	GO:0002376	Immune system process	2.71E-05
8	GO:0045766	Positive regulation of angiogenesis	2.75E-05
4	GO:0045898	Regulation of rna polymerase ii transcription preinitiation complex assembly	2.77E-05
7	GO:0060560	Developmental growth involved in morphogenesis	2.93E-05
10	GO:0060562	Epithelial tube morphogenesis	3.02E-05
13	GO:0006351	Transcription, dna-templated	3.14E-05
8	GO:0009743	Response to carbohydrate	3.35E-05
10	GO:0051271	Negative regulation of cellular component movement	3.35E-05
25	GO:0010467	Gene expression	3.35E-05
7	GO:0014013	Regulation of gliogenesis	3.40E-05
4	GO:0097345	Mitochondrial outer membrane permeabilization	3.43E-05
15	GO:0016032	Viral process	3.43E-05
8	GO:0048639	Positive regulation of developmental growth	3.58E-05
22	GO:0009059	Macromolecule biosynthetic process	3.70E-05
6	GO:0046902	Regulation of mitochondrial membrane permeability	3.78E-05
7	GO:0014066	Regulation of phosphatidylinositol 3-kinase signaling	4.11E-05
15	GO:0006974	Cellular response to dna damage stimulus	4.39E-05
12	GO:0051301	Cell division	4.49E-05
11	GO:0006366	Transcription by rna polymerase ii	4.65E-05
7	GO:0030879	Mammary gland development	4.75E-05
9	GO:0003007	Heart morphogenesis	4.88E-05
10	GO:0050863	Regulation of t cell activation	5.27E-05
10	GO:0010721	Negative regulation of cell development	5.40E-05
8	GO:0071383	Cellular response to steroid hormone stimulus	5.58E-05
14	GO:0051347	Positive regulation of transferase activity	5.78E-05
17	GO:0032269	Negative regulation of cellular protein metabolic process	5.81E-05
5	GO:0022602	Ovulation cycle process	5.96E-05
16	GO:0032880	Regulation of protein localization	6.48E-05

10	GO:0002521	Leukocyte differentiation	6.56E-05
9	GO:0030336	Negative regulation of cell migration	6.71E-05
11	GO:0019216	Regulation of lipid metabolic process	6.80E-05
7	GO:0001936	Regulation of endothelial cell proliferation	6.82E-05
10	GO:0051047	Positive regulation of secretion	6.85E-05
5	GO:0010863	Positive regulation of phospholipase c activity	7.18E-05
5	GO:0050999	Regulation of nitric-oxide synthase activity	7.18E-05
13	GO:0051223	Regulation of protein transport	7.25E-05
8	GO:0010821	Regulation of mitochondrion organization	7.29E-05
8	GO:0030278	Regulation of ossification	7.54E-05
7	GO:0009749	Response to glucose	8.04E-05
8	GO:1901654	Response to ketone	8.04E-05
9	GO:0071375	Cellular response to peptide hormone stimulus	8.04E-05
21	GO:0034645	Cellular macromolecule biosynthetic process	8.09E-05
6	GO:0001892	Embryonic placenta development	8.43E-05
10	GO:0051222	Positive regulation of protein transport	8.95E-05
5	GO:0090199	Regulation of release of cytochrome c from mitochondria	9.26E-05
5	GO:0097194	Execution phase of apoptosis	9.26E-05
5	GO:1904645	Response to amyloid-beta	9.26E-05
13	GO:0010638	Positive regulation of organelle organization	9.26E-05
7	GO:0007623	Circadian rhythm	9.41E-05
7	GO:0030217	T cell differentiation	9.41E-05
5	GO:0090342	Regulation of cell aging	1.00E-04
11	GO:0051249	Regulation of lymphocyte activation	1.00E-04
8	GO:0050870	Positive regulation of t cell activation	1.10E-04
9	GO:0090287	Regulation of cellular response to growth factor stimulus	1.10E-04
11	GO:0062012	Regulation of small molecule metabolic process	1.10E-04
11	GO:0043254	Regulation of protein-containing complex assembly	1.10E-04
12	GO:0043410	Positive regulation of mapk cascade	1.10E-04
5	GO:0048146	Positive regulation of fibroblast proliferation	1.20E-04
6	GO:0001938	Positive regulation of endothelial cell proliferation	1.20E-04
8	GO:1901215	Negative regulation of neuron death	1.20E-04
9	GO:0051251	Positive regulation of lymphocyte activation	1.20E-04
12	GO:0045860	Positive regulation of protein kinase activity	1.20E-04
5	GO:0001541	Ovarian follicle development	1.30E-04
5	GO:0051148	Negative regulation of muscle cell differentiation	1.40E-04
7	GO:0048762	Mesenchymal cell differentiation	1.40E-04
9	GO:0070372	Regulation of erk1 and erk2 cascade	1.40E-04
4	GO:0001782	B cell homeostasis	1.50E-04
6	GO:0022612	Gland morphogenesis	1.50E-04
12	GO:0007423	Sensory organ development	1.50E-04
3	GO:0061419	Positive regulation of transcription from rna polymerase ii promoter in response to hypoxia	1.60E-04
5	GO:1903428	Positive regulation of reactive oxygen species biosynthetic process	1.60E-04
7	GO:1902107	Positive regulation of leukocyte differentiation	1.60E-04

4	GO:0055022	Negative regulation of cardiac muscle tissue growth	1.70E-04
5	GO:0051353	Positive regulation of oxidoreductase activity	1.70E-04
12	GO:0007610	Behavior	1.70E-04
13	GO:0007346	Regulation of mitotic cell cycle	1.70E-04
8	GO:0051896	Regulation of protein kinase b signaling	1.80E-04
10	GO:0030900	Forebrain development	1.80E-04
4	GO:0060317	Cardiac epithelial to mesenchymal transition	1.90E-04
8	GO:0042063	Gliogenesis	1.90E-04
11	GO:0050769	Positive regulation of neurogenesis	1.90E-04
16	GO:0060341	Regulation of cellular localization	1.90E-04
18	GO:0051336	Regulation of hydrolase activity	1.90E-04
5	GO:0071385	Cellular response to glucocorticoid stimulus	2.00E-04
10	GO:0046649	Lymphocyte activation	2.00E-04
13	GO:0051046	Regulation of secretion	2.00E-04
46	GO:0043170	Macromolecule metabolic process	2.00E-04
3	GO:0001844	Protein insertion into mitochondrial membrane involved in apoptotic signaling pathway	2.20E-04
6	GO:2000134	Negative regulation of g1/s transition of mitotic cell cycle	2.20E-04
5	GO:0003170	Heart valve development	2.30E-04
7	GO:2000241	Regulation of reproductive process	2.30E-04
9	GO:0001655	Urogenital system development	2.40E-04
4	GO:0031069	Hair follicle morphogenesis	2.50E-04
4	GO:0060706	Cell differentiation involved in embryonic placenta development	2.50E-04
4	GO:0090200	Positive regulation of release of cytochrome c from mitochondria	2.50E-04
7	GO:0032869	Cellular response to insulin stimulus	2.50E-04
9	GO:0050900	Leukocyte migration	2.50E-04
24	GO:0090304	Nucleic acid metabolic process	2.50E-04
5	GO:0046637	Regulation of alpha-beta t cell differentiation	2.60E-04
5	GO:0046686	Response to cadmium ion	2.60E-04
6	GO:0003279	Cardiac septum development	2.60E-04
6	GO:0051341	Regulation of oxidoreductase activity	2.60E-04
7	GO:0001763	Morphogenesis of a branching structure	2.60E-04
7	GO:0003205	Cardiac chamber development	2.60E-04
4	GO:0045736	Negative regulation of cyclin-dependent protein serine/threonine kinase activity	2.70E-04
6	GO:0021782	Glial cell development	2.70E-04
8	GO:0030098	Lymphocyte differentiation	2.70E-04
49	GO:0006807	Nitrogen compound metabolic process	2.70E-04
3	GO:0032071	Regulation of endodeoxyribonuclease activity	2.80E-04
3	GO:0062043	Positive regulation of cardiac epithelial to mesenchymal transition	2.80E-04
8	GO:0042110	T cell activation	2.80E-04
13	GO:0051129	Negative regulation of cellular component organization	2.80E-04
4	GO:0051150	Regulation of smooth muscle cell differentiation	3.00E-04
5	GO:0010660	Regulation of muscle cell apoptotic process	3.10E-04
8	GO:0045165	Cell fate commitment	3.20E-04
7	GO:0010001	Glial cell differentiation	3.30E-04

8	GO:0031334	Positive regulation of protein-containing complex assembly	3.30E-04
4	GO:0032770	Positive regulation of monooxygenase activity	3.40E-04
4	GO:0046627	Negative regulation of insulin receptor signaling pathway	3.40E-04
5	GO:0045844	Positive regulation of striated muscle tissue development	3.50E-04
6	GO:0010906	Regulation of glucose metabolic process	3.50E-04
10	GO:0061564	Axon development	3.50E-04
6	GO:0042542	Response to hydrogen peroxide	3.60E-04
6	GO:2000736	Regulation of stem cell differentiation	3.60E-04
10	GO:0001558	Regulation of cell growth	3.60E-04
4	GO:0045907	Positive regulation of vasoconstriction	3.70E-04
4	GO:1902692	Regulation of neuroblast proliferation	3.70E-04
6	GO:0035270	Endocrine system development	3.70E-04
7	GO:0046328	Regulation of jnk cascade	3.80E-04
5	GO:0001837	Epithelial to mesenchymal transition	3.90E-04
6	GO:0010822	Positive regulation of mitochondrion organization	3.90E-04
8	GO:2000027	Regulation of animal organ morphogenesis	3.90E-04
12	GO:1903530	Regulation of secretion by cell	3.90E-04
8	GO:0050730	Regulation of peptidyl-tyrosine phosphorylation	4.00E-04
13	GO:0001817	Regulation of cytokine production	4.00E-04
5	GO:0008630	Intrinsic apoptotic signaling pathway in response to dna damage	4.10E-04
5	GO:1903524	Positive regulation of blood circulation	4.10E-04
8	GO:0007611	Learning or memory	4.10E-04
12	GO:0009617	Response to bacterium	4.10E-04
5	GO:0003208	Cardiac ventricle morphogenesis	4.30E-04
5	GO:0014015	Positive regulation of gliogenesis	4.30E-04
7	GO:0030308	Negative regulation of cell growth	4.30E-04
4	GO:0003203	Endocardial cushion morphogenesis	4.40E-04
4	GO:0070059	Intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	4.40E-04
7	GO:0042593	Glucose homeostasis	4.40E-04
3	GO:0045899	Positive regulation of rna polymerase ii transcription preinitiation complex assembly	4.50E-04
3	GO:0048103	Somatic stem cell division	4.50E-04
5	GO:0003151	Outflow tract morphogenesis	4.50E-04
5	GO:0003281	Ventricular septum development	4.50E-04
5	GO:0001942	Hair follicle development	4.70E-04
5	GO:0060411	Cardiac septum morphogenesis	4.70E-04
4	GO:0017145	Stem cell division	4.80E-04
4	GO:1904707	Positive regulation of vascular associated smooth muscle cell proliferation	4.80E-04
6	GO:0030326	Embryonic limb morphogenesis	4.80E-04
11	GO:0044089	Positive regulation of cellular component biogenesis	4.80E-04
13	GO:0010564	Regulation of cell cycle process	4.90E-04
6	GO:0003206	Cardiac chamber morphogenesis	5.00E-04
6	GO:0003231	Cardiac ventricle development	5.10E-04
6	GO:1905477	Positive regulation of protein localization to membrane	5.10E-04
11	GO:0006935	Chemotaxis	5.10E-04

26	GO:0044249	Cellular biosynthetic process	5.10E-04
3	GO:1902237	Positive regulation of endoplasmic reticulum stress-induced intrinsic apoptotic signaling pathway	5.50E-04
6	GO:0050680	Negative regulation of epithelial cell proliferation	5.50E-04
10	GO:0007005	Mitochondrion organization	5.60E-04
13	GO:0051345	Positive regulation of hydrolase activity	5.60E-04
4	GO:0002437	Inflammatory response to antigenic stimulus	5.70E-04
7	GO:1902275	Regulation of chromatin organization	5.70E-04
9	GO:0051052	Regulation of dna metabolic process	5.70E-04
6	GO:0008584	Male gonad development	5.90E-04
14	GO:0050776	Regulation of immune response	5.90E-04
4	GO:0010907	Positive regulation of glucose metabolic process	6.20E-04
5	GO:0045913	Positive regulation of carbohydrate metabolic process	6.40E-04
6	GO:0019827	Stem cell population maintenance	6.40E-04
8	GO:0007162	Negative regulation of cell adhesion	6.50E-04
8	GO:0014706	Striated muscle tissue development	6.50E-04
8	GO:0042493	Response to drug	6.60E-04
4	GO:0031018	Endocrine pancreas development	6.80E-04
4	GO:0048066	Developmental pigmentation	6.80E-04
4	GO:0051205	Protein insertion into membrane	6.80E-04
4	GO:0071634	Regulation of transforming growth factor beta production	6.80E-04
4	GO:1904646	Cellular response to amyloid-beta	6.80E-04
4	GO:1905898	Positive regulation of response to endoplasmic reticulum stress	6.80E-04
12	GO:0031347	Regulation of defense response	6.80E-04
5	GO:0034644	Cellular response to uv	7.40E-04
8	GO:0050768	Negative regulation of neurogenesis	7.50E-04
6	GO:0007050	Cell cycle arrest	7.80E-04
17	GO:0007049	Cell cycle	7.80E-04
4	GO:0007566	Embryo implantation	8.00E-04
4	GO:0071548	Response to dexamethasone	8.00E-04
6	GO:0030595	Leukocyte chemotaxis	8.10E-04
4	GO:0042149	Cellular response to glucose starvation	8.60E-04
4	GO:0060412	Ventricular septum morphogenesis	8.60E-04
10	GO:0061061	Muscle structure development	8.60E-04
6	GO:0062013	Positive regulation of small molecule metabolic process	8.70E-04
9	GO:0007409	Axonogenesis	8.90E-04
5	GO:0008286	Insulin receptor signaling pathway	9.00E-04
5	GO:0014068	Positive regulation of phosphatidylinositol 3-kinase signaling	9.00E-04
5	GO:2000106	Regulation of leukocyte apoptotic process	9.00E-04
6	GO:0010212	Response to ionizing radiation	9.00E-04
7	GO:0006109	Regulation of carbohydrate metabolic process	9.10E-04
27	GO:0006725	Cellular aromatic compound metabolic process	9.10E-04
4	GO:2000772	Regulation of cellular senescence	9.20E-04
3	GO:0030949	Positive regulation of vascular endothelial growth factor receptor signaling pathway	9.40E-04
3	GO:0045722	Positive regulation of gluconeogenesis	9.40E-04

3	GO:0060038	Cardiac muscle cell proliferation	9.40E-04
3	GO:0060253	Negative regulation of glial cell proliferation	9.40E-04
3	GO:0060707	Trophoblast giant cell differentiation	9.40E-04
3	GO:0070243	Regulation of thymocyte apoptotic process	9.40E-04
3	GO:1900103	Positive regulation of endoplasmic reticulum unfolded protein response	9.40E-04
3	GO:1905063	Regulation of vascular associated smooth muscle cell differentiation	9.40E-04
6	GO:0016458	Gene silencing	9.40E-04
6	GO:0045580	Regulation of t cell differentiation	9.40E-04
9	GO:0045787	Positive regulation of cell cycle	9.40E-04
15	GO:0019438	Aromatic compound biosynthetic process	9.50E-04
4	GO:0045429	Positive regulation of nitric oxide biosynthetic process	9.90E-04
4	GO:0055025	Positive regulation of cardiac muscle tissue development	9.90E-04
5	GO:0001678	Cellular glucose homeostasis	1.00E-03
26	GO:1901576	Organic substance biosynthetic process	1.00E-03
3	GO:0048070	Regulation of developmental pigmentation	1.10E-03
3	GO:0060044	Negative regulation of cardiac muscle cell proliferation	1.10E-03
3	GO:0090343	Positive regulation of cell aging	1.10E-03
3	GO:1902043	Positive regulation of extrinsic apoptotic signaling pathway via death domain receptors	1.10E-03
4	GO:0051402	Neuron apoptotic process	1.10E-03
6	GO:0031056	Regulation of histone modification	1.10E-03
7	GO:0061448	Connective tissue development	1.10E-03
7	GO:0006839	Mitochondrial transport	1.10E-03
7	GO:0043583	Ear development	1.10E-03
7	GO:2001020	Regulation of response to dna damage stimulus	1.10E-03
8	GO:0045930	Negative regulation of mitotic cell cycle	1.10E-03
9	GO:0022411	Cellular component disassembly	1.10E-03
29	GO:0043412	Macromolecule modification	1.10E-03
30	GO:0006996	Organelle organization	1.10E-03
3	GO:0006309	Apoptotic dna fragmentation	1.20E-03
5	GO:0031058	Positive regulation of histone modification	1.20E-03
5	GO:1901655	Cellular response to ketone	1.20E-03
8	GO:1903532	Positive regulation of secretion by cell	1.20E-03
29	GO:0034641	Cellular nitrogen compound metabolic process	1.20E-03
4	GO:0048546	Digestive tract morphogenesis	1.30E-03
5	GO:0014020	Primary neural tube formation	1.30E-03
5	GO:0043255	Regulation of carbohydrate biosynthetic process	1.30E-03
6	GO:0031099	Regeneration	1.30E-03
6	GO:0001816	Cytokine production	1.30E-03
6	GO:0061138	Morphogenesis of a branching epithelium	1.30E-03
8	GO:0001525	Angiogenesis	1.30E-03
8	GO:0009895	Negative regulation of catabolic process	1.30E-03
14	GO:0022402	Cell cycle process	1.30E-03
18	GO:0044271	Cellular nitrogen compound biosynthetic process	1.30E-03
4	GO:0045646	Regulation of erythrocyte differentiation	1.40E-03

4	GO:0046622	Positive regulation of organ growth	1.40E-03
6	GO:0021915	Neural tube development	1.40E-03
6	GO:0048738	Cardiac muscle tissue development	1.40E-03
4	GO:0035094	Response to nicotine	1.50E-03
5	GO:0045639	Positive regulation of myeloid cell differentiation	1.50E-03
5	GO:2001243	Negative regulation of intrinsic apoptotic signaling pathway	1.50E-03
6	GO:0006367	Transcription initiation from rna polymerase ii promoter	1.50E-03
9	GO:1901990	Regulation of mitotic cell cycle phase transition	1.50E-03
14	GO:0034654	Nucleobase-containing compound biosynthetic process	1.50E-03
3	GO:1904706	Negative regulation of vascular associated smooth muscle cell proliferation	1.60E-03
4	GO:0033077	T cell differentiation in thymus	1.60E-03
8	GO:0010948	Negative regulation of cell cycle process	1.60E-03
25	GO:0006139	Nucleobase-containing compound metabolic process	1.60E-03
4	GO:0003179	Heart valve morphogenesis	1.70E-03
5	GO:0008593	Regulation of notch signaling pathway	1.70E-03
6	GO:0043409	Negative regulation of mapk cascade	1.70E-03
2	GO:0097324	Melanocyte migration	1.80E-03
3	GO:0003215	Cardiac right ventricle morphogenesis	1.80E-03
3	GO:0050765	Negative regulation of phagocytosis	1.80E-03
3	GO:1902176	Negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway	1.80E-03
5	GO:0062014	Negative regulation of small molecule metabolic process	1.80E-03
6	GO:0030324	Lung development	1.80E-03
6	GO:0071478	Cellular response to radiation	1.80E-03
8	GO:0050708	Regulation of protein secretion	1.80E-03
10	GO:0009611	Response to wounding	1.80E-03
5	GO:0048640	Negative regulation of developmental growth	1.90E-03
5	GO:2001237	Negative regulation of extrinsic apoptotic signaling pathway	1.90E-03
6	GO:0021543	Pallium development	1.90E-03
6	GO:0051216	Cartilage development	1.90E-03
9	GO:0007389	Pattern specification process	1.90E-03
4	GO:0048708	Astrocyte differentiation	2.00E-03
6	GO:0016241	Regulation of macroautophagy	2.00E-03
9	GO:0051090	Regulation of dna-binding transcription factor activity	2.00E-03
3	GO:1904996	Positive regulation of leukocyte adhesion to vascular endothelial cell	2.10E-03
4	GO:0070228	Regulation of lymphocyte apoptotic process	2.10E-03
5	GO:0097549	Chromatin organization involved in negative regulation of transcription	2.10E-03
5	GO:0021761	Limbic system development	2.10E-03
9	GO:0042060	Wound healing	2.10E-03
4	GO:0043551	Regulation of phosphatidylinositol 3-kinase activity	2.20E-03
4	GO:2000378	Negative regulation of reactive oxygen species metabolic process	2.20E-03
4	GO:2000242	Negative regulation of reproductive process	2.30E-03
8	GO:0071902	Positive regulation of protein serine/threonine kinase activity	2.30E-03
7	GO:0045637	Regulation of myeloid cell differentiation	2.40E-03
8	GO:0050727	Regulation of inflammatory response	2.40E-03

4	GO:1902041	Regulation of extrinsic apoptotic signaling pathway via death domain receptors	2.50E-03
6	GO:0030177	Positive regulation of wnt signaling pathway	2.50E-03
8	GO:0048871	Multicellular organismal homeostasis	2.50E-03
11	GO:0007276	Gamete generation	2.50E-03
11	GO:0030855	Epithelial cell differentiation	2.50E-03
3	GO:0001836	Release of cytochrome c from mitochondria	2.60E-03
3	GO:0035162	Embryonic hemopoiesis	2.60E-03
3	GO:0051000	Positive regulation of nitric-oxide synthase activity	2.60E-03
6	GO:0022408	Negative regulation of cell-cell adhesion	2.60E-03
6	GO:2001252	Positive regulation of chromosome organization	2.60E-03
7	GO:0001503	Ossification	2.60E-03
13	GO:0007155	Cell adhesion	2.60E-03
4	GO:0032722	Positive regulation of chemokine production	2.70E-03
4	GO:2000514	Regulation of cd4-positive, alpha-beta t cell activation	2.70E-03
6	GO:0071222	Cellular response to lipopolysaccharide	2.70E-03
8	GO:0033044	Regulation of chromosome organization	2.70E-03
10	GO:0000904	Cell morphogenesis involved in differentiation	2.70E-03
49	GO:0044237	Cellular metabolic process	2.70E-03
2	GO:0046668	Regulation of retinal cell programmed cell death	2.80E-03
2	GO:1900147	Regulation of schwann cell migration	2.80E-03
2	GO:1902263	Apoptotic process involved in embryonic digit morphogenesis	2.80E-03
2	GO:1903984	Positive regulation of trail-activated apoptotic signaling pathway	2.80E-03
2	GO:2000619	Negative regulation of histone h4-k16 acetylation	2.80E-03
3	GO:0034391	Regulation of smooth muscle cell apoptotic process	2.80E-03
3	GO:0043586	Tongue development	2.80E-03
3	GO:0097150	Neuronal stem cell population maintenance	2.80E-03
3	GO:1903798	Regulation of production of mirnas involved in gene silencing by mirna	2.80E-03
5	GO:0045667	Regulation of osteoblast differentiation	2.80E-03
7	GO:0046883	Regulation of hormone secretion	2.80E-03
7	GO:0034612	Response to tumor necrosis factor	2.80E-03
10	GO:1903827	Regulation of cellular protein localization	2.80E-03
20	GO:0003008	System process	2.80E-03
27	GO:1901360	Organic cyclic compound metabolic process	2.80E-03
7	GO:0001822	Kidney development	2.90E-03
7	GO:0051091	Positive regulation of dna-binding transcription factor activity	2.90E-03
12	GO:0019953	Sexual reproduction	2.90E-03
50	GO:0071704	Organic substance metabolic process	2.90E-03
4	GO:0050805	Negative regulation of synaptic transmission	3.00E-03
4	GO:1905268	Negative regulation of chromatin organization	3.00E-03
5	GO:0007219	Notch signaling pathway	3.00E-03
5	GO:0042476	Odontogenesis	3.00E-03
14	GO:0001775	Cell activation	3.00E-03
15	GO:1901362	Organic cyclic compound biosynthetic process	3.00E-03
3	GO:0003181	Atrioventricular valve morphogenesis	3.10E-03

3	GO:0051570	Regulation of histone h3-k9 methylation	3.10E-03
4	GO:0060135	Maternal process involved in female pregnancy	3.10E-03
5	GO:0019218	Regulation of steroid metabolic process	3.10E-03
8	GO:0032102	Negative regulation of response to external stimulus	3.10E-03
11	GO:0000278	Mitotic cell cycle	3.10E-03
6	GO:0002064	Epithelial cell development	3.40E-03
6	GO:0050731	Positive regulation of peptidyl-tyrosine phosphorylation	3.40E-03
6	GO:0046890	Regulation of lipid biosynthetic process	3.50E-03
7	GO:0016579	Protein deubiquitination	3.50E-03
4	GO:1903201	Regulation of oxidative stress-induced cell death	3.60E-03
8	GO:0045862	Positive regulation of proteolysis	3.60E-03
6	GO:0060348	Bone development	3.70E-03
3	GO:0010614	Negative regulation of cardiac muscle hypertrophy	3.80E-03
3	GO:0060045	Positive regulation of cardiac muscle cell proliferation	3.80E-03
3	GO:0071887	Leukocyte apoptotic process	3.80E-03
3	GO:0090344	Negative regulation of cell aging	3.80E-03
4	GO:0070828	Heterochromatin organization	3.80E-03
2	GO:0002051	Osteoblast fate commitment	3.90E-03
2	GO:0003274	Endocardial cushion fusion	3.90E-03
2	GO:0061082	Myeloid leukocyte cytokine production	3.90E-03
2	GO:1904684	Negative regulation of metalloendopeptidase activity	3.90E-03
4	GO:0071230	Cellular response to amino acid stimulus	3.90E-03
5	GO:1903038	Negative regulation of leukocyte cell-cell adhesion	3.90E-03
6	GO:0040029	Regulation of gene expression, epigenetic	3.90E-03
8	GO:0043588	Skin development	3.90E-03
3	GO:0003148	Outflow tract septum morphogenesis	4.10E-03
3	GO:0010661	Positive regulation of muscle cell apoptotic process	4.10E-03
3	GO:1900740	Positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway	4.10E-03
4	GO:0003143	Embryonic heart tube morphogenesis	4.10E-03
4	GO:0061035	Regulation of cartilage development	4.10E-03
7	GO:0060828	Regulation of canonical wnt signaling pathway	4.10E-03
5	GO:0048754	Branching morphogenesis of an epithelial tube	4.20E-03
6	GO:0006338	Chromatin remodeling	4.20E-03
4	GO:0045739	Positive regulation of dna repair	4.30E-03
4	GO:0070301	Cellular response to hydrogen peroxide	4.30E-03
5	GO:0046887	Positive regulation of hormone secretion	4.30E-03
7	GO:0031647	Regulation of protein stability	4.30E-03
7	GO:0090068	Positive regulation of cell cycle process	4.30E-03
3	GO:0038179	Neurotrophin signaling pathway	4.40E-03
4	GO:0001570	Vasculogenesis	4.40E-03
5	GO:0045598	Regulation of fat cell differentiation	4.40E-03
5	GO:0046425	Regulation of receptor signaling pathway via jak-stat	4.40E-03
8	GO:0010959	Regulation of metal ion transport	4.40E-03
14	GO:0048878	Chemical homeostasis	4.40E-03

6	GO:0070374	Positive regulation of erk1 and erk2 cascade	4.50E-03
5	GO:0050671	Positive regulation of lymphocyte proliferation	4.60E-03
8	GO:0010639	Negative regulation of organelle organization	4.60E-03
9	GO:0098609	Cell-cell adhesion	4.80E-03
4	GO:0010827	Regulation of glucose transmembrane transport	4.90E-03
4	GO:0072655	Establishment of protein localization to mitochondrion	4.90E-03
5	GO:0043280	Positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	4.90E-03
4	GO:0030279	Negative regulation of ossification	5.10E-03
9	GO:0050878	Regulation of body fluid levels	5.10E-03
2	GO:0034349	Glial cell apoptotic process	5.20E-03
2	GO:0045636	Positive regulation of melanocyte differentiation	5.20E-03
2	GO:0051097	Negative regulation of helicase activity	5.20E-03
2	GO:1905007	Positive regulation of epithelial to mesenchymal transition involved in endocardial cushion formation	5.20E-03
3	GO:0033032	Regulation of myeloid cell apoptotic process	5.20E-03
3	GO:0045737	Positive regulation of cyclin-dependent protein serine/threonine kinase activity	5.20E-03
3	GO:0048873	Homeostasis of number of cells within a tissue	5.20E-03
3	GO:2000108	Positive regulation of leukocyte apoptotic process	5.20E-03
4	GO:0008306	Associative learning	5.20E-03
4	GO:1903747	Regulation of establishment of protein localization to mitochondrion	5.20E-03
5	GO:0097746	Regulation of blood vessel diameter	5.20E-03
6	GO:0043281	Regulation of cysteine-type endopeptidase activity involved in apoptotic process	5.20E-03
11	GO:0030162	Regulation of proteolysis	5.20E-03
17	GO:0016070	RNA metabolic process	5.20E-03
6	GO:0050670	Regulation of lymphocyte proliferation	5.40E-03
11	GO:0055082	Cellular chemical homeostasis	5.40E-03
9	GO:0090066	Regulation of anatomical structure size	5.50E-03
3	GO:0071549	Cellular response to dexamethasone stimulus	5.60E-03
13	GO:0070647	Protein modification by small protein conjugation or removal	5.70E-03
4	GO:0042509	Regulation of tyrosine phosphorylation of stat protein	5.90E-03
4	GO:0071260	Cellular response to mechanical stimulus	5.90E-03
6	GO:0071241	Cellular response to inorganic substance	5.90E-03
3	GO:0045648	Positive regulation of erythrocyte differentiation	6.00E-03
3	GO:0061036	Positive regulation of cartilage development	6.00E-03
3	GO:1902230	Negative regulation of intrinsic apoptotic signaling pathway in response to dna damage	6.00E-03
3	GO:1902253	Regulation of intrinsic apoptotic signaling pathway by p53 class mediator	6.00E-03
8	GO:0042176	Regulation of protein catabolic process	6.00E-03
47	GO:0044238	Primary metabolic process	6.10E-03
4	GO:0014031	Mesenchymal cell development	6.20E-03
5	GO:0033135	Regulation of peptidyl-serine phosphorylation	6.30E-03
8	GO:0052548	Regulation of endopeptidase activity	6.30E-03
3	GO:0071354	Cellular response to interleukin-6	6.40E-03
3	GO:0090398	Cellular senescence	6.40E-03
4	GO:0021766	Hippocampus development	6.40E-03
4	GO:0042475	Odontogenesis of dentin-containing tooth	6.40E-03

8	GO:0008544	Epidermis development	6.40E-03
2	GO:0002326	B cell lineage commitment	6.50E-03
2	GO:0006501	C-terminal protein lipidation	6.50E-03
2	GO:0007406	Negative regulation of neuroblast proliferation	6.50E-03
2	GO:0010626	Negative regulation of schwann cell proliferation	6.50E-03
2	GO:0051136	Regulation of nk t cell differentiation	6.50E-03
2	GO:0070141	Response to uv-a	6.50E-03
2	GO:0070245	Positive regulation of thymocyte apoptotic process	6.50E-03
4	GO:0010507	Negative regulation of autophagy	6.60E-03
4	GO:0043502	Regulation of muscle adaptation	6.60E-03
4	GO:1901224	Positive regulation of nik/nf-kappab signaling	6.60E-03
3	GO:0007202	Activation of phospholipase c activity	6.80E-03
3	GO:0031663	Lipopolysaccharide-mediated signaling pathway	6.80E-03
3	GO:0034405	Response to fluid shear stress	6.80E-03
3	GO:0060969	Negative regulation of gene silencing	6.80E-03
3	GO:0110111	Negative regulation of animal organ morphogenesis	6.80E-03
3	GO:1901797	Negative regulation of signal transduction by p53 class mediator	6.80E-03
3	GO:2000516	Positive regulation of cd4-positive, alpha-beta t cell activation	6.80E-03
5	GO:0030856	Regulation of epithelial cell differentiation	7.10E-03
5	GO:1905952	Regulation of lipid localization	7.10E-03
5	GO:0045834	Positive regulation of lipid metabolic process	7.20E-03
6	GO:0097305	Response to alcohol	7.20E-03
3	GO:0043516	Regulation of dna damage response, signal transduction by p53 class mediator	7.30E-03
4	GO:0010631	Epithelial cell migration	7.30E-03
4	GO:0046427	Positive regulation of receptor signaling pathway via jak-stat	7.30E-03
51	GO:0008152	Metabolic process	7.30E-03
3	GO:0001893	Maternal placenta development	7.80E-03
3	GO:0014065	Phosphatidylinositol 3-kinase signaling	7.80E-03
3	GO:0060416	Response to growth hormone	7.80E-03
4	GO:1901532	Regulation of hematopoietic progenitor cell differentiation	7.90E-03
4	GO:1904035	Regulation of epithelial cell apoptotic process	7.90E-03
6	GO:0021700	Developmental maturation	7.90E-03
2	GO:0001547	Antral ovarian follicle growth	8.00E-03
2	GO:0035234	Ectopic germ cell programmed cell death	8.00E-03
5	GO:0045931	Positive regulation of mitotic cell cycle	8.00E-03
5	GO:1903707	Negative regulation of hemopoiesis	8.00E-03
12	GO:0045321	Leukocyte activation	8.00E-03
4	GO:0045778	Positive regulation of ossification	8.10E-03
6	GO:0045732	Positive regulation of protein catabolic process	8.10E-03
3	GO:0014002	Astrocyte development	8.30E-03
3	GO:0051154	Negative regulation of striated muscle cell differentiation	8.30E-03
3	GO:0071276	Cellular response to cadmium ion	8.30E-03
3	GO:1905314	Semi-lunar valve development	8.30E-03
3	GO:2001240	Negative regulation of extrinsic apoptotic signaling pathway in absence of ligand	8.30E-03

4	GO:0003158	Endothelium development	8.40E-03
4	GO:0045582	Positive regulation of t cell differentiation	8.40E-03
4	GO:0050810	Regulation of steroid biosynthetic process	8.40E-03
5	GO:0007369	Gastrulation	8.50E-03
3	GO:0007259	Receptor signaling pathway via jak-stat	8.70E-03
3	GO:0043491	Protein kinase b signaling	8.70E-03
4	GO:0001843	Neural tube closure	8.70E-03
4	GO:0002244	Hematopoietic progenitor cell differentiation	8.70E-03
4	GO:0030509	BMP signaling pathway	8.70E-03
4	GO:0048588	Developmental cell growth	8.70E-03
6	GO:0090092	Regulation of transmembrane receptor protein serine/threonine kinase signaling pathway	8.70E-03
9	GO:0034762	Regulation of transmembrane transport	8.70E-03
4	GO:0070542	Response to fatty acid	8.90E-03
5	GO:0043467	Regulation of generation of precursor metabolites and energy	8.90E-03
7	GO:1903829	Positive regulation of cellular protein localization	8.90E-03
8	GO:0002683	Negative regulation of immune system process	9.20E-03
4	GO:0050764	Regulation of phagocytosis	9.30E-03
5	GO:0032874	Positive regulation of stress-activated mapk cascade	9.50E-03
12	GO:0006468	Protein phosphorylation	9.50E-03
2	GO:0001955	Blood vessel maturation	9.60E-03
2	GO:0032909	Regulation of transforming growth factor beta2 production	9.60E-03
2	GO:0060353	Regulation of cell adhesion molecule production	9.60E-03
2	GO:0090400	Stress-induced premature senescence	9.60E-03
2	GO:1903896	Positive regulation of ire1-mediated unfolded protein response	9.60E-03
2	GO:1905064	Negative regulation of vascular associated smooth muscle cell differentiation	9.60E-03
2	GO:1990314	Cellular response to insulin-like growth factor stimulus	9.60E-03
5	GO:0042129	Regulation of t cell proliferation	9.70E-03
6	GO:0043122	Regulation of i-kappab kinase/nf-kappab signaling	9.70E-03
3	GO:0000422	Autophagy of mitochondrion	9.80E-03
3	GO:0071392	Cellular response to estradiol stimulus	9.80E-03

Supplementary table 9: Gene Ontology (GO) Cellular Component enrichment analysis for potentially downregulated proteins. Only the terms with FDR p <0.01 were considered.

Number of genes	Term name	Description	FDR value
48	GO:0005654	Nucleoplasm	2,88E-10
50	GO:0031981	Nuclear lumen	5,57E-09
54	GO:0070013	Intracellular organelle lumen	5,29E-08
61	GO:0005634	Nucleus	5,29E-08
24	GO:0000785	Chromatin	5,45E-08
21	GO:0000790	Nuclear chromatin	6,04E-07
48	GO:0005829	Cytosol	6,73E-07
46	GO:0032991	Protein-containing complex	3,53E-06
13	GO:0005667	Transcription regulator complex	7,56E-06

69	GO:0043231	Intracellular membrane-bounded organelle	1,99E-05
70	GO:0005737	Cytoplasm	1,10E-04
73	GO:0043227	Membrane-bounded organelle	1,70E-04
41	GO:0043232	Intracellular non-membrane-bounded organelle	2,10E-04
73	GO:0043229	Intracellular organelle	2,20E-04
7	GO:0090575	RNA polymerase II transcription regulator complex	7,00E-04
4	GO:0005942	Phosphatidylinositol 3-kinase complex	7,20E-04
5	GO:0000792	Heterochromatin	1,90E-03
7	GO:0005741	Mitochondrial outer membrane	1,90E-03
74	GO:0043226	Organelle	2,90E-03
13	GO:0070161	Anchoring junction	3,40E-03
10	GO:0005911	Cell-cell junction	3,50E-03
76	GO:0005622	Intracellular	4,00E-03
2	GO:0034274	Atg12-Atg5-Atg16 complex	6,10E-03

Supplementary table 10: Gene Ontology (GO) Molecular Function data enrichment analysis for potentially downregulated proteins. Only the terms with FDR p <0.01 were considered.

Number of genes	Term name	Description	FDR value
46	GO:0098772	Molecular function regulator	5,31E-11
64	GO:0005515	Protein binding	1,11E-10
28	GO:0043565	Sequence-specific dna binding	9,43E-10
35	GO:0019899	Enzyme binding	1,78E-09
24	GO:0001067	Regulatory region nucleic acid binding	4,61E-09
17	GO:0001228	DNA-binding transcription activator activity, RNA polymerase II-specific	5,68E-09
24	GO:1990837	Sequence-specific double-stranded dna binding	5,68E-09
25	GO:0003690	Double-stranded dna binding	5,68E-09
20	GO:0000987	Cis-regulatory region sequence-specific dna binding	5,76E-09
29	GO:0140110	Transcription regulator activity	7,65E-09
23	GO:0000976	Transcription regulatory region sequence-specific dna binding	1,32E-08
19	GO:0000978	RNA polymerase II cis-regulatory region sequence-specific DNA binding	1,80E-08
22	GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific	6,74E-08
18	GO:0019901	Protein kinase binding	7,94E-08
19	GO:0019900	Kinase binding	7,94E-08
18	GO:0008134	Transcription factor binding	1,10E-07
20	GO:0000977	RNA polymerase II transcription regulatory region sequence-specific DNA binding	1,42E-07
33	GO:0003677	DNA binding	1,42E-07
78	GO:0005488	Binding	1,42E-07
18	GO:0019904	Protein domain specific binding	2,37E-07
23	GO:0003700	DNA-binding transcription factor activity	2.57E-07
12	GO:0140297	DNA-binding transcription factor binding	9,87E-06

14	GO:0003682	Chromatin binding	2,27E-05
38	GO:0003676	Nucleic acid binding	2,73E-05
5	GO:0035035	Histone acetyltransferase binding	3,98E-05
22	GO:0005102	Signaling receptor binding	7,90E-05
46	GO:1901363	Heterocyclic compound binding	2,00E-04
8	GO:0019902	Phosphatase binding	2,70E-04
5	GO:0001221	Transcription cofactor binding	2,90E-04
46	GO:0097159	Organic cyclic compound binding	2,90E-04
9	GO:0061629	RNA polymerase II-specific DNA-binding transcription factor binding	4,40E-04
7	GO:0019903	Protein phosphatase binding	4,60E-04
4	GO:0001223	Transcription coactivator binding	8,50E-04
5	GO:0004860	Protein kinase inhibitor activity	1,10E-03
4	GO:0051721	Protein phosphatase 2a binding	1,40E-03
17	GO:0044877	Protein-containing complex binding	1,40E-03
5	GO:0070491	Repressing transcription factor binding	1,70E-03
3	GO:0004861	Cyclin-dependent protein serine/threonine kinase inhibitor activity	2,70E-03
6	GO:0002020	Protease binding	2,80E-03
15	GO:0030234	Enzyme regulator activity	2,90E-03
5	GO:0008013	Beta-catenin binding	3,30E-03
7	GO:0019207	Kinase regulator activity	3,50E-03
3	GO:0046935	1-phosphatidylinositol-3-kinase regulator activity	4,20E-03
4	GO:0016538	Cyclin-dependent protein serine/threonine kinase regulator activity	5,10E-03

Supplementary table 11: KEGG pathway enrichment analysis for potentially upregulated proteins. Only the terms with FDR p <0.01 were considered.

Number of genes	Term name	Description	FDR value
24	hsa05206	MicroRNAs in cancer	5,87E-27
26	hsa05200	Pathways in cancer	2,20E-18
12	hsa04933	AGE-RAGE signaling pathway in diabetic complications	4,63E-12
13	hsa05162	Measles	6,35E-12
11	hsa01522	Endocrine resistance	5,95E-11
12	hsa04218	Cellular senescence	2,48E-10
10	hsa05210	Colorectal cancer	3,06E-10
10	hsa05222	Small cell lung cancer	6,90E-10
11	hsa04068	FoxO signaling pathway	6,90E-10
10	hsa05215	Prostate cancer	9,01E-10
15	hsa04151	PI3K-Akt signaling pathway	1,15E-09
11	hsa05224	Breast cancer	1,75E-09
11	hsa05161	Hepatitis B	4,08E-09
10	hsa04915	Estrogen signaling pathway	1,27E-08

11	hsa05169	Epstein-Barr virus infection	2,47E-08
8	hsa04917	Prolactin signaling pathway	2,71E-08
11	hsa05205	Proteoglycans in cancer	2,71E-08
8	hsa04115	p53 signaling pathway	3,34E-08
9	hsa04668	TNF signaling pathway	4,16E-08
11	hsa05166	Human T-cell leukemia virus 1 infection	4,52E-08
8	hsa01521	EGFR tyrosine kinase inhibitor resistance	5,14E-08
11	hsa05131	Shigellosis	5,69E-08
11	hsa05163	Human cytomegalovirus infection	5,69E-08
8	hsa04211	Longevity regulating pathway	9,99E-08
9	hsa04140	Autophagy - animal	1,08E-07
9	hsa05418	Fluid shear stress and atherosclerosis	1,08E-07
6	hsa04215	Apoptosis - multiple species	1,11E-07
9	hsa04210	Apoptosis	1,11E-07
10	hsa05167	Kaposi sarcoma-associated herpesvirus infection	1,37E-07
7	hsa04137	Mitophagy - animal	2,18E-07
12	hsa05165	Human papillomavirus infection	2,18E-07
9	hsa04932	Non-alcoholic fatty liver disease	2,44E-07
7	hsa05223	Non-small cell lung cancer	3,23E-07
7	hsa05230	Central carbon metabolism in cancer	3,44E-07
9	hsa05160	Hepatitis C	3,44E-07
7	hsa01524	Platinum drug resistance	3,57E-07
7	hsa05220	Chronic myeloid leukemia	5,39E-07
8	hsa04935	Growth hormone synthesis, secretion and action	5,86E-07
8	hsa04380	Osteoclast differentiation	7,28E-07
8	hsa04926	Relaxin signaling pathway	1,01E-06
6	hsa05213	Endometrial cancer	2,32E-06
8	hsa05226	Gastric cancer	2,32E-06
6	hsa04213	Longevity regulating pathway - multiple species	3,22E-06
7	hsa04928	Parathyroid hormone synthesis, secretion and action	3,45E-06
7	hsa04066	HIF-1 signaling pathway	4,05E-06
7	hsa04931	Insulin resistance	4,15E-06
10	hsa04010	MAPK signaling pathway	4,15E-06
8	hsa04630	JAK-STAT signaling pathway	4,24E-06
8	hsa05164	Influenza A	5,19E-06
7	hsa04071	Sphingolipid signaling pathway	6,47E-06
8	hsa05202	Transcriptional misregulation in cancer	6,47E-06
6	hsa05214	Glioma	6,61E-06
6	hsa05218	Melanoma	6,61E-06
6	hsa05212	Pancreatic cancer	6,86E-06
7	hsa04919	Thyroid hormone signaling pathway	6,93E-06
7	hsa04110	Cell cycle	7,17E-06
8	hsa05203	Viral carcinogenesis	9,05E-06
6	hsa05235	PD-L1 expression and PD-1 checkpoint pathway in cancer	1,78E-05
8	hsa04024	cAMP signaling pathway	2,27E-05

6	hsa05231	Choline metabolism in cancer	2,77E-05
7	hsa04934	Cushing syndrome	3,07E-05
6	hsa05142	Chagas disease	3,17E-05
6	hsa04659	Th17 cell differentiation	3,48E-05
7	hsa05225	Hepatocellular carcinoma	3,88E-05
7	hsa05152	Tuberculosis	5,18E-05
6	hsa04725	Cholinergic synapse	5,29E-05
6	hsa04722	Neurotrophin signaling pathway	6,32E-05
6	hsa04152	AMPK signaling pathway	8,23E-05
7	hsa05170	Human immunodeficiency virus 1 infection	1,60E-04
5	hsa04012	ErbB signaling pathway	1,80E-04
8	hsa05016	Huntington disease	2,30E-04
7	hsa04014	Ras signaling pathway	2,90E-04
5	hsa04922	Glucagon signaling pathway	4,20E-04
5	hsa05145	Toxoplasmosis	4,90E-04
4	hsa05211	Renal cell carcinoma	1,00E-03
4	hsa05221	Acute myeloid leukemia	1,00E-03
4	hsa04520	Adherens junction	1,10E-03
6	hsa04015	Rap1 signaling pathway	1,20E-03
5	hsa04910	Insulin signaling pathway	1,30E-03
3	hsa04136	Autophagy - other	1,40E-03
4	hsa05133	Pertussis	1,40E-03
3	hsa05143	African trypanosomiasis	2.50E-03
4	hsa04914	Progesterone-mediated oocyte maturation	3,30E-03
3	hsa05219	Bladder cancer	3,50E-03
7	hsa05010	Alzheimer disease	3,60E-03
4	hsa04620	Toll-like receptor signaling pathway	4,20E-03
6	hsa05020	Prion disease	4,20E-03
8	hsa05168	Herpes simplex virus 1 infection	4,30E-03
3	hsa04930	Type II diabetes mellitus	4,50E-03
3	hsa05144	Malaria	4,50E-03
5	hsa04062	Chemokine signaling pathway	5,00E-03
4	hsa04670	Leukocyte transendothelial migration	5,10E-03
3	hsa05030	Cocaine addiction	5,20E-03
5	hsa04510	Focal adhesion	6,40E-03
3	hsa04923	Regulation of lipolysis in adipocytes	6,60E-03
3	hsa05134	Legionellosis	6,90E-03
4	hsa05135	Yersinia infection	7,80E-03
5	hsa05132	Salmonella infection	7,80E-03
4	hsa04728	Dopaminergic synapse	8,40E-03

Supplementary table 12: Reactome pathway enrichment analysis for potentially upregulated proteins. Only the terms with FDR p <0.01 were considered.

Number of genes	Term name	Description	FDR value
14	HSA-6785807	Interleukin-4 and Interleukin-13 signaling	2.50E-13
31	HSA-74160	Gene expression (Transcription)	2.56E-11
11	HSA-9009391	Extra-nuclear estrogen signaling	4.09E-11
19	HSA-449147	Signaling by Interleukins	4.09E-11
28	HSA-212436	Generic Transcription Pathway	4.09E-11
22	HSA-1280215	Cytokine Signaling in Immune system	4.59E-11
13	HSA-8939211	ESR-mediated signaling	8.70E-10
7	HSA-9634638	Estrogen-dependent nuclear events downstream of ESR-membrane signaling	1.18E-08
10	HSA-2219528	PI3K/AKT Signaling in Cancer	1.32E-08
36	HSA-162582	Signal Transduction	2.71E-08
13	HSA-1257604	PIP3 activates AKT signaling	3.04E-08
15	HSA-5663202	Diseases of signal transduction by growth factor receptors and second messengers	3.04E-08
7	HSA-9607240	FLT3 Signaling	1.17E-07
25	HSA-1643685	Disease	6.11E-07
7	HSA-109606	Intrinsic Pathway for Apoptosis	6.15E-07
14	HSA-9006934	Signaling by Receptor Tyrosine Kinases	4.19E-06
9	HSA-2559583	Cellular Senescence	5.85E-06
26	HSA-168256	Immune System	1.14E-05
5	HSA-5674400	Constitutive Signaling by AKT1 E17K in Cancer	1.95E-05
4	HSA-198693	AKT phosphorylates targets in the nucleus	2.84E-05
5	HSA-114452	Activation of BH3-only proteins	2.84E-05
6	HSA-1236394	Signaling by ERBB4	2.84E-05
6	HSA-9614085	FOXO-mediated transcription	4.88E-05
8	HSA-109581	Apoptosis	8.57E-05
4	HSA-9661069	Defective binding of RB1 mutants to E2F1,(E2F2, E2F3)	1.30E-04
5	HSA-1433557	Signaling by SCF-KIT	1.30E-04
7	HSA-453279	Mitotic G1 phase and G1/S transition	2.80E-04
12	HSA-2262752	Cellular responses to stress	2.80E-04
5	HSA-8848021	Signaling by PTK6	3.10E-04
6	HSA-199418	Negative regulation of the PI3K/AKT network	6.70E-04
3	HSA-111453	BH3-only proteins associate with and inactivate anti-apoptotic BCL-2 members	6.90E-04
4	HSA-1251985	Nuclear signaling by ERBB4	9.20E-04
4	HSA-2559585	Oncogene Induced Senescence	1.00E-03
3	HSA-9617629	Regulation of FOXO transcriptional activity by acetylation	1.10E-03
9	HSA-3700989	Transcriptional Regulation by TP53	1.50E-03
3	HSA-8866910	TFAP2 (AP-2) family regulates transcription of growth factors and their receptors	1.60E-03
3	HSA-9614399	Regulation of localization of FOXO transcription factors	1.60E-03
5	HSA-69202	Cyclin E associated events during G1/S transition	1.60E-03
5	HSA-69656	Cyclin A:Cdk2-associated events at S phase entry	1.60E-03
7	HSA-8878171	Transcriptional regulation by RUNX1	1.60E-03

8	HSA-5688426	Deubiquitination	1.60E-03
3	HSA-6804116	TP53 Regulates Transcription of Genes Involved in G1 Cell Cycle Arrest	2.10E-03
3	HSA-9614657	FOXO-mediated transcription of cell death genes	2.50E-03
5	HSA-8878159	Transcriptional regulation by RUNX3	2.50E-03
15	HSA-1266738	Developmental Biology	2.50E-03
4	HSA-69231	Cyclin D associated events in G1	2.60E-03
3	HSA-9617828	FOXO-mediated transcription of cell cycle genes	3.10E-03
2	HSA-6804754	Regulation of TP53 Expression	4.00E-03
5	HSA-6811558	PI5P, PP2A and IER3 Regulate PI3K/AKT Signaling	4.00E-03
3	HSA-9670439	Signaling by phosphorylated juxtamembrane, extracellular and kinase domain KIT mutants	4.50E-03
3	HSA-8862803	Deregulated CDK5 triggers multiple neurodegenerative pathways in Alzheimers disease models	5.70E-03
4	HSA-2559586	DNA Damage/Telomere Stress Induced Senescence	5.90E-03
3	HSA-982772	Growth hormone receptor signaling	6.10E-03
5	HSA-8878166	Transcriptional regulation by RUNX2	6.10E-03
5	HSA-9018519	Estrogen-dependent gene expression	6.20E-03
4	HSA-69615	G1/S DNA Damage Checkpoints	6.90E-03
3	HSA-1266695	Interleukin-7 signaling	7.20E-03
3	HSA-9006115	Signaling by NTRK2 (TRKB)	7.20E-03
4	HSA-400253	Circadian Clock	7.40E-03
2	HSA-211163	AKT-mediated inactivation of FOXO1A	8.10E-03
6	HSA-157118	Signaling by NOTCH	8.10E-03
6	HSA-5689880	Ub-specific processing proteases	8.10E-03
10	HSA-109582	Hemostasis	8.20E-03
5	HSA-166520	Signaling by NTRKs	9.00E-03
5	HSA-6807070	PTEN Regulation	9.90E-03