

ROLE OF PLA2G2A rs11573156 C>G POLYMORPHISM IN PHOSPHOLIPASE
ACTIVITY AND PROSTATE CANCER PROGRESSION



by
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Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2022

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my thesis advisor Prof. Dr. Dilek Telci Temeltaş for her support and guidance through my thesis studies. I appreciate all the advice, help, and opportunities she has provided. Working with Prof. Dr. Dilek Telci and her wisdom and commitment always inspired and motivated me. It is a privilege to work with her and to be her student.

I also would like to thank all lab members Selen Tiryaki, Serra Şener, Fartma Sertgöz and Halime İlhan Sığınç for their endless support and help during my studies. Especially, I would like to thank Ece Nezir for her guidance and priceless friendship. In addition, I am so thankful to Kaan Öztürk for the contributions he made to my thesis. I also would like to thank to Utku Özbey for his support in flow cytometry. I am also thankful to Deniz Uzunoğlu and Zehra Çobandede for their help, support, and believing in me at every single step of my thesis.

Furthermore, I would like to thank my friends Berk Uluğ, Duygu Orak, Özge Deniz Yeşil, Fulya Şahin, Gökçeçişek Özdemir, and İsmet Can Bulut for their time, support, patience, and help to overcome the difficulties.

Most importantly, I want to express my greatest gratitude to my family, Funda, Fethi, Yağız Kaan, Filiz and Mertgün. Their endless belief, support, and patience through all my life is everything for me. Without the love they gave me, it would be impossible for me to start and finish my thesis. Finally, I would like to express my gratitude and love to my cat Luna for touching my soul.

ABSTRACT

Role of PLA2G2A rs11573156 C>G Polymorphism in Phospholipase Activity and Prostate Cancer Progression

Prostate cancer (PCa) is one of the most common health problems and cause of cancer-related mortality among men worldwide. Despite the existence of many treatment options, PCa is a recurrent disease with highly metastatic characteristics, often resistant to chemotherapy. The phospholipase A2 (PLA2) enzyme promotes the hydrolysis of the fatty acid acyl ester bond of phosphoglycerides, leading to the release of free acids and formation of lysophospholipids. Secretory PLA2 group II (sPLA2G2A) is one of the secreted forms in the PLA2 family members, which acts as an extracellular enzyme. PLA2G2A is responsible for the biosynthesis of prostaglandins and leukotrienes, which have potent inflammatory properties. Inflammatory responses have important roles at different stages of tumor development. PLA2G2A was associated with various types of cancer and malignancies, including prostate cancer. Previous studies have shown that elevated serum levels of PLA2G2A in patients with prostate cancer was in association with the tumor grade. This elevation in serum levels of PLA2G2A may in part be due to an SNP on the 5'UTR region (rs11573156 C>G polymorphism) on the gene. A previous study of our group suggested that patients carrying the G allele on the 5'UTR region of the PLA2G2A gene were 2.5 times less susceptible to development of metastatic PCa compared with those carrying the C allele. The aim of this thesis was to model the 5'UTR (rs11573156 C>G) polymorphism of PLA2G2A in metastatic PC-3 prostate cancer cell lines by editing GC genotype to the homozygous GG genotype using the CRISPR/Cas9 technology. Following gene editing, functional analysis of the PC-3 prostate cancer cell line with GG genotype was performed. This study is the first to investigate the effect of PLA2G2A rs11573156 SNP in PLA2G2A expression as well as proliferation and cell cycle regulation in PCa. Our findings demonstrate for the first time in literature that the homozygous G allele elevates the PLA2G2A expression, decreases the proliferation rate of PC-3 cells by attenuating the transition from G1 phase to S phase in response to hydrogen peroxide and interferon- γ that mimics the inflammatory tumor microenvironment.

ÖZET

PLA2G2A rs11573156 C>G Polimorfizminin Fosfolipaz Aktivitesi ve Prostat Kanseri Gelişimindeki Rolü

Prostat kanseri (PCa), dünya çapında erkeklerde kansere bağlı ölümlerin en yaygın nedenidir. Günümüzde yaygın olarak kullanılan birçok tedavi yöntemi bulunmasına rağmen PCa kemoterapiye karşı direnç gösteren, yüksek metastatik özelliklere sahip ve tekrarlaması olası bir hastalıktır. Fosfolipaz A2 (PLA2), fosfogliseritlerdeki asil ester bağının hidrolizinden, bu hidroliz sonucunda oluşan yağ asitleri ve lizofosfolipidlerin sentezinden sorumludur. PLA2 enzim grubunun tanımlanmış üyelerinden biri olan sekretuar fosfolipaz A2 grup II (PLA2G2A), hücre dışı bir enzim olarak işlev gören, salgılanan bir PLA2 formudur. PLA2G2A, güçlü inflammatuar özelliklere sahip prostaglandinlerin ve lökotrienlerin biyosentezinden sorumludur. İnflammatuar yanıtlar, tümör gelişiminin farklı aşamalarında çok önemli rol oynar. PLA2G2A, prostat kanseri de dahil olmak üzere farklı kanser türleri ile ilişkilendirilmiştir. Önceki çalışmalar, prostat kanseri hastalarında yüksek PLA2G2A serum seviyesinin tümör derecesi ile ilişkili olduğunu göstermiştir. PLA2G2A serum seviyesinin yükselmesi kısmen 5'UTR gen bölgesindeki bir SNP'ye (rs11573156 C>G polimorfizmi) bağlı olabilir. Grubumuz tarafından daha önce yapılan bir çalışma, PLA2G2A geninin 5'UTR bölgesinde G aleli taşıyan hastaların, C aleli taşıyan hastalara kıyasla metastaz gelişimine 2,5 kat daha az meyilli olduğunu göstermiştir. Bu tezin amacı, metastatik PC-3 prostat kanseri hücre dizilerinde PLA2G2A'nın 5'UTR (rs11573156 C>G) polimorfizmini, CRISPR/Cas9 teknolojisini kullanarak GC genotipinden homozigot GG genotipine çevirerek modellemektir. Gen düzenlemesinin ardından GG genotipli PC-3 prostat kanseri hücre dizisinin fonksiyonel analizi yapılmıştır ve buna göre bulgularımız literatürde ilk kez homozigot G alelinin PLA2G2A ekspresyonunu yükselttiğini, inflammatuar tümör mikroçevresini taklit eden interferon- γ ve hidrojen perokside yanıt olarak G1 fazından S fazına geçişi ve PC-3 hücrelerinin proliferasyon hızını azalttığını göstermektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

β	Beta
$^{\circ}\text{C}$	Degree centigrade
M	Molar
mL	Millilitre
mM	Millimolar
mm	Millimetre
μL	Microliter
μM	Micromolar
μm	Micrometer
5'UTR	5' untranslated region
AAV	Viral vectors adeno-associated virus
AdPLA2	Adipose-specific PLA2
ADT	Androgen deprivation therapy (ADT)
AKT	Protein Kinase B
AR	Androgen receptor
BGH pA	Bovine growth hormone polyadenylation signal
BIR	Break-induced repair
bp	Base pair
Cas	CRISPR-associated
CBh	Chicken β -actin
CMV	Cytomegalovirus
COX-2	Cyclooxygenase 2
cPLA2	Cytosolic PLA2
CPPS	Chronic pelvic pain syndrome (CPPS)
CRISPR	Clustered regularly interspaced short palindromic repeats
CRPS	Castration-resistant PCa
crRNA	CRISPR RNA
DAG	Diacylglycerol
DAMP	Danger-associated molecular patterns
dbSNP	SNP database

dHJs	Double Holliday junctions
DRE	Digital rectal examination
DSB	Double-strand break
DSBR	DSB repair
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EVs	Extracellular vesicles
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
gRNA	Guide RNA
GWAS	Genome-wide association study
H ₂ O ₂	Hydrogen peroxide
HDR	Homology directed repair
IFN	Interferon
IFN- γ	Interferon- γ
IKK	kappaB kinase
IL-6	Interleukin-6
iPLA2	Ca ²⁺ -independent PLA2
JAK-STAT	Janus kinase and signal activators and transducers
JNK	c-Jun N-terminal kinases
LDL	Low-density lipoprotein
LHRH	Luteinizing hormone-releasing hormone
LPS	Lipopolysaccharide
LyPLA2l	Lysosomal PLA2
MAPK	Mitogen-activated protein kinase
mCRPC	Metastatic CRPC
NF- κ B	Nuclear factor-kappa B
NHEJ	Non-homologous end joining
PAF-AH	Platelet-activation factor acetylhydrolase
PAM	Protospacer adjacent motif
PCa	Prostate Cancer
PCR	Polymerase chain reaction
PGE	Prostaglandin E2

PI3K	Phosphatidylinositol 3-kinase
PIN	Prostatic intraepithelial neoplasia
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLA2G6	Phospholipase A2 group VI
PLA2G15	Lysosomal phospholipase A2
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
PNPLA	Patatin-like phospholipase
PSA	Penicillin, Streptomycin and Amphotericin B
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen
PTEN	Phosphatase and tensin homolog
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl Sulfate
SDSA	Synthesis-dependent strand-annealing
SFM	Serum-free medium
sgRNA	Single guide RNA
SNP	Single nucleotide polymorphism
SpCas9	CRISPR-associated protein-9 from <i>Streptococcus pyogenes</i>
sPLA2	Secretory PLA2
sPLA2-IIA	Secretory PLA2 group II-A
SSA	single-strand annealing
ssDNA	Single-stranded DNA
SV40 late PA	Polyadenylation signal
TAMs	Tumor-associated macrophages
TGF- β	Transforming growth factor- β
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF- α	Tumor necrosis factor- α
TNF- β	Tumor necrosis factor- β
TNM	Tumor (T), Lymph node (N), Metastasis (M)

VEGF	Vascular endothelial growth factor
WST-1	Water Soluble Tetrazolium
XRCC	X-ray repair cross-complementing protein



1. INTRODUCTION

1.1. THE ANATOMY AND FUNCTION OF THE PROSTATE GLAND

The prostate is a small-sized tubuloalveolar gland located beneath the bladder, deep inside the groin, and surrounding the urethra (Figure 1.1.). The prostate is important gland of the male reproductive system.

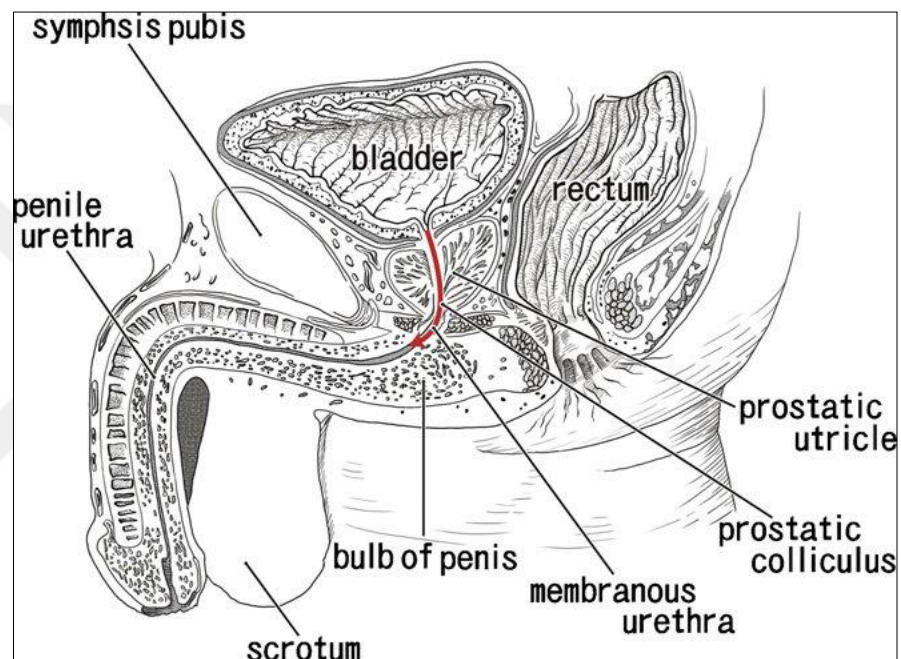


Figure 1.1. The anatomical regions of the prostate gland in male anatomy [1]

The prostate gland is raised from the posterior part of urogenital sinus by the effect of the underlying mesenchyme during the third month of gestation [2]. The prostate gland can be described by divided into five main anatomical parts: the central zone, periurethral region, transition zone, peripheral zone, and stroma (fibromuscular region). All compartments of the prostate gland continuously communicate with each other by exchanging chemical messages and growth signals [3]. Each of the regions arises from acini and ducts are embedded in the stroma. The acini and ducts are surrounded by the basal epithelial cells. The epithelial cells produce basal membrane layer of the extracellular matrix (ECM) anchored to the stromal cells (Figure 1.2). Each acinus is composed of epithelial cells embedded by central lumen. which is filled with the secretion fluid. The secretion fluid is generated by the epithelial cells.

This secretion joins the system both by the branching epithelial tubules and epithelial ducts that is ended in the urethra. The gland epithelial site is basically made-up from the two types of epithelial cells, basal and glandular. The functions of epithelial cells include the synthesis and secretion of components of basement membrane of the acini. While the majority of the prostate gland is made-up from stromal cells, epithelial cells are lesser in number and morphologically not fully developed for the bulk secretion and protein synthesis compared to the glandular epithelial cells. These cells are found attached to the basal cells. They are secretory active cells that is responsible for the production of prostatic fluid and also express the androgen receptor (AR). Moreover, glandular epithelial cells secrete the prostatic fluid into acinar lumen which contains the glycoprotein enzyme prostate-specific antigen (PSA), pro-inflammatory cytokines, lipids prostaglandins, and prostatic acid phosphatase. A developed basal membrane acts as a separation of the epithelial ducts and acini from the fibromuscular stromal tissue. This tissue is made-up from endothelial cells, fibroblasts, nerves, smooth muscle cells, and assorted infiltrating cells such as lymphocytes. Therefore, glandular and basal cells of the prostate gland access the nutrients and androgen so that the stromal compartment can modify the epithelial cell microenvironment [4]. The prostate ECM, which is connected to stromal cells, is fundamentally composed of myocyte cells of the smooth muscles that are responsible for the spontaneous contractility and prevention of fluid stagnation [5]. 80 percent of the prostate tumors arise from the stromal region that promotes the secretion of semen [6].

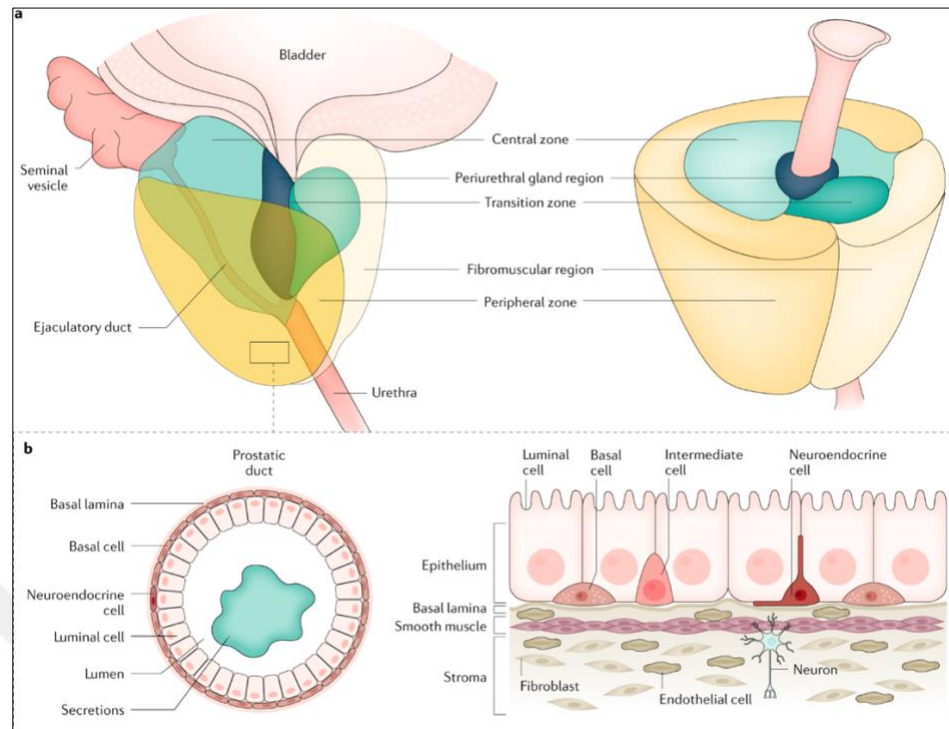


Figure 1.2. Overview of prostate gland and cell structure. a. Anatomical regions of the prostate gland. b. Cell layers in the prostate gland [7]

1.2. PROSTATE CANCER

PCa is the second ranked cancer worldwide. PCa is a disease that mostly affects the elderly male population [8]. PCa is raised by the uncontrolled growth of cells within the prostate gland. When the prostate gland becomes cancerous, it causes pressure on the urethra, which results in frequent painful urination with a burning sensation. The pressure may also cause difficulty in urination, weakness in urine flow, and sometimes blood in the urine. Rare symptoms of PCa can be swollen lymph nodes in the groin and painful aches in the pelvis, hips, ribs, and back [9].

The risk of developing PCa is related with the advanced age, ethnicity, and family history. For instance, in the United States, PCa is the second-leading cause of cancer death; 13 out of 100 men will develop PCa during their lifetime, whereas in Turkey, according to recent studies, the age-adjusted incidence rate of PCa is 35 cases per 100,000 [10–12]. The American Cancer Society has reported the PCa incidence and mortality rates (Figure 1.3.) by geographical area in 2012. The incidence rate of PCa increased sharply after the

development of widespread screening for PSA enabling the early-stage diagnosis [13]. Although PCa is one of the most treatable malignancies worldwide when diagnosed in the early stages, in the late stages, tumors become androgen resistant and progress to a lethal stage [14].

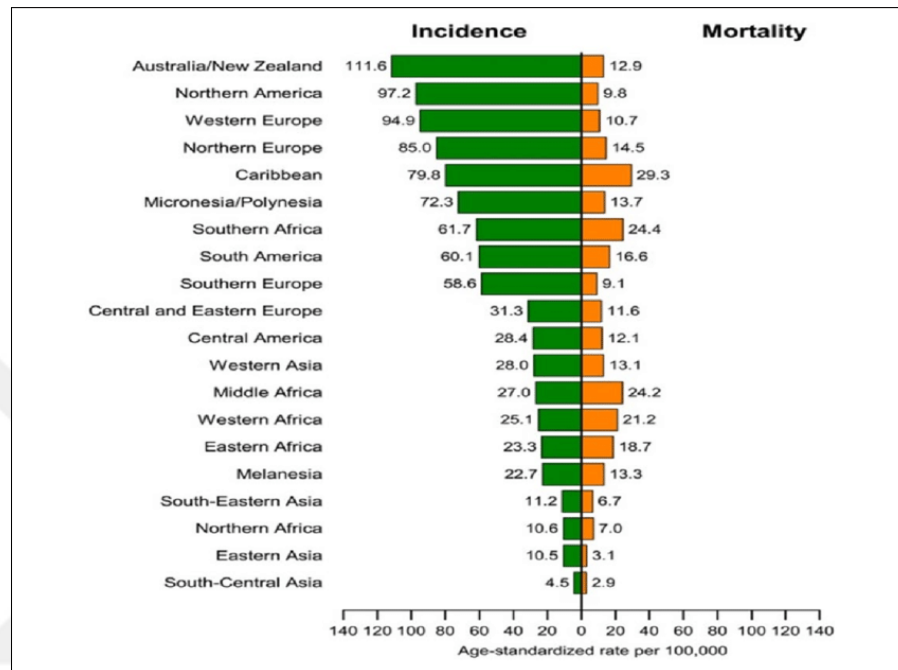


Figure 1.3. Incidence rates of PCa by geographical area [15]

Chronic infections arise from sexually transmitted diseases and prostatitis are associated with the enhanced rate of PCa [14]. In the progression of the disease, aberrant cell signaling involving the male androgen hormones, especially testosterone and its metabolites are the contributing factors [16]. Testosterone may be converted into two active compounds, estradiol or dihydrotestosterone in certain tissues. Estradiol promotes the proliferation of PCa cells through its binding capacity towards intracellular AR, which prevents the apoptosis. Dihydrotestosterone also has a vital role in preventing the apoptosis when aberrant cells arise; however, the mechanism and contribution of testosterone and its active derivatives have not been fully elucidated [17].

1.2.1. Pillars of Prostate Cancer

Tumorigenesis is a dynamic process which rises as a result of clusters of tumor cells. The fundamental hallmarks of cancer are acquired during the tumorigenesis [18]. There are ten fundamental hallmarks/pillars of cancer including unresponsiveness to growth suppressors, unlimited division capacity, resistance to cell death, deregulation of cellular energy, avoiding immune system destruction, invasion and metastasis to other tissues, resistance to apoptosis, angiogenic stimulation, genomic instability and inflammation [19].

Tumors present various dimensions of complexity. For instance, tumor cells recruit the ostensibly normal cells to gain the cellular transformation by modifying the tumor microenvironment (TME) [20]. The TME of PCa includes the complicated interactions between the prostate gland epithelial cell and the surrounding stromal epithelial cells. The interaction between these cells has function in the development of angiogenesis, inflammation, metastatic dissemination, and also in survival of the PCa cells [21]. The reactive stromal cells are generally identified by the existence of cancer-associated fibroblasts and myofibroblast cells, following the loss of smooth muscle cells, which cause intratumor heterogeneity [22]. Adipocytes also has a distinctive function in the prostate tumor modulation by secreting various growth factors, such as interleukin (IL)-6, tumor necrosis factor (TNF)- α , and TNF- β [23]. There are three unique factors that can be considered as pillars of PCa. The first one is the aberrant functioning of AR, the molecular mechanisms of which can be investigated under three major categories: *(I)* hyper-activation of AR by an increase in AR synthesis, mutation rate, and ligand-dependent activation, *(II)* the overexpression of the AR gene, *(III)* alternative AR activation pathways mediated by transactivation, providing the self-sustenance in growth signaling [24,25]. The second pillar of PCa is the alteration in growth factor receptor activity, resulting in deregulated proliferation of the cells. Although epithelial cells of the prostate gland have a low capacity to proliferate, in prostatic intraepithelial neoplasia (PIN) and early-stage prostate carcinoma they show a higher rate of proliferation [26]. The last pillar is reprogramming or find an alternative intracellular signaling pathways that associate with the viability and proliferation of cells [27,28]. These factors significantly not support to the drug-resistant and androgen-independent PCa phenotypes but also represent the major signaling pathways related to growth, proliferation, and viability of PCa cells and tumorigenesis (described in section

1.2.3.). In Human PCa Hallmarks Map [29], the pillars of PCa are described by presenting a protein network with different interactions, signaling pathways that involve various proteins, and pathway-based functional modules. Silencing of apoptotic signaling, resistance to chemotherapeutic drugs, epithelial-mesenchymal transition (EMT), aberrant interactions of cell adhesion molecules, enhanced inflammatory response, and unlimited replicative potential can also be considered as pillars of PCa [30]. The pillars of cancer are acquired prior to and during the tumor development stage, building an organized plan for the neoplastic disease [31]. Thus, early diagnosis is essential to prevent the spreading of tumor cells (metastasis) to the other tissues and organs.

1.2.2. Genetics, Diagnosis and Prognosis of Prostate Cancer

Studies on PCa and its progression have been ongoing since the early ages; however, the mechanisms underlying PCa remain an important medical question. Malignant transformation of the prostate comprises multistep processes [32]. The disease is initiated as PIN followed by localized PCa, and lastly, it is transformed to the advanced PCa with zonary invasions and metastasis [26]. The PIN is defined by proliferation of the luminal cells with dysplasia along the ducts. Afterward, PIN transforms to the local prostate tumor, that eventually becomes locally invasive adenocarcinoma [33]. Later, the basal cell part is partially degraded, thus the cancer cells gained invasive feature. So that, cancer cells can to migrate through the basal lamina. In the local advanced PCa, lymph nodes are the first location that the cancer cells metastasis through. Afterward, the local cancer cells migrate to the nearest organs such as lungs, livers and bones [34]. In bone metastasis of PCa, essential interaction occurs between the invading cancer cells and osteoblasts, resulting in a bone formation and destruction process called “the vicious cycle.” This process ensures the cell survival and growth of the tumor [35]. Androgen deprivation therapy (ADT) is the routinely preferred therapy in PCa, which blocks the androgen pathway. AR-dependent localized advanced adenocarcinoma is able to respond to ADT initially. However, thereafter AR-dependent localized advanced adenocarcinoma is transforms into the castration-resistant PCa (CRPC) or metastatic CRPC (mCRPC), where it becomes resistant to the ADT therapy. Locally advanced adenocarcinoma is also able to alter *de novo* resistance to ADT, similar to the CRPC formation (Figure 1.4.). The main feature of PCa is hormone responsiveness; thus,

the treatment options must be determined according to the tumor stage and histological pattern [36].

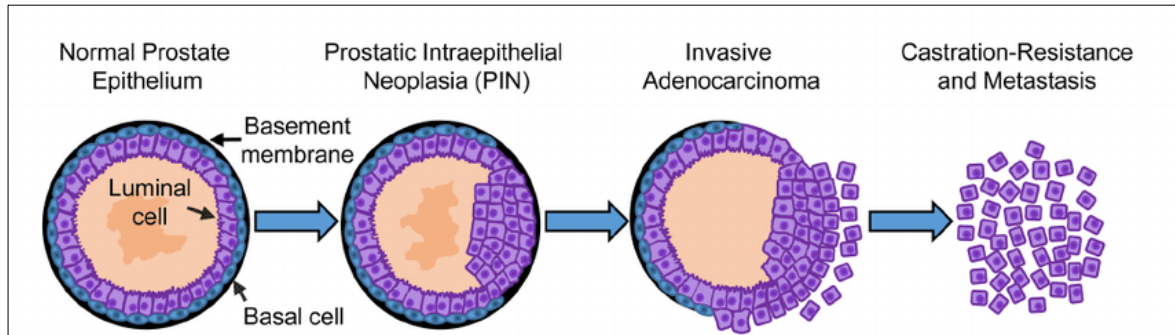


Figure 1.4. The stages of PCa development [37]

Based on the epidemiological studies and large-scale genome-wide association studies (GWASs), environmental and genetic factors that impact PCa initiation and progression were defined [38]. Especially in epidemiological studies, it was found that the family history of PCa remarkably increased the risk. In twin studies, it was indicated that PCa is one of the most heritable cancers [38]. The presence of single nucleotide polymorphisms (SNPs) also triggers the inflammation-related pathways. Categorizing the genetic drivers of PCa and defining the subtypes of the disease is also important. In primary prostate tumorigenesis, various large-scale genomic studies identified mutations, the altered copy number of genes along with gene fusions and rearrangements in mCRPS [39–41]. It was found that primary tumors and mCRPC showed remarkably increased alterations of genome-wide copy numbers and, accordingly, increased mutations. Genetic alterations in PCa frequently alter the AR, phosphatidylinositol 3-kinase (PI3K), and phosphatase and tensin homolog (PTEN) proteins in the AR pathway [42].

In AR pathway, signals initially conducted by the dihydrotestosterone results in the AR dimerization and nucleus relocation, initiating the metabolic activities and growth (Figure 1.5.). However, in tyrosine kinase receptors activation mediated by AR activation, receptor dimerization process triggers PI3K activation and protein kinase B (AKT) phosphorylation. The phosphorylated AKT promotes the dimerization of AR, which results in the relocation of AR to the nucleus. Under normal conditions, functional PTEN inhibits the dimerization of AR by hydrolyzing the phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5) P3) and

terminating the AKT activation. Mutations in PTEN therefore cause insensitivity to ADT [43].

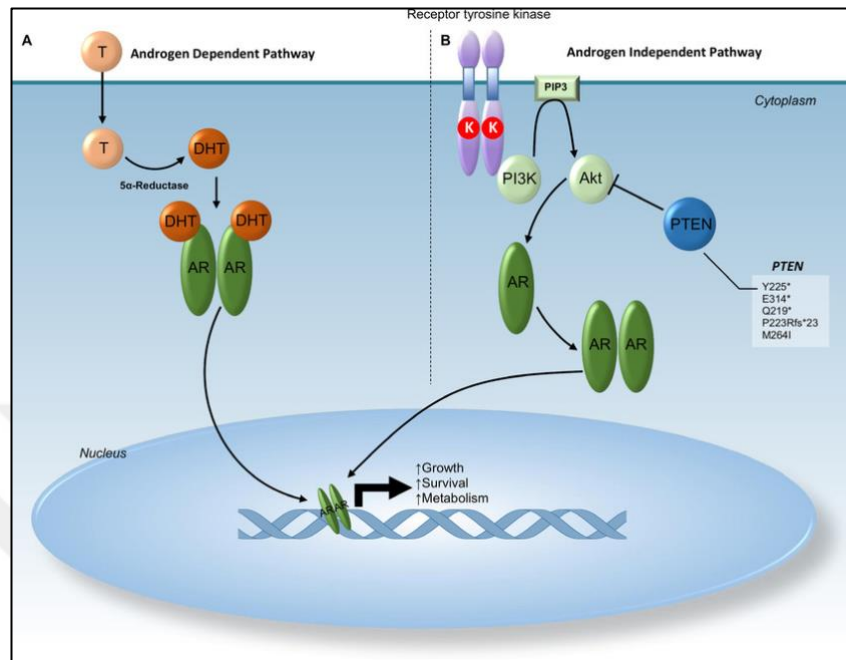


Figure 1.5. The AR pathway [44]. Section A represents the AR activation by testosterone under the normal conditions. Section B demonstrates the tyrosine kinase receptors activation mediated by AR activation

Common genomic alterations that cause the initiation of or contribute to the progression of PCa have also been identified. These alterations directly give rise to the poor prognosis of PCa [44]. A various studies correlate the PCa and SNPs. For instance, a recent study has already investigated 140 SNPs in 11 of inflammation-related genes and, as a result, it was found that men carrying multiple SNPs in cytokine-related genes have an elevated risk of PCa [45]. These studies underline the significance of SNPs in PCa. The diagnosis and treatment modality of PCa can be determined based on these findings.

PCa is diagnosed by a screening method including two tests: digital rectal examination (DRE) and prostate-specific antigen (PSA) blood test. A DRE is a test in which the physician examines the rectum by finger and feels the surface of the prostate. PSA is a type of a protein produced by prostate cells and right after the formation it was secreted into the bloodstream. Level of PSA in body is used to prognose early-stage PCa. Patients with PCa generally have PSA levels above the reference range 2.5 to 5.5 according to age of the individual. However,

patients with the infected prostate gland which is referred as prostatitis also have a high level of PSA [46]. In addition, some sexual activities can also elevate the PSA levels in the blood [46]. Thus, PSA level alone is not preferred to be used in the prognosis of PCa [47]. Physicians recommend biopsy shortly after diagnosis for the active surveillance. After the biopsy, the pathologist investigates the pathological tissue according to a special grading system based on the prostate adenocarcinoma histological pattern. [48]. The grading system was defined by Donald Gleason is the used grading system to determine the PCa stages and aggressiveness [49]. In Gleason grading system, pathologist investigates the arrangement of cancer cells by giving a score on a scale of 3 to 5 according to two different locations of the prostate. Cancer cells that have similar morphological features to healthy cells receive a low score, whereas ostensibly less healthy cells receive a higher Gleason score [50]. The grading system is based on the main pattern of prostate cell growth where the cancer is detected. According to the area of cancerous cells, the pathologist assigns a grade from 1 to 5. Then, the grades are added together, and the overall score is determined, which ranges from 6 to 10. Grade 3+3 (Gleason score 1) which indicates the lower risk. Grade 3+4 (Gleason score 2) represents the medium-grade interval. Grade 3+5 (Gleason score 3), grade 3+6 (Gleason score 4) and higher grades (Gleason score 5) considered as high-grade cancer [51,52].

After the determination of the Gleason score, the physicians try to decide whether the cancer is metastatic. The staging process also involves the PSA level. Tumor (T), lymph node (N), or metastasis (M) stages are assigned, according to TNM staging system [53]. The staging of PCa helps to determine the level of severity and spreading of cancer and to design the best treatment. In PCa, according to the aggressiveness of the tumor, three stages are defined. These are localized PCa, regional PCa of local extension, and metastatic PCa, corresponding to stage I to stage IIIA, stage IIIB to stage IVA, and stage IVB, respectively. Stage IVB is the most advanced and aggressive type among all stages [54]. At stage I, the cancer has a slow growth rate, and invades through half or one side of the prostate [55]. PSA levels in bloodstream are low at this stage, and cancer cells resemble to the healthy cells; thus, it is the hardest stage to be defined as cancer. At stage II, part of the tumor is still small but has a higher rate of growth and spread. Stage II is sub-categorized into stage IIA, stage IIB, and stage IIC. At stage IIA, the tumor cannot be felt, and PSA levels are moderate; however, PCa cells are differentiated. At stage IIB, the tumor can be detected inside the prostate gland during DRE, and PSA levels in bloodstream are at medium-high levels and PCa cells are

moderately differentiated at this level. At the stage IIC, the tumor is larger than in stage IIB but not spread to the distal sides, PSA level in bloodstream is moderately high, and PCa cells are poorly differentiated. At stage III, the PSA level appears as high, and the tumor grows and spreads. Cancer grade is high at this level. Stage III is also sub-categorized as stage IIIA, stage IIIB, and stage IIIC. At stage IIIA, cancer has spread into the seminal vesicles, and the PSA levels are high. At stage IIIB, the tumor has spread to the nearby tissues such as the liver. At stage IIIC, the PCa cells are morphologically distinct from the healthy cells, and can be identified easily. Further, the nearby healthy cells are poorly differentiated. Stage IV is the last PCa stage. Cancer invades through the distant parts of the body. This stage is sub-categorized as stage IVA and stage IVB. At stage IVA, cancer has invaded to the lymph nodes, and at stage IVB, it has invaded to the bones [56]. After determining the stage of cancer by considering the PSA levels and Gleason score, the best treatment is suggested by the physicians. Sometimes, although cancer seems to be cured upon treatment, it can re-develop in the prostate or other metastatic sites of the body; this is called recurrent cancer [57]. For this reason, it is highly recommended that PSA test should be given in every 3 to 6 months and DRE should be done at least once a year [58].

1.2.3. Inflammation in Prostate Cancer

Inflammation is the reaction that results from the activation of some cellular actions and immune system [59]. Inflammation is important feature in host defense against infections. Moreover, it has a very important role in regeneration, tissue repair, homeostasis, and the progression of diseases such as cancer. The development of cancer is regulated, and its response to therapy is maintained by inflammation. Recent studies have reported that inflammation is crucial for tumor progression. However, it can either suppress or promote the progression of tumors [60]. Many cancers occur due to chronic inflammation, which facilitates both the tumor progression and the resistance to treatment, whereas acute inflammation induction sometimes triggers the maturation of dendritic cell and presenting antigens which leads to form anti-tumor immune responses [60]. Inflammatory cells largely contribute to the tumor microenvironment fostering the survival, proliferation, and migration of the cells. In addition, various signaling pathways are contributed during inflammation [61]. Nuclear factor-kappa B (NF- κ B), Janus kinase, mitogen-activated protein kinase (MAPK), and signal activators and transducers (JAK-STAT), and toll-like receptor (TLR)

pathways are the major pathways that involved in inflammation. In response to inflammatory factors, interferon (IFN), IL, chemokines, and TNF- α , these pathways are activated. Vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)- β and some inflammatory metabolites including leukotrienes, prostaglandins, and thromboxane is responsible in the regulation of inflammation [62]. Main inflammatory cytokines and TNF- α , which triggers the NF- κ B pathway, and TGF- β , which triggers to epithelial-mesenchymal transition (EMT) and disruption of ECM secreted by the tumor-associated macrophages (TAMs) that alter the tumor microenvironment. The TAMs have role in the angiogenic response via VEGF upregulation. Consequential disruption of ECM, resulting the mesenchymal prostate cells not only gain resistance to apoptosis (anoikis) but also gain invasive characteristic to proceed through metastasis (Figure 1.6.).

Another inflammation-related agent is cyclooxygenase 2 (COX-2), an inducible enzyme. COX-2 isoforms convert arachidonic acid (AA) to proinflammatory prostaglandins. Recent studies have suggested that elevated COX-2 expression, increases the Gleason histological grading of PCa; thus, COX-2 remains a linker molecule between inflammation and PCa [63–65].

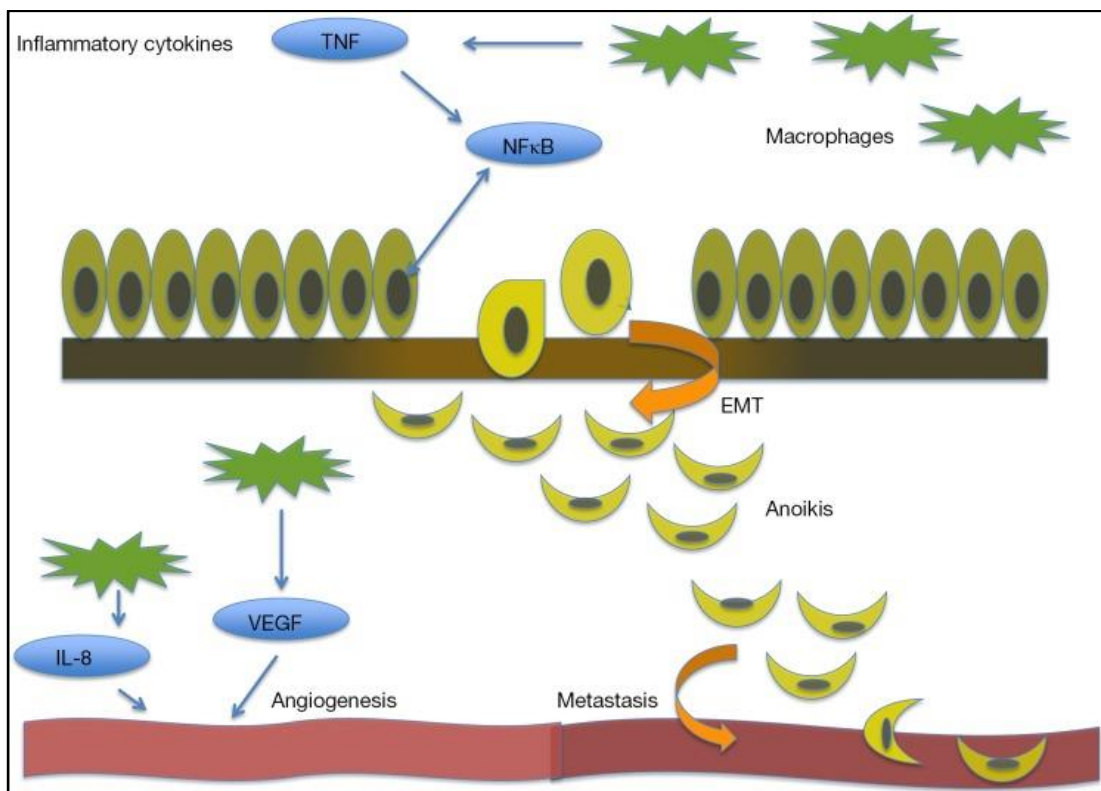


Figure 1.6. Inflammatory metabolites act of action on cancer progression [14]

Chronic inflammation is also related to the progression of PCa including angiogenesis, EMT and metastasis. These processes also impact the dynamics of the tumor microenvironment. Proliferative inflammatory atrophy refers to the existence of activated inflammatory cells and also proliferating epithelial cells, which are the pathological precursors to PIN lesions and also to PCa [66]. The relationship between the PIN lesions and PCa can provide information about the relationship between PCa and inflammation. When determining the tumor profile of patients with PCa, the state of inflammation and its impact on the tumor microenvironments must be considered. In benign prostate tissues, the chronic inflammation is correlated with high-grade PCa [67]. Consequentially, Pentraxin which is one of inflammatory effector has been revealed as a biomarker for comprehending the progression of tumor due to inflammation in patients with PCa [68]. There are also multiple studies suggesting that within prostate, inflammation is common in adult men, which is related to the subsequent initiation of PCa [69].

Prostatitis is heterogeneous and a complicated concept that the National Institutes of Health suggested its classification as divided into two, chronic prostatitis and chronic pelvic pain syndrome (CPPS). CPPS is categorized into four different classes; three of these are related to men with symptoms of the disease: (I) acute bacterial prostatitis, (II) chronic bacterial prostatitis, (III) chronic prostatitis or CPPS, and (IV) asymptomatic inflammatory prostatitis [70]. It has been suggested that racial and geographical differences also impact the inflammation in adult men. Many different bacterial species that cause the infection in prostate gland of men and cause inflammation are identified. In prostatitis, the most commonly seen microorganisms are *Klebsiella* spp., *Enterococcus*, *Pseudomonas* spp., *E. coli*, *Proteus mirabilis*, and *Serratia* spp. Studies attempting to understand the correlation between prostatitis and PCa identified an elevated in the risk of PCa with the history of prostatitis. There are also several studies that support the correlation between asymptomatic prostatic inflammation caused by infected microorganisms and PCa development [69,71,72].

1.2.4. Therapeutic Strategies Against Prostate Cancer

In the last decade, androgen targeting therapy has been the most commonly used strategy against PCa. The first androgen targeting therapy was developed in 1789, by which androgen

ablation was used to control PCa [73]. Later, prostatectomy, the removal of the entire prostate gland, was performed as a treatment. In the 19th century, a number of reports described a link between the prostate and testes. Thereafter, castration was performed as PCa therapy [74]. After castration, prostate weight and atrophy of the glandular elements decreases. Thus, castration therapy become the treatment of PCa. In the late 1930s, hormonal manipulations such as estrogen injection was performed as castration. At the beginning of the 1940s, Charles Huggins reported the beneficial effects of castration and estrogen in patients with advanced PCa [73]. In 1947, radical retropubic prostatectomy was introduced by Terrence Millin as a new treatment modality [75]. In 1962, megavoltage radiation for localized PCa was described by Malcolm Bagshaw [76]. Finally, in 1969, the Gleason grading system was developed. Thus, treatments were developed according to the stages of PCa [77].

PCa treatment must be specified according to the patient's PSA levels, Gleason score, life expectancy, and current medical conditions. Thus, physicians must collect as much data as possible about the patient to serve the best treatment [78]. Treatment against PCa is basically divided into four categories; local treatments, radiation therapy, focal therapies, and systemic treatments. Local treatments involve the removal of the prostate gland (prostatectomy) along with the surrounding lymph nodes. Radiation therapy is the utilization of high-energy rays to damage and cause the destruction of cancer cells [79]. External-beam radiation therapy, intensity-modulated radiation therapy, internal radiation therapy and proton therapy are the generally used types of radiation therapy. Focal therapies are less invasive compared to the other treatment options. Here, prostate tumors are destroyed without harming the rest of the prostate gland and nearby structures [80].

Systemic therapies are the utilization of medications in order to destroy cancer cells. Hormonal therapy, targeted therapy, chemotherapy, and immunotherapy, are the most common systemic therapies against PCa [52]. In hormonal therapies, luteinizing hormone-releasing hormone (LHRH) agonists are the most utilized therapy in cancer [81]. The LHRH agonists block the signals that the testicles receive from the body to secrete testosterone; however, sometimes, their effect can be irreversible. Moreover, LHRH antagonists are widely used to reduce testosterone levels; although it is faster than LHRH, severe allergic reactions may be triggered [81]. As for ADT, AR inhibitors are used as hormonal therapy [81]. While LHRH agonists and antagonists inhibit testosterone secretion, AR inhibitors

block the secreted testosterone that would bind to the AR by lowering the androgen level. Sometimes, AR inhibitors are combined with LHRH agonists to maximize the effect according to the stage of cancer [82].

Targeted therapy is another PCa treatment method that targets specific genes and proteins of cancer. Targeted therapy aims to prevent cancer cell growth and survival and limits damage to non-cancerous cells. In order to determine the most suitable targeted therapy, genomic sequencing of tumor tissues and tumor DNA in the bloodstream are employed [83]. Generally, PARP inhibitors olaparib and rucaparib are used as targeted therapy against metastatic PCa with DNA repair defects. Moreover, in the past two decades, prostate-specific membrane antigen (PSMA) has emerged as an important molecular therapeutic target in PCa [84,85].

Chemotherapy is the dynamic treatment method which is basically the utilization of drugs that can targets actively dividing cells including cancer cells. Chemotherapy involves medicines that are effective against advanced and castration-resistant PCa [86]. Chemotherapeutic medicines are usually administered in a certain number of cycles over a set of periods. There are several agents preferred for use against PCa [87]. Generally, chemotherapy begins with docetaxel, a microtubule inhibitor, which can be combined with prednisone [88]. However, for metastatic castration-resistant PCa, cabazitaxel, which is also a taxane is preferred [89]. According to recent studies, the combination of docetaxel with hormonal therapy shows a significant decrease in the growth and spreading of cancer cells [90].

Immunotherapy, or biologic therapy, is developed to boost the immune defense to fight against cancer cells. In recent years, immunotherapy made a huge impact on metastatic cancer treatments, as well as many different types of tumors. For example, vaccine therapy with sipuleucel-T (Provenge) is recommended as an initial option before chemotherapy for patients with metastatic PCa showing only a few cancer symptoms [91].

1.3. PHOSPHOLIPASE ENZYME FAMILY

1.3.1. Description and Classification of Phospholipase Enzymes

Phospholipases are ubiquitous enzymes that are responsible for the hydrolysis of membrane phospholipids into fatty acids and lipophilic molecules [92]. There are various subtypes of a phospholipase enzymes categorized according to their structure and act of action (Figure 1.7.). Different groups of phospholipases have important role in multifunctional activities that are fundamental for the biological systems. The removal of acyl chains from the phospholipid head group and remodeling of the hydrolytic products are crucial for the lipid signaling, membrane trafficking, and digestion [93]. Most cells contain most of the phospholipase subtypes that can either be found on the membrane, in the intracellular space, or in secreted forms [92]. The roles of phospholipases in the cellular system are diverse and depend on their localization. Phospholipases are enzymes that are divided into two general classes, phosphodiesterases and acylhydrolases, and groups within each set of enzyme groups are divided into groups based on the site of cleavage in their phospholipid substrates [94]. Phospholipase A1 (PLA1), phospholipase A2 (PLA2), and phospholipase B (PLB) are defined as acylhydrolases, while phospholipase C (PLC) and phospholipase D (PLD) are identified as phosphodiesterases. PLA1 and PLA2 catalyze the formation of 1-acyl or 2-acyl lysophospholipids and free fatty acids. PLB is responsible for the hydrolysis of sn-1 and sn-2 position fatty acids of the phospholipids. PLC cleaves the glycerophosphate linkage that connects the polar heads of the glycerol backbone producing diacylglycerol (DAG) and inositol triphosphate (IP₃). The DAG and IP₃ are involved in the signaling of protein kinase C and also in release of the calcium ions from the intracellular stores [95]. PLD is type of a phosphodiesterase, which removes head groups of the phospholipids [96].

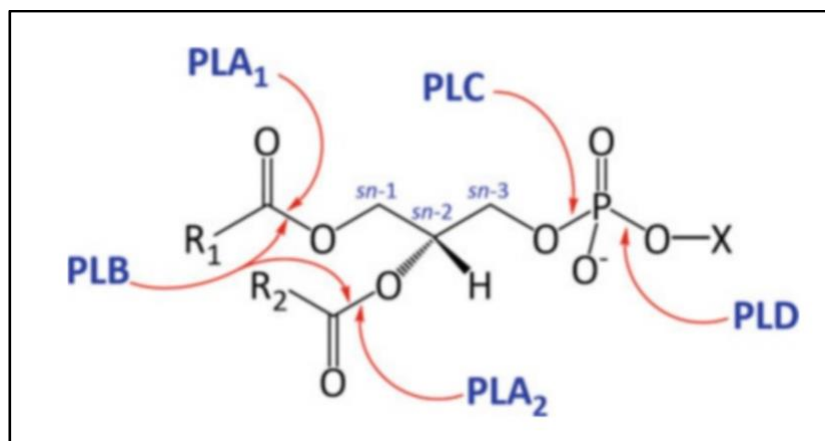


Figure 1.7. The molecular structure of glycerophospholipid, and the site of action of phospholipases [93]

The PLA, PLB, PLC, and PLD enzymes can be classified into sub-groups with a complex taxonomy [97]. This classification is based on the phospholipase distribution, structural characteristics, sequential similarity, and optimal activity *in vitro*, whereas these properties are directly related to the activity of phospholipase enzymes [93]. Based on the hydrolysis sites within lipid mediators, PLA is classified into the two subgroups PLA1 and PLA2 [98]. The growing superfamily of PLC consists of 11 isoforms specific to phosphatidylinositol and has additional four sub-families, PLC-beta, PLC-gamma, PLC-delta, and PLC-epsilon [95].

1.3.2. Phospholipase A₂ (PLA₂) Enzyme Family

The PLA₂ enzyme group consists of more than 50 groups of lipolytic enzymes that have different structures and functions in mammals [99]. The PLA₂ enzymes are basically responsible for the catalysis of phospholipids which are in the sn-2 position (glycerol), resulting in the synthesis of lysophospholipids, fatty acids, and arachidonic acid. The role of PLA₂ in signal transduction is essential because release of fatty acids by PLA₂ can be converted to active lipid molecules called as lipid mediators such as, prostaglandin, leukotrienes and PAF [98]. Lipid mediators act in various biological functions in cells through activation of G-protein coupled receptors [100]. Moreover, the PLA₂ enzyme family has also been affected in membrane homeostasis, membrane remodeling, and energy production [101].

The PLA2 superfamily is divided into six major sub-groups based on structure, location, calcium requirement, and substrate specificity (Table 1.1.). These are the secreted PLA2 (sPLA2), cytosolic PLA2 (cPLA2), Ca^{2+} -independent PLA2 (iPLA2), also called as patatin-like phospholipase (PNPLA), platelet-activation factor acetylhydrolase (PAF-AH), lysosomal PLA2 (LyPLA2 or PLA2G15), and adipose-specific PLA2 (AdPLA2) [102].

Table 1.1. Major classification of PLA2 superfamily enzymes [103]

Families	Enzymes	Other names	Size (kDA)	Ca^{2+} requirement	Catalytic site	Human chromosome
sPLA2	IIB	Pancreatic PLA2	14	mM	His/Asp dyad	12q23-24
	IIA	Synovial PLA2	14	mM	His/Asp dyad	1p34-36
	IIC		15	mM	His/Asp dyad	1p34-36
	IID		14	mM	His/Asp dyad	1p34-36
	IIE		14	mM	His/Asp dyad	1p34-36
	IIF		16	mM	His/Asp dyad	1p34-36
	V		14	mM	His/Asp dyad	1p34-36
	X		14	mM	His/Asp dyad	16p12-13
	III		55	mM	His/Asp dyad	22q
	XII		19	mM	His/Asp dyad	4q25
cPLA2	IVA	cPLA α	85	< μM	Ser/Asp dyad Ser/Asp	1q25
	IVB	cPLA2 β	110	< μM	dyad	15
	IVC	cPLA2 γ	60		Ser/Asp dyad	19
iPLA2	VIA	iPLA2 β	85-88	None	Ser/His/Asp Triad	22q13
	VIB	iPLA2 γ	90	None	Ser/His/Asp Triad	7q31
PAF-AH	VIIA	Plasma PAF-AH	45	None	Ser/His/Asp Triad	N.D.
	VIIIB	PAF-AH-II	40	None	Ser/His/Asp Triad	N.D.
	VIIIA	PAF-AH-I α 1 subunit	30	None	Ser/His/Asp Triad	N.D.
	VIIIB	PAF-AH-I α 2 subunit	30	None		11q23

Since each subgroup of the enzyme family contains various isoforms, it is estimated that more than 30 types of PLA2 are encoded by the human genome [102]. sPLA2 enzyme is one of the important members of the PLA2 enzyme family. It is the only PLA2 enzyme that demonstrates a distinct preference for arachidonic acid (AA) rather than other fatty acids. sPLA2 is activated via sub-micromolar Ca^{2+} concentrations and phosphorylation. All known ten types of sPLA2s in mammalian cells have a binding loop of Ca^{2+} and a highly conserved catalytic domain [104].

The fourth identified type of PLA2 is cPLA also named as group IV or IVA [105]. The cPLA2 was discovered by the cloning of two novel cPLA2 paralogs that have conserved catalytic sites with a serine residue at the catalytic center [105]. The name is based on the distribution of enzyme as it is found in the cytosol of cells in a resting state. However, naming the cPLA2 is problematic since it is translocated to the cell membrane, nuclear envelope and

endoplasmic reticulum in biologically active cells, despite cytoplasm being the most popular designation [106,107]. As per the historical order, iPLA2 with its various alternatively spliced functional forms was the fifth enzyme that was identified and shown to be independent of calcium to be activated [108].

Another subgroup of the PLA2 enzyme family is PAF-AHs with a specific substrate preference toward unique phospholipid molecules. As PAF-AHs catalyze the deacetylation of phospholipids, they are also named as PAF-acetylhydrolases [109]. PAF-AHs are classified according to their catalytic subunits. There is a secreted plasma-type group and an intracellular form of PAF-AH enzyme group [110]. These two subunits are highly homologous, have significant sequence identity and were classified as group VIIA and VIIB, respectively. The intracellular form of PAF-AHs shows one regulatory and two catalytic subunits [110,111]. The plasma-type PAF-AH has a crucial role in the oxidized phospholipids hydrolysis which contains *sn*-2 residues as a short chain [110]. Moreover, the plasma-type PAF-AH consists of a polypeptide that primarily originates from macrophages. PAF-AH in human plasma, generally associated with low-density lipoprotein (LDL) thus PAF-AH can be called as lipoprotein-associated PLA2 [111,112].

The lysosomal phospholipase A2 (PLA2G15) is another characterized ubiquitous enzyme, which locates to the lysosomes and late endosomes. The crystal structure of this enzyme has been recently characterized [113]. PLA2G15 is calcium-independent and have a transacylase role in the existence of N-acetyl-sphingosine [113].

The last identified group of enzymes is AdPLA, which is responsible for the generation of free fatty acids and lysophospholipids from phosphatidylcholine [114]. Calcium is not essential for AdPLA activity, and it has the lowest lysophospholipase activity among all phospholipases. Further, AdPLA demonstrated no significant activity against other lipid substrates [114].

The structural characteristics of mammalian PLA2s have been established clearly however their physiological role remain a debated subject. Each group of PLA2s has distinct enzymatic properties displayed in tissue expression patterns. This suggests that each group of enzymes have a different phospholipid membrane moiety *in vivo* [101]. In the past twenty years, studies not only identified the roles of PLA2s but also the disorders caused as a result of mutations occurred on these enzymes [115,116]. For example, bee venom was tested as a

therapy against SARS-Cov-2 infection due to the presence of PLA2 [117]. It has been suggested that PLA2 may have long-term therapeutic effects since it tends to bind to the membrane *in vivo*, causes antibody binding to the membrane of the cells, and triggers the stimulation of cytotoxic CD8⁺ T cells, a key mediator of the innate immune system [117].

1.3.3. Secretory Phospholipase A2 Group Enzymes

Secretory phospholipase A2 (sPLA2) is a Ca²⁺-dependent lipolytic enzyme family with low molecular mass and conserved a Histidine-Asparagine (His-Asp) catalytic side. The sPLA2 enzymes are found in a various bacteria, viruses, plants, and vertebrate and invertebrate animals. There are 10 mammalian sPLA2 enzyme groups (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA, and XIIB) that have been discovered so far [118]. The structures sPLA2s contains a well-conserved catalytic domain and Ca²⁺ loops (Figure 1.8.). While disulfides are present in Group I (Cys¹¹-Cys⁷⁷) and group II (Cys⁵⁰-C terminal Cys) sPLA2s, a unique pattern of insertion which can be named as the pancreatic loop exists in sPLA2-IB as well as a cleavable prepropeptide. Group II sPLA2 genes are clustered in the same locus of the chromosome and all members possess a C-terminal extension [103]. In sPLA2-IIC structure additional disulfide bonds exist while sPLA2-IIF has a long chain of C-terminal that contains a free cysteine residue. It has been found that sPLA2-X has a structural feature shared by the group I and II enzymes. sPLA2-IB, -II, -V, and -X subfamilies are similar in their structure [103].

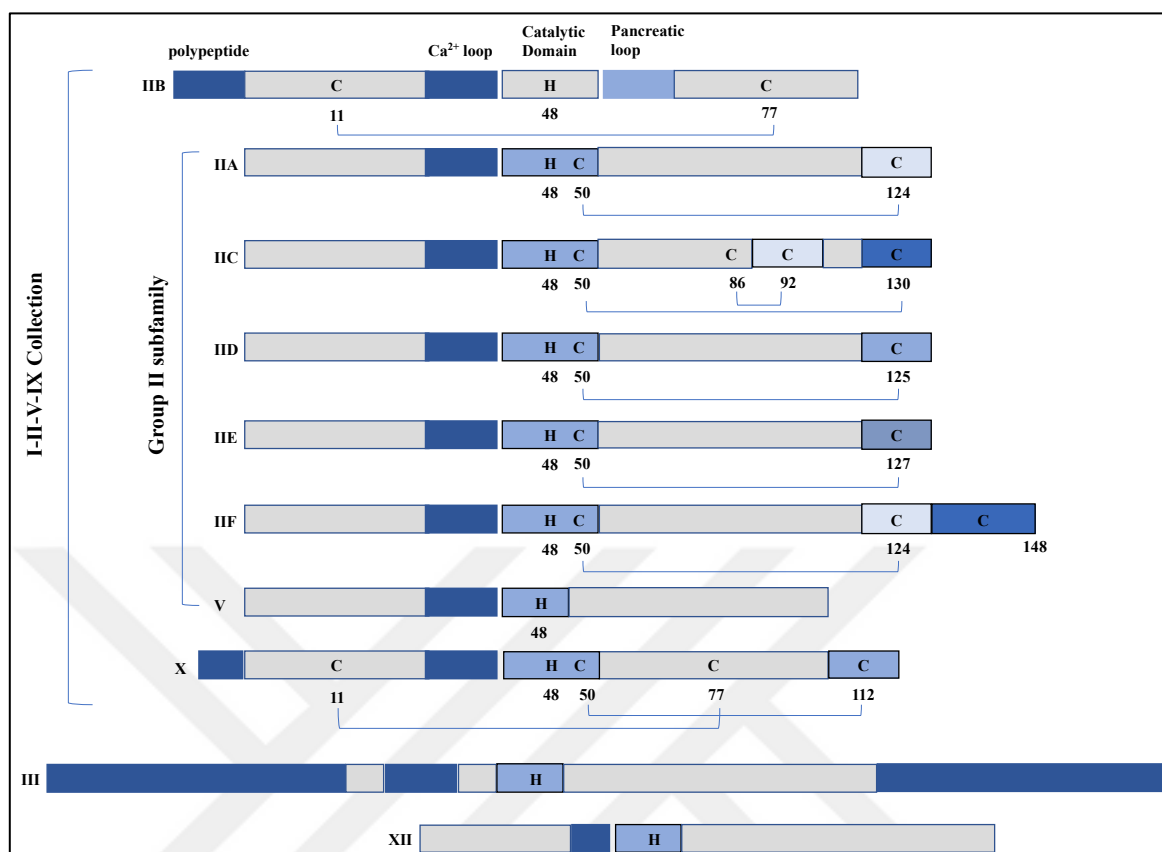


Figure 1.8. The structures of sPLA2 subfamily enzymes [103]

Individual sPLA2s perform their functions by altering the lipid microenvironment and membrane phospholipid compositions through the degradation of foreign phospholipids in microorganisms. The best-known type of sPLA2 which is fundamentally responsible in host defense is sPLA2 group II-A (sPLA2-IIA), which is encoded by the PLA2G2A gene [119]. The sPLA2-IIA have unique role in the degradation of bacterial cell membranes, especially that of Gram-positive bacteria, which is why it is also called the bactericidal sPLA2. The phosphatidylglycerol hydrolysis ability of sPLA2-IIA compromise the bacterial membrane integrity hence appears to fit with the enzyme's antibacterial function [120]. Beside its role in the disruption of bacterial membranes, sPLA2-IIA has an important function in the induction of proinflammatory cytokines and lipopolysaccharide (LPS), hence the enzyme is also called "inflammatory sPLA2." sPLA2-IIA targets the extracellular vesicles' (EVs) phospholipids released from leukocytes at the sites of inflammation or activated platelets [119]. Especially, the release of AA from platelet-derived EVs by sPLA2-IIA is responsible in host defense against foreign microorganisms and triggering the innate immune system [120]. In brief, stimulation of inflammation and the role of sPLA2-IIa in defense against

bacteria relies on not only the degradation of the bacterial membrane but also releasing pro-inflammatory lipid mediators and eicosanoids from EVs (Figure 1.9.) [119]. sPLA₂-IIA also targets the phospholipids in EVs. For instance, sPLA₂-IIA acts on the phospholipids of EVs in extracellular mitochondria that are released from leukocytes or activated platelets at the site of inflammation, since mitochondria have evolved from bacteria. Hydrolysis of these EV phospholipids result in the release of mitochondrial DNA, leading to danger-associated molecular patterns (DAMP) that amplifies the inflammation [99].

Oxidative stress also impacts the number of proinflammatory responses in mammalian cells. It is suggested that under the oxidant stress, cPLA₂ and sPLA₂ cross-talk, resulting in AA synthesis [121]. Moreover, hydrogen peroxide (H₂O₂), one of the reactive oxygen species, triggers the expression of sPLA₂-IIA through the NF- κ B signaling pathway. Thus, H₂O₂ also triggers the AA release [121].

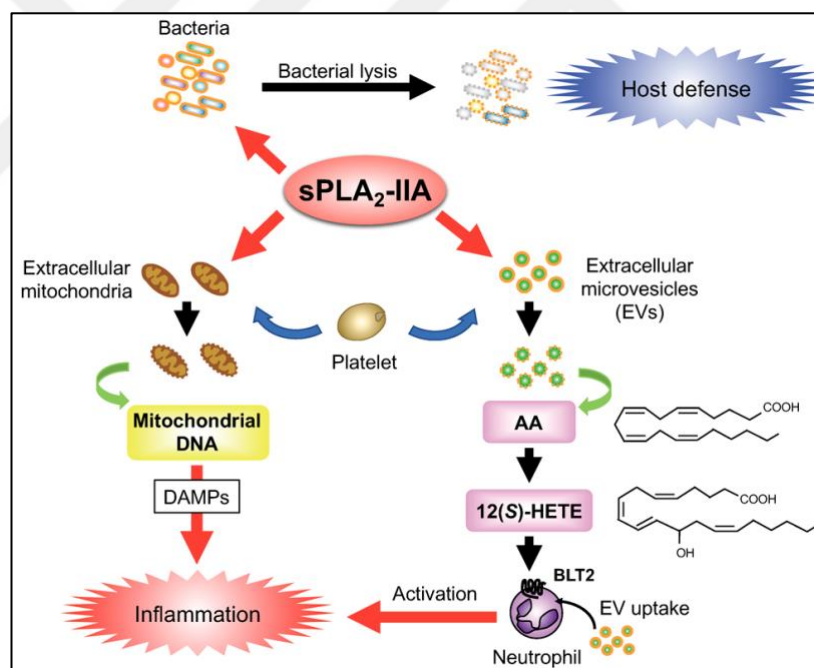


Figure 1.9. Defense mechanism of sPLA₂-IIA against bacteria [99]

The role of sPLA₂-IIA also appears in defense against the pathogen *Plasmodium falciparum*, which causes malaria [122]. Various sPLA₂s hydrolyze the plasma lipoproteins in order to release fatty acids, meaning that they have the capacity to prevent the growth of the parasite. Although this was shown only *in vitro* but not in human plasma, sPLA₂-IIA was found to increase in the plasma of patients with malaria and sPLA₂-IIA hydrolysis of oxidized

lipoproteins was found to prevent the growth of Plasmodium [123]. Recent studies also demonstrated that sPLA2-IIA triggers the growth of hair follicles through the intracellular signaling pathway of c-Jun N-terminal kinases (JNK) [124]. Additionally, sPLA2-IIA has been recognized as a mediator in tumor formation in *in vivo* models [125]. While the action mechanism of sPLA2-IIA in cancer has not been fully comprehended yet; recent studies indicated a potential mechanism where sPLA2-IIA expression in the small intestine prevents the growth of colon cancer [126]. In order to maintain homeostasis, sPLA2 enzyme suppresses the Wnt signaling through Yap1 activation [126]. However, upon initiation of inflammation, the secretion of sPLA2-IIa from the intestinal lumen promotes the synthesis of prostaglandin E2 (PGE) and Wnt signaling [126]. Moreover, it has been reported that sPLA2-IIA increases the susceptibility of gastrointestinal cancer to therapy, even though the underlying mechanism is still being explored [127]. Thus, sPLA2-IIA might have dual effects in tumorigenesis depending on the cellular state and cancer type.

1.3.4. Secretory Phospholipase A2 Group-IIA Enzyme and Cancer

The sPLA2-IIA expression is maintained through various growth peptides, transcription factors, and cytokines, based on the type of the cell [128]. Although sPLA2-IIA has a role in inflammation, the effects of the cytokine-related signaling pathways on sPLA2-IIA are still being investigated. This question is important in many terms since the relationship between proinflammatory factors interferon- γ , TNF- α , and interleukin-6 and the progression of cancer is well known. Thus, it was suggested that transcription factors have a pivotal role in transmitting the cytokine signals of proinflammatory cytokines during the development of tumors [129]. NF- κ B and STAT family transcription factors are involved in cytokine release and are essential for the sPLA2-IIA promoter activity [130].

NF- κ B regulates the synthesis of arachidonic acids from eicosanoid precursors [131]. NF- κ B also involved the growth of PCa cells. Moreover, overexpression of NF- κ B has been detected in most of the prostate tumors [132]. Elevated sPLA2-IIA serum levels are also associated with advanced-stage PCa and a high Gleason score [133]. In related studies, it has also been demonstrated that in androgen-independent PCa, the level of sPLA2-IIA is overexpressed [134]. According to these findings, it has been suggested that elevated levels of sPLA2-IIA lead to increased HER/HER2-elicited, NF- κ B- and PI3K-Akt-mediated

signaling pathways, contributing to the PCa progression. In turn, it was found that overexpression of PI3K-Akt, HER/HER2 and NF- κ B signaling induce the elevation of sPLA2-IIA serum level in PCa [135]. Thus, it can be suggested that elevated serum sPLA2-IIA levels can be important in PCa progression [134].

The NF- κ B-mediated pathway cross talks with PI3K-Akt signaling by phosphorylation of Akt and promotes the activation of an upstream kinase of NF- κ B signaling called inhibitory-kappa B kinase (IKK). Small molecule inhibitors gefitinibi bortezomib, bapatinib, c(2NapA), LS(2NapA) R, cFLSYR CI-1033, LY294002 act against the EGFR, PI3K, HER2, NF- κ B, and sPLA2-mediated pathways (Figure 1.10.) [132,134].

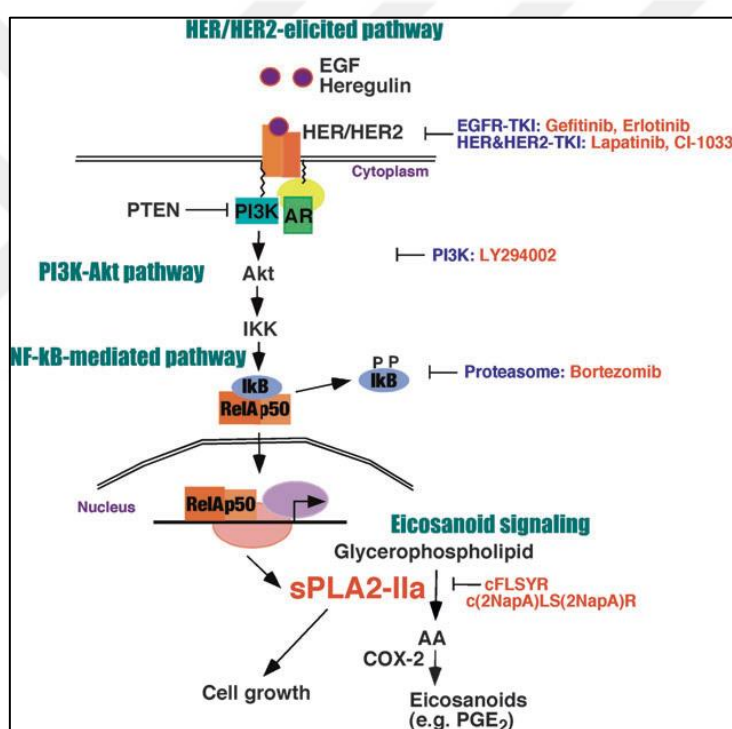


Figure 1.10. HER/HER2 signaling pathway in PCa cells [136]

According to the recent findings, eicosanoid signaling elevation has been identified in PCa pathogenesis and progression [134]. Various genes which are responsible such as NF- κ B, sPLA2-IIA, and cyclooxygenase-2 have a unique role in the eicosanoid biosynthesis pathway and found to be overexpressed in PCa hence implicated in the PCa progression [65,132].

Most of sPLA2-IIA molecules are stored in the granules found in the cytoplasm and released into the extracellular microenvironment by the activation of the cell under inflammatory conditions [123]. Furthermore, gene expression of sPLA2-IIA can be also amplified by priming inflammatory markers such as IL-1, IFN- γ , IL-6 and TNF- α under pathological conditions [137]. In PCa cells, elevated serum level of sPLA2-IIA suggests that there can be various alternative cell signaling pathways stimulating the sPLA2-IIA and supporting the progression of androgen-independent PCa [138].

1.4. SINGLE NUCLEOTIDE POLYMORPHISM

Single nucleotide polymorphism (SNP) is the most frequent type of genetic variation among all species. An SNP is a sequence variation of DNA where a single nucleotide differs from the expected nucleotide in the genome [139]. Each SNP represents a difference in DNA building blocks. GWASs suggested that the occurrence probability of SNPs is once in every 1000 nucleotides on average in the human genome [140]. A nucleotide variation is identified as a SNP when the frequency of the minor allele exceeds one percent in at least one population; otherwise, it is referred to as a mutation or variant [141]. The variations of SNPs may be unique to an individual, or they may be common for many individuals. These common variations can act as biological markers that can be associated with the prevalence of diseases [142]. SNPs do not always affect the health and development of organisms; however, they have a remarkable role in association with the risk of developing particular diseases such as heart diseases, diabetes, and cancer [143]. Moreover, SNPs can be utilized for tracking the inheritance of disease genes within generations [144].

The first systematic study about single base variants was performed through the identification of endonucleases' digestion site in which a single base pair (bp) alters the restriction site. Digestion of the relevant part of the DNA with appropriate endonucleases could distinguish variants or alleles based on the fragment size in electrophoresis. This polymorphism was called as restriction fragment length polymorphism (RFLP) [145]. Although the occurrence probability of SNPs is greater than that of RFLPs alone, precise estimations cannot be done, especially considering the populations and genomic regions.

SNPs can be found in any region of the genome, such as introns, exons, promoters, enhancers, and intergenic regions. SNPs found in the coding region can be directly effective

on the function or structure of a protein [146]. An intronic SNP can also influence the splicing mechanism [147]. The degree of phenotypic expressions is determined based on the location and type of the SNP, which is still being studied [142]. In the normal human genome, two copies of each autosome and two copies of sex chromosomes exist. These two alleles have both maternal and paternal origins that are ninety-nine percent identical in their DNA sequence [148]. A difference between these two alleles is genetic variations, which include the SNPs. If the maternal and paternal alleles have identical nucleotides, it is called a homozygous SNP, and if the parental alleles have different nucleotides, it is called a heterozygous SNP (Figure 1.11.).

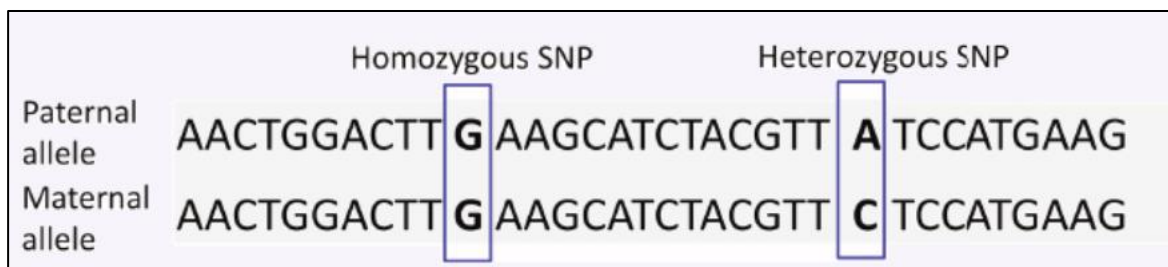


Figure 1.11. Homozygous and heterozygous SNPs in human genome [148]

Homozygous SNPs are also referred to as non-informative since the two alleles cannot be distinguished. According to recent studies, nearly thirty million SNPs are registered in the SNP database (dbSNP) [149] and this genome-wide variation is associated with many types of cancers [150]. Accordingly, the role of SNPs in the PCa progression has gained increasing attention. The rising number of SNPs identified in PCa is reported by various studies [128,151].

1.5. THE CRISPR/CAS9 SYSTEM

The concept of genome engineering was born in 1970s by the time various research groups demonstrated that fragments of exogenous DNA could be possibly obtained from bacteria or yeast and can be integrated into the genome randomly [152]. Subsequently, it was discovered that this process could be used in a targeted manner. In 1989, Martin Evans and Oliver Smithies formed the knockout *in vivo* model for the first time and the experiment was a resulted successfully for genome engineering [153]. Genome engineering, especially the

use of endonucleases as a tool, is a powerful and robust technique for high precision genome alteration [154,155].

Clustered regularly interspaced short palindromic repeats (CRISPR) is regarded as revolutionary innovation in genome engineering [155]. CRISPR offers multiplexing and scaling, as well as flexibility, making it the most promising gene editing tool developed so far. Prokaryotes utilize CRISPR/Cas as the main defense against viral invaders [156]. The sequences of CRISPR were initially discovered in bacterial genome in 1978; however, their function and mechanism were not fully elucidated until 2007 [157]. The first experiment was performed using *S. thermophilus*, a bacterium responsible for cheese and yogurt production [158]. The bacterium was exposed to predatory phages in order to test the incorporation of their genetic material into the bacterial genome in the form of CRISPR repeats.

CRISPR-associated (Cas) genes encode the nucleases, helicases, and polymerases in bacteria. Scientists suggested that prokaryotes have an improved immune system that uses different Cas proteins to record the gene fragments of invading phages and destroy them [158]. Specialized Cas proteins chop foreign DNA by an endonuclease activity, and, as a result, small fragments of approximately 30 bp are formed. The chopped fragments are then pasted into the CRISPR sequence in the bacterial genome. In the meantime, distinct Cas enzymes operate the CRISPR loci and form the CRISPR RNAs (crRNAs). By the aid of sequence homology, the generated crRNAs guide Cas endonucleases to the target site of the DNA or RNA, which is located three bp upstream of a species-specific protospacer adjacent motif (PAM). Consequently, the CRISPR/Cas complex binds to the invasive organism's genetic material and destruct the invader by cleaving its genetic material (Figure 1.12.) [159].

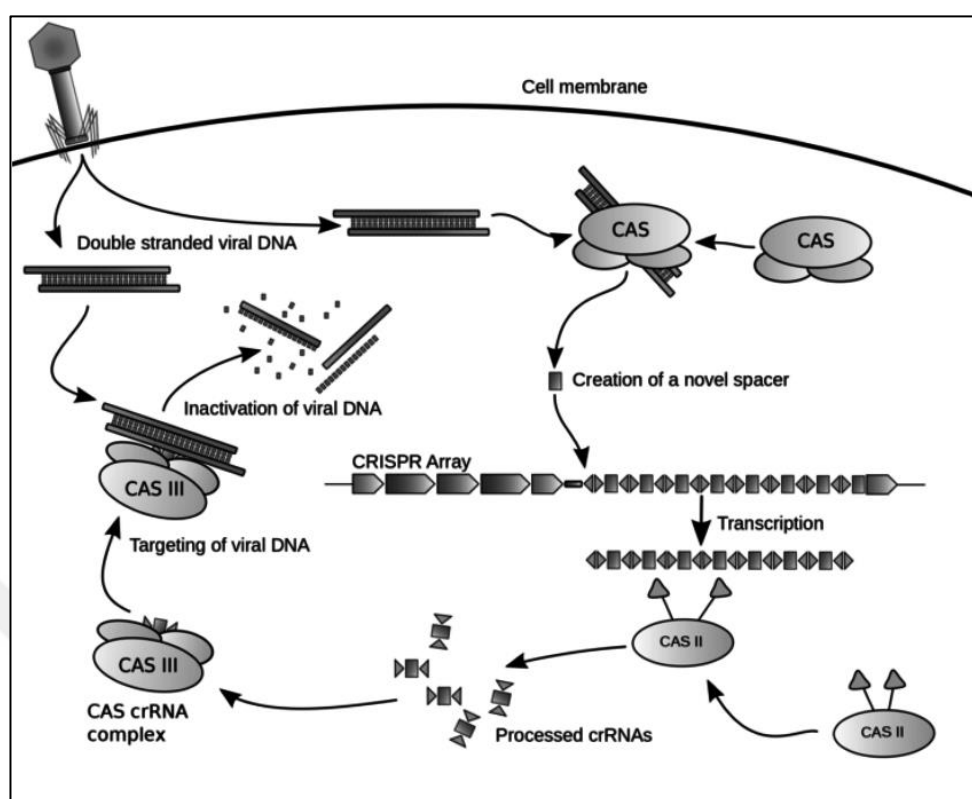


Figure 1.12. CRISPR/Cas complex act of action activity in bacterial immune system [159]

The CRISPR system is found in forty-eight percent of bacterial and ninety-five percent of archaeal genomes, with variations in the PAM sequences and Cas proteins types [160].

1.5.1. Components of the CRISPR/Cas9 System

Basically, the CRISPR/Cas9 genome editing system utilizes non-specific endonucleases. Cas9 and Cpf1 are the most common enzymes that cleave DNA, and a small RNA sequence called guide RNA (gRNA), as the name implies, guides endonucleases to the site of cleavage [161]. Together, gRNA and Cas9 enzyme constitute the scaffold (Figure 1.12.). While the native CRISPR/Cas system employs various alternatives of endonucleases, in genome editing, Cas9 or its variants are the only endonucleases that CRISPR requires. The reason for that is, Cas9 requires three basic properties that are necessary for the system to work. First, the endonuclease should have the capacity to bind to gRNA. The gRNA leads Cas9 to cut the genome at a specific target of various possible loci; without gRNA, Cas9 is not able to cleave any region of the DNA. The second characteristic that is essential for the CRISPR/Cas

complex is the presence of the PAM [162]. The binding of the Cas9 enzyme to the target site of the genome, is mediated by the gRNA which recognizes the target sequence and the sequence known as PAM [163]. For the formation of the double-strand break (DSB), the PAM sequence located in downstream (3') of the site of DSB [164,165]. In the absence of either the PAM sequence or gRNA, the Cas9 enzyme cannot bind or cut the target region. The PAM sequence consists of the NGG bases, which is the most abundant genotype in the whole genome. Thus, CRISPR/Cas9 can be used at any desirable genomic locus just upstream of a PAM sequence, which can be found in large range of regions [166]. While PAM sequences are abundant for genome editing, they are not always in a correct position for CRISPR/Cas9-mediated gene editing. Thus, a target sequence may be found elsewhere in genome. The sequence that can be targeted for CRISPR/Cas9 system with the wrong position called as off-targets. Off-targets can also be altered non-specifically during the genome editing [167]. The third and last basic characteristic that the endonuclease should have, is the cleavage of DNA in a targeted manner. Cas9 and its variant enzymes have two nuclease domains close to the protein center which are n-terminal RuvC and HNH catalytic domain. When Cas9 nuclease binds to the target genomic region, the Cas9 endonuclease undergoes a conformational change, thus the positions of nuclease domains are changed and cut the target DNA's opposite strand. As a result, a Cas9-mediated DNA DSB occurs within the target DNA from 3 to 4 nucleotides upstream of the PAM sequence [168]. In the prokaryotic CRISPR/Cas system, Cas endonuclease is guided to the target gene region by two RNAs: the crRNA and tracrRNA. The crRNA defines the target sites for the Cas9 endonuclease, while tracrRNA acts as scaffold by linking the crRNA in order to facilitate the processing of mature crRNAs. In most utilized CRISPR-mediated gene editing systems, the crRNAs and tracrRNAs are combined into all-in-one RNA sequence, gRNA or single gRNA (sgRNA). The gRNA consists of a 20-nucleotide that directs Cas9 to cleave the target genomic locus and a sequence of the scaffold for Cas9 binding. Next, through the interactions of positively charged surface grooves on Cas9 and gRNA scaffold, a ribonucleoprotein complex is formed [169]. Thus, Cas9 undergoes a conformational change by binding the gRNA that alters the molecular form of the Cas9 protein. The conformation of Cas9 protein shifts from an inactive, non-DNA binding form to an active DNA-binding motif. Upon this change, Cas9 can efficiently bind to the target genomic loci. Once the Cas9 enzyme binds to the target region of genome, at the 3' end of gRNA, it will initiate the annealing of the DNA in 3' to 5' direction. As a result, DSB is initiated. The resulting DSB

is repaired by two general pathways; the non-homologous end joining (NHEJ) and homology directed repair (HDR) pathways [50].

1.5.2. CRISPR/Cas9 System Repair Mechanisms

1.5.2.1. NHEJ Repair Pathway

The NHEJ repair is the canonical repair mechanism in CRISPR; however, it is error-prone, and sometimes causes insertions or deletions (indels) at the sites of DSB [170]. Mostly, the indels that occur in the target site of DNA cause alterations in amino acid structure, leading to the formation of premature stop codons [171]. There are three basic factors that are required for the NHEJ repair system. Initially, target DNA sequences are identified by the formation of the Ku70/Ku80 protein heterodimer [172]. As a second step, the formed heterodimer acts as a scaffold to recruit unique DNA-dependent protein kinases and also the two subunits of DNA ligase IV. Together, they form a paired end complex, holding the pair of DNA ends of the DSB, with the aid of X-ray repair cross-complementing protein (XRCC) and the paralog of XRCC. Lastly, the paired end sites of the DNA fragments are ligated, and the repairing of the DSB is accomplished [173,174].

1.5.2.2. HDR Pathway

While NHEJ is the canonical way of repair in CRISPR, HDR is a more error-free and accurate repair system that preserves the DNA (Figure 1.13.) [175]. In this mode of DNA repair, a donor template sequence is introduced to the system, which promotes precise homology to the damaged strand of DNA. There are three basic steps for the HDR pathway to progress. Initially, the 5' end of the target strand of DNA is cleaved to form a 3' overhang. This overhang serves as a primer required for the polymerase, as well as a substrate for proteins that act in the “strand invasion” process. The invasive strand of the donor DNA displaces one strand of the homologous target DNA while the pairing is initiated using the other. This results in a DNA hybrid formation and displacement of the D loop. As a last step, the 3' single-stranded DNA (ssDNA) invades through the homologous DNA complex. At this point, the HDR is completed [176].

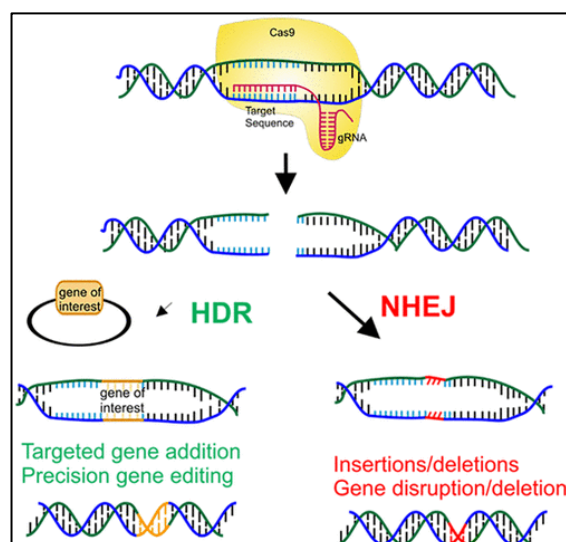


Figure 1.13. Repair pathways of DSB [177]

The HDR can occur conservatively or non-conservatively. The non-conservative system involves the single-strand annealing (SSA) pathway, while the conservative method is identified as the precise repair of the DSB by introducing a homologous template, such as the sister chromatid or an engineered-plasmid [178]. The conservative method consists of three main pathways: synthesis-dependent strand-annealing (SDSA), the DSB repair (DSBR), and break-induced repair (BIR) (Figure 1.14.). Mostly, crossover takes part in the canonical DSB pathway that occurs semi-conservatively. The DSBS initiates the invasion of the 3' end to an intact homologous template. The 3' ends provide primers for DNA synthesis, which leads to the formation of double Holliday junctions (dHJs). The four-stranded branches of dHJs are formed by the time while the invasive strand elongation and DNA synthesis occur from the end of DSB. Each of the dHJs are then separated via cleavage [179]. Resolution of each junction occurs on either the crossing or non-crossing strand. If cleavage occurs dissimilarly, crossover will occur, while if both dHJs are resolved similarly, resulting in a non-crossover process. The SDSA is a fully conservative event, and particularly results in non-crossover events. Therefore, the newly synthesized strand sequence exists on the same molecule. Different than DSBR, the formation of a D loop in SDSA and invasion of the strand promote the synthesis of invasive strand, which is substituted from the donor template. The newly synthesized invasive strand appears as a D loop in SDSA which is replaced from the old template, and returns to the 3' protruding end

of the non-invading strand at the other DSB. Thus, the strand is elongated and ligated and SDSA is completed [178].

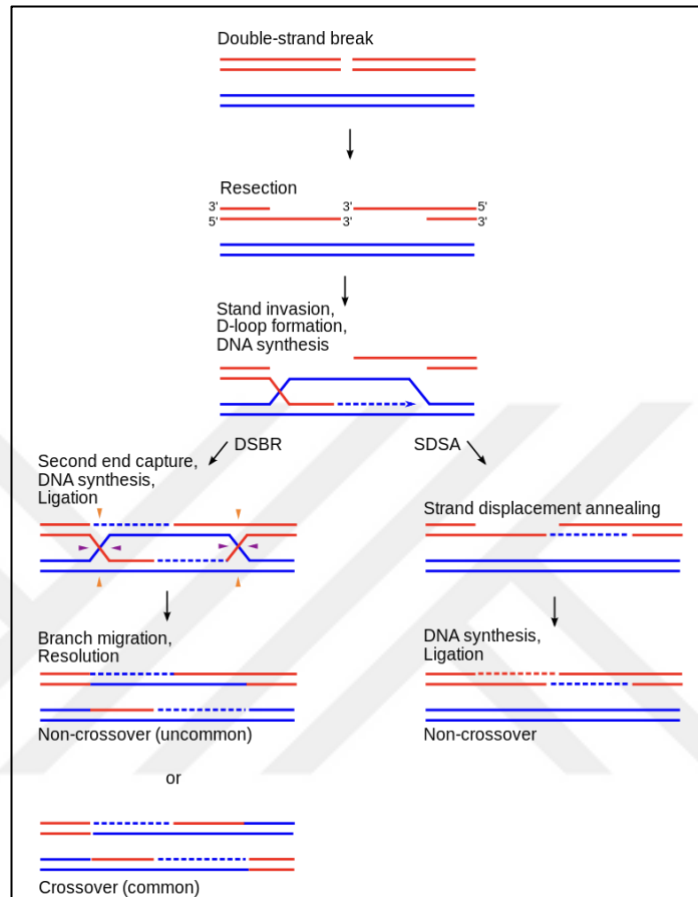


Figure 1.14. Illustration of DNA repair of DSB by DSBR and SDSA [179]

The BIR pathway has not been fully characterized. A crucial feature of the BIR pathway is the presence of a single invasive strand at the end of the DSB, which can be used for the repair [180]. The single invasive strand then migrates to the homologous sequence; thus, the synthesis of lagging and leading strands is initiated, which ends up with the formation of a single Holliday junction [181]. The Holliday junction is resolved by cleaving the crossed strand. Although the pathway is not instantly applicable in DSB-induced gene editing, it has a biological importance, for instance, in chromosomal repair, where no second end is present to enable the SDSA repair or DSBR [182].

The HDR mechanism and donor templates are utilized to form point mutations or insert new elements into the gene of interest. Scientists have been successfully utilizing homology arms, which start at the site of DSB by CRISPR induction [182]. The homology arm length is a

crucial feature to be considered when designing the donor templates. However, there are several limitations [179]. Generally, the modification sites of insertion should be less than 100 bp proximity from the site of the DSB, while a 10 bp proximity is the optimum value by means of efficiency [183]. Another important point is that once DSB occurs, the Cas9 enzyme does not stop cleaving as long as the gRNA, the target site of DSB, and the PAM site remain the same. The Cas9 enzyme keeps cleaving the DNA, and the DSB keeps being repaired via either NHEJ or HDR [184]. This situation can cause a problem as it can create specific mutations leading to the off-target effect. To overcome this problem, the HDR template can be designed in a special way that it will prevent the cleavage activity of Cas9 after the initial DSB occurs [181,185].

The HDR efficiency is generally very low (less than ten percent in modified alleles) even in cells that already express the gRNA, Cas, and donor repair template. Therefore, in scientific studies, cells are usually synchronized at the S or G2 phases to enhance the HDR efficiency, since HDR occurs during progression from S to G2 phase [184].

1.5.3. CRISPR Expression Systems and Delivery Methods

There are many factors that is required to be considered, while designing CRISPR-based expression systems. First, the delivery method should be selected. Usually, one of the two major methods, delivery vehicles or cargo molecules, is selected based on their suitability for the cargo molecules to be used. Regarding the CRISPR cargo molecules, there are three common approaches that are used; (i) a plasmid DNA which encodes both gRNA and Cas9 endonuclease, (ii) a mRNA for the translation of Cas9 endonuclease along with the gRNA and (iii) a ribonucleoprotein (gRNA and Cas9 endonuclease) complex [186]. The delivery vehicles are tested for their packing ability to determine which of the three cargo molecules can be carried and for their suitability to be used in *in vitro* or *in vivo* investigations [187]. Common delivery methods include physical delivery and the use of viral or non-viral vectors (Figure 1.15.) [188,189].

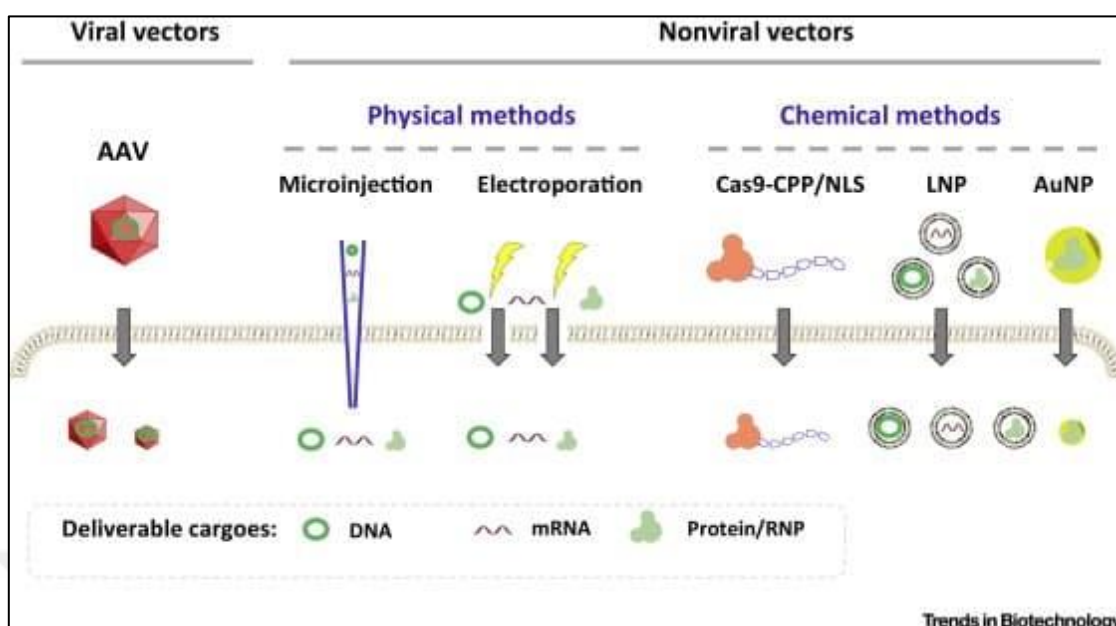


Figure 1.15. Schematic representation of CRISPR delivery systems [189]

The fundamental physical delivery methods are the electroporation and microinjection. In the electroporation technique, high-power electrical currents are used to transiently create nano-meter-scale pores within the membranes of the cells. Thus, the target cargo molecule can enter into the cell. Microinjection is the transferring of cargo molecules into cells using a microinjection needle that ranges between 0.1 to 10 μm in diameter [190].

Viral vectors such as adeno-associated viruses (AAVs) and lentiviruses, are particularly preferred for *in vivo* CRISPR/Cas9 studies. As non-viral vector systems, lipid nanoparticles, DNA ‘nanoclews,’ conjugated gold nanoparticles, and cell-penetrating peptides are commonly chosen for the mammalian cell manipulation [191,192]. Since nucleic acids are highly anionic and unstable in nature, delivery of the CRISPR/Cas9 system through the membrane is not easy. However, by the encapsulation of nucleic acids within liposomes, cationic lipid coated molecules, transport through the cell membrane is facilitated. Moreover, nanoparticle-based delivery methods are safer than viral delivery methods, considering immunological concerns [193].

1.6. AIM OF THE STUDY

PLA2 enzymes control the regulation of inflammation by triggering the secretion of lipid mediators through the phospholipid hydrolysis [194]. PLA2G2A is a member of group II of the sPLA2 enzyme family, which acts as a secreted molecule and controls the actions of the adaptive immune system [195].

Deficiencies in the control mechanism of the immune system impacts cellular homeostasis, which promotes the development of cancer [196]. Our recent study investigated the association of PLA2G2A rs11573156 C>G polymorphism in 5' untranslated region (5'UTR) with PCa progression and showed that G allele shows an anti-metastatic effect on PCa [197]. Previous reports implicated that the G allele causes exon 2 skipping during the transcription process and leads to enhanced mRNA and, eventually, serum levels of PLA2G2A [198]. Based on these findings, this thesis aimed to identify effects of the polymorphic G allele on PCa by establishing a model PCa cell line with GG genotype using the CRISPR/Cas9 gene editing system. In this context, the effects of the homozygous G allele on PLA2G2A gene expression were investigated in a response of H₂O₂ and interferon- γ that mimics the inflammatory TME. Moreover, the effect of gene-editing on cell proliferation was investigated using the human PCa cell line PC-3, harboring the homozygous G allele.

2. MATERIALS

2.1. INSTRUMENTS

CO₂ incubator (Nuair NU5510/E/G, USA), Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet 2A, Singapore), Waterbath (Stuart, SBS40, UK), Zeiss PrimoVert Microscopy with Axio Cam 105 color (GmbH 37081, Germany), Microcentrifuge (Beckman Coulter, A46473, Germany), Vortex (Stuart SA8, UK), Centrifuge (Gyrozen M416, USA), pH meter (Hanna instruments PH211, Germany), -80°C freezer (Thermo Forma -86 C ULT Freezer, USA), ChemiDOC XRS+Gel Imaging System (Biorad Universal Hood 2, USA), Heater (Bioer, MB102, China), SpectraMax Paradigm Spectrometry Multi-Mode Plate Reader, Weighing Scale (Shimadzu, TW423L, Japan), StepOne™ Real-Time PCR System, BD FACS Aria™ III Cell Sorter, Maxicell Electrophoretic Gel System (EC360M), Thermal Cycler (Applied Biosystems).

2.2. EQUIPMENT

Cell culture flasks (T-25 and T-75, 96 well-plate, 6 well-plate, 24 well-plate) (TPP Switzerland or Grenier-Bio, Germany), falcon tubes (15 mL, 50 mL Isolab, Germany), Electronic pipette (CAPP aid, Denmark), Polypropylene eppendorf tubes (1ml, 1.5 ml, 2 ml Germany), PCR Tubes (0.2 ml, Axygen). Micropipettes (10µL, 200µL, 1000µL, Thermo Scientific, USA) and Pipette Tips (10 µL, 200 µL, 1000 µL, CAPP Expell Plus, Denmark), Serological pipettes (2ml, 5 mL, 10 mL, 25 mL, SPL Life Sciences, USA), cryovial (2 mL, NEST, China), Hemocytometer (Marienfeld, Germany), Cover slip (Sigma Aldrich, USA), Graduated Cylinder (50 mL, 250 mL, 500 mL, 1000mL Isolab, Germany), 0.22 µm filter (TPP, Switzerland), PCR Plate 96-well (ThermoFisher Scientific), Liquid Nitrogen Tank (Arpece 110, France), Thermal Cycler (Applied Biosystems, UK).

2.3. CELL LINES AND CHEMICALS

PC-3 Cell Line (ATCC CRLR-1435, USA), RPMI-1640 Medium (Gibco, BE12-702F, UK), Hydrogen Peroxide (Brtr Chemistry, Turkey), Fetal Bovine Serum (FBS) (Sigma F9665, Germany), 100X Penicillin-Streptomycin (Gibco, UK), Recombinant Human IFN- γ (Biolegends, 570204), (50 bp DNA Ladder (New England BioLabs, B7025), s_PLA2G2A_2_SG QuantiTect Primer Assay (Qiagen, 249900), Sodium Chloride 71376 (Sigma), Q5 High Fidelity DNA Polymerase (New England BioLabs, M0491L), Agarose (Sigma, A9536), Absolute Ethyl Alcohol (MERCK, 1.00983.2511), Isopropanol (Sigma, 24137), 0.5 percent Trypsin-EDTA (Lonza, BE02-007E, Belgium), Cell Proliferation Reagent WST-1 (Merck, 11644807001, Germany), Sensiscript RT Kit (Qiagen, 205213, USA), Trizol (Invitrogen, 15596-018), 6X Loading Dye (Thermo Scientific, R0611) Triton-100X (Biomatik Corporation, A4025), SYBR Safe DNA gel stain (Invitrogen, Germany), TE Buffer (Invitrogen, Germany), VB180618-1024xfd (VectorBuilder, USA), donor DNA (IDT, USA), Dulbecco's Phosphate Buffered Saline (DPBS) (PAN Biotech P04-53500, Germany), Applied Biosystems™ Power SYBR™ Green PCR Master Mix (4367659, UK), E.Z.N.A.® Tissue DNA Kit (Omega, D3396), QIAamp DNA Micro Kit (Qiagen, 69504), Ampliqon PCR Enzymes and Reagents (Denmark, 5200350), sPLA2 Activity Kit (Enzo, 907-002), LB Broth (Sigma, L3522, USA), Dimethyl sulfoxide (Santa Cruz sc-202581, USA), Propidium Iodide (Invitrogen, P1304MP).

3. METHODS

3.1. CELL CULTURE METHODS

3.1.1. Cell Lines and Culturing Conditions

For cell culture, PCa cell line acquired from bone metastasis of PCa, PC-3, was acquired from American Type Cell Collection (ATCC-CRL1435). PC-3 cell were cultured in RPMI 1640 media complemented with 100 units/ml Penicillin, 100 µg/ml Streptomycin, and 5 µg/mL Amphotericin B (1 percent PSA), and 10 percent (v/v) fetal bovine serum (FBS). PC-3 was kept at 37°C, 5 percent CO₂ and 95 percent humidified air conditions.

3.1.2. Cell Sub-Culturing

The sub-culturing procedure of PC-3 was initiated by the time the cells reached to the 70 to 80 percent confluency. The existing medium was discarded and cells were washed with 1X DPBS (pH 7.4). Afterward, the cells were detached using 1 percent (w/v) trypsin with 2 mM EDTA and incubated at incubator at 37°C for 5 minutes. In order to stop the activity of trypsin, 10 percent (v/v) FBS containing growth medium was added at two times the volume of trypsin and then the cell suspension was collected to be centrifuged at 300 *xg* for 5 minutes. Finally, cell pellet was resuspended in the growth media.

3.1.3. Cell Counting and Determination of Cell Number

Following the sub-culturing procedure, 10 µl from the cell suspension was taken and loaded into a hemocytometer to count the cells. Afterward, the cells located in the middle square of the hemocytometer were counted under an inverted light microscope using a 10X objective. The total cell number in one-milliliter volume was calculated by using the following formula: The number of counted cells x dilution factor / mm² x chamber depth.

3.1.4. Cell Cryopreservation

Following the cell sub-culturing procedure, the number of the cells were counted as described in Section 3.1.3. Afterward, an appropriate amount (about 1×10^6) of the cells were centrifuged once again and resuspended in 1 ml of the cell freezing mixture composed of 10 percent (v/v) dimethyl sulfoxide and 90 percent (v/v) FBS. The cell suspension was then transferred into a fresh cryovial and frozen initially at -80°C . Next day, the cells were placed into the liquid nitrogen for the long-term storage.

3.1.5. Cell Thawing

Cryovials containing the frozen cells were rapidly warmed to 37°C . Afterward, the cell suspension was placed into a sterile 15 ml centrifuge tube, and 5 ml of growth medium was gently placed into the cell suspension, drop by drop, in order to prevent the damage to the cells due to a rapid increase in the osmotic pressure. Next, the suspension of cells was centrifuged at $300 \times g$ for 5 minutes, and cell pellet was collected to be gently resuspended in the growth medium. Following the cell seeding into a tissue culture flask, the medium was replenished by fresh medium to remove the cell debris and DMSO released from the cells.

3.2. GENOTYPE ANALYSIS OF PC-3 CELLS

As an initial step, DNA isolation from PC-3 cells was performed based on the EZNA DNA Isolation Kit procedure. 300,000 cells were harvested using the 1 percent (w/v) trypsin-EDTA solution, washed with cold (4°C) 1X PBS as twice, and resuspended in 200 μl of 1X PBS. Next, 25 μl OB Protease Solution was added and vortexed to mix thoroughly, and 220 μl BL Buffer was placed into the cell suspension. The sample solution was incubated at 70°C for 10 minutes and briefly vortexed once during incubation, at the 5th minute of incubation. The sample was then washed with 220 μl of absolute (100 percent) ethanol and vortexed to mix thoroughly. Following the loading of the sample solution into HiBind[®] DNA Mini Column, and the elute was collected into a 2 ml collection tube, which was centrifuged at $14,000 \times g$ for 1 minute. The filtrate was discarded and the column was washed with 500 μl

of HBC Buffer to be centrifuged again at 14,000 xg for 30 seconds. The column was then placed onto a fresh 2 ml microcentrifuge tube and 700 μ l of DNA Wash Buffer was added into the column. Following two consecutive washing steps followed by centrifugation at 14,000 xg for 30 seconds, the column was placed onto a fresh 1.5 ml nuclease-free tube and 100 μ l of Elution Buffer pre-heated to 70°C was added onto the column. After a 2-minute incubation and a consecutive centrifugation at 14,000 xg for 30 seconds, the elution step was performed once again with 50 μ l of Elution Buffer. The eluted DNA was then measured using the NanoDrop. Afterward, the target PLA2G2A region containing the rs11573156 polymorphism was amplified using Q5 High Fidelity PCR enzyme, 1 μ g template DNA, and forward (5'-CCCAATCCTCAACTCTGTCCTG-3') and reverse (5'-GAGGTAAAGAGCCAGCGAAGC-3') primers with the given amounts in Table 3.1. For identify the success of PCR experiment, the visualization of the amplified PCR products was performed by agarose gel electrophoresis. To prepare a 1.8 percent (w/v) agarose gel, 1.8 grams of agarose powder was dissolved in 100 ml of 1X TAE buffer (40 mM Tris-Base, 5 mM NaAc, 1 mM EDTA and pH 8.0;). 5 μ l SYBR Safe (10000X) was added to the solution. 20 μ l of the PCR products were loaded into the gel by mixing with 4 μ l of 6X gel loading dye. The gel was run at 80V for 100 minutes. Afterward, the run was completed, images were taken using Molecular Imager® (Bio-Rad ChemiDoc™). The PCR product was sent to Sanger Sequencing by Sentebiolab, Turkey. The acquired sequence result of 5' UTR PLA2G2A gene region, 236 bp, was analyzed by SnapGene and then sequence results were aligned with the theoretical gene region by online tool Genome Compiler.

Table 3.1. PCR Master Mix Reagents

Nuclease Free H ₂ O	10.75 µl
Forward Primer	1,25 µl
Reverse Primer	1,25 µl
5X Buffer	5 µl
dNTP	0,5 µl
Template	1 µl
Q5 High Fidelity Polymerase	0,25 µl
GC Enhancer	5 µl

3.3. CONSTRUCTION AND ISOLATION OF THE CRISPR PLASMID AND DONOR TEMPLATE

3.3.1. Designing the sgRNA- and Cas9-containing Plasmid Vector and the Donor Template

pRP[CRISPR]-EGFP-hCas9-U6>{PLA2G2A sgRNA1} plasmid vector (pRP-CRISPR/Cas9) was designed and purchased from VectorBuilder. The plasmid vector contains the origin of replication PUC; human U6 small nuclear 1 promoter, which promotes the small RNA molecules expression. In the system human U6 small nuclear 1 promoter was responsible for the expression of coding sequence of the single guide RNA (sgRNA) for recognition of the target sequence. The cytomegalovirus (CMV) early enhancer fused to modified chicken β -actin promoter (CBh promoter) was placed in the 5' of human codon-optimized CRISPR-associated protein-9 obtained from *Streptococcus pyogenes* (SpCas9), which generates DNA double-strand breaks. CBh is a strong promoter that promotes the transcription, and polyadenylation by Pol II RNA polymerase. The vector also contained a bovine growth hormone polyadenylation signal (BGH pA), which is not only terminates the transcription but also allows the polyadenylation of mRNA that is transcribed by Pol III

RNA polymerase. Following the BGH pA, a human CMV enhancer/promoter which is responsible for the expression of enhanced green fluorescent protein (EGFP) sequences, a Simian virus 40 late polyadenylation signal (SV40 late pA), which facilitate the function of Pol II RNA polymerase; and ampicillin antibiotic resistance gene to enable selection take place in the vector from 5' to 3'. The plasmid map is shown in Figure 3.1. The optimal guide-RNA (gRNA) sequence (5'-GGAGTGCAGAACAAACAAGA-3') was selected using the online tool ChopChop, considering the DSB site and the location of the polymorphism. In order to repair the DSB, the homology-directed repair (HDR) mechanism was selected, so that the ssODN template/donor DNA with the sequence of 5'C*C*C*grTCCCAGAGGGAGGAGCTATTTAAGGGGAGCAGGAGTGCAGAACAAACAAGACGGCCTGGGGATACAACCTCTGGAGTCCTCTGAGA*G*G*T3' was acquired from Integrated DNA Technologies (IDT). The asymmetric ssODN/single-stranded donor DNA was designed according to the site of the DSB and distal side of the PAM sequence. The donor DNA template was designed according to site of polymorphism and it has phosphorothioate modifications. Since CRISPR/Cas9 and donor DNA template introduced to cells at the same time, in order to prevent Cas9 endonuclease activity on itself, donor DNA template have phosphorothioate modifications.

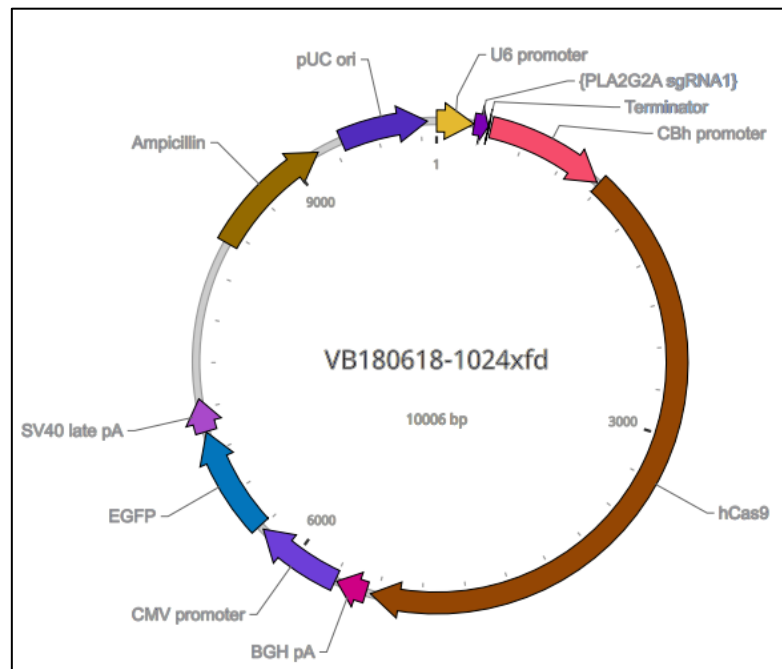


Figure 3.1. B180618-1024xfd plasmid vector map

3.3.2. Inoculation of Bacterial Culture

The Luria–Bertani (LB) broth base and LB Agar powder were purchased as ready to use. As an initial step, 35 grams of LB Agar was dissolved in 1 liter of distilled water and autoclaved at 121°C for 15 minutes. The solution was left for cooling down approximately to 55°C and 100 µg/ml of ampicillin was added by gently swirling. Working inside a laminar flow hood, the solution was distributed into 10 ml per Petri dish to cover the bottom of the dishes. After the LB agar plates were solidified, using a sterilized loop, bacteria taken from the bacterial stock stored at -80°C were seeded into the LB agar plates by the streaking plate method. Agar plates were then placed into 37°C incubator and incubated for 18 hours. Following day, 25 grams of LB Broth was weighed and dissolved in 1 liter of distilled water, then the top of the Erlenmeyer Flask was covered with aluminum foil. After that, the solution was autoclaved, and the solution remained at room temperature for cooling. By the time the LB Broth was ready, 100 µg/ml of ampicillin was added. Using a sterile pipette tip, a single bacteria colony was selected from the LB agar plate and transferred into the broth. Single colonies on the LB agar plate were shown in Figure 3.2. The bacterial culture was incubated at 37°C in a shaking incubator for 16 hours. After incubation, the OD of the culture was measured by spectrophotometer, and plasmid isolation was initiated at OD 0.2 to 0.4.

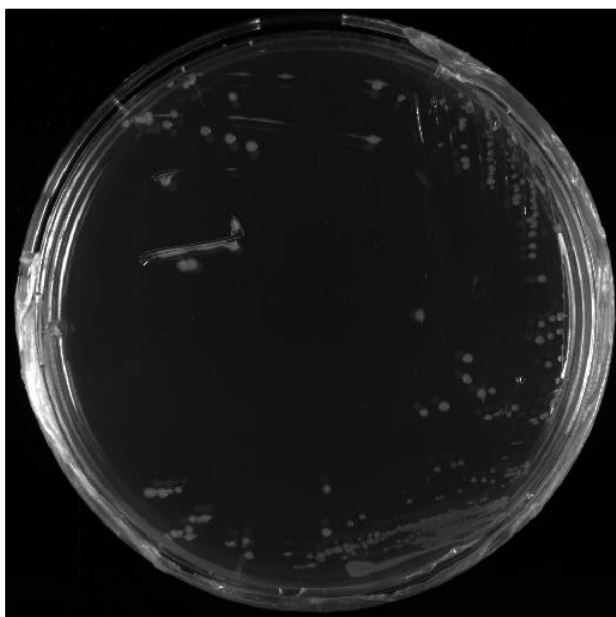


Figure 3.2. Colony formation of bacterial cells

3.3.3. Isolation of the Vector Plasmid

Plasmid isolation was performed based on the PureLink® Quick Plasmid Midiprep Kit protocol. First of all, 30 ml of Equilibration Buffer was placed into the column, and the solution was allowed to drain by gravity flow. The grown bacterial culture was placed into 50 ml collection tubes and centrifuged at 4,000 $\times g$ for 10 minutes to harvest the cells to obtain bacterial pellet which was next suspended in 10 ml of Resuspension Buffer containing RNase A. The bacterial suspension was then mixed until the solution became homogeneous and 10 ml of Lysis buffer was introduced to the bacterial suspension by gentle inversion until the mixture became homogeneous. Mixture was incubated at room temperature for 5 minutes for homogenization. Afterward, 10 ml of Precipitation Buffer was introduced into the mixture and centrifuged at 12,000 $\times g$ for 10 minutes at 25°C. Next, the supernatant was loaded onto the equilibration column, and the suspension was drained by gravity flow as a binding step. In the washing step, 60 ml of Wash Buffer was added onto the column. After Wash Buffer was completely drained, the liquid in the collection tube was discarded and then a sterile 50 ml collection tube was placed under the column. The DNA was eluted by addition 15 ml of Elution Buffer onto the column. into a fresh collection tube. Afterward, 10.5 ml of isopropyl alcohol was placed onto the DNA eluate and mixed well. The collection tube was centrifuged at 1,250 $\times g$ for 5 minutes at 4°C, and the pellet was collected to be air dried at room temperature for 35 minutes. Finally, DNA was resuspended in 220 μ l of TE Buffer and the plasmid concentration was measured using NanoDrop. Plasmid DNA was stored at -20° for later use.

3.4. TRANSFECTION OF PC-3 CELLS

3.4.1. Dose Optimization of Nocodazole by WST-1 Cell Viability Assay followed by Cell Cycle Analysis

Nocodazole dose was determined using WST-1 cell proliferation assay. For the experiment, the standard curve plate set-up was prepared by seeding 2,500, 5,000, 7,500, 10,000, 12,500, and 15,000 cells per well in a 96-well plate. The seeded cells were allowed to attach overnight. Next day, in order to measure the cell viability, each well was treated with 10

percent (v/v) WST-1 reagent in complete RPMI media and incubated for 1 hour in the dark at 37°C. After incubation period was completed, the absorbances of wells were measured with a microplate reader at 450 nm wavelength, using 630 nm values as a reference. In order to select the most convenient dose of nocodazole, five different doses (10 ng/ml, 20 ng/ml, 30 ng/ml, 40 ng/ml, and 50 ng/ml) were tested. To identify the optimal dose of nocodazole, 5,000 cells per well were seeded into separate 96-well plates simultaneously during the preparation of the standard curve plate. After an overnight incubation, the cells were treated with the indicated doses of nocodazole. After a 16-hour incubation, each well was treated with WST-1 reagent, and the absorbance values were measured as described. After the determination of the effective dose of nocodazole (50 ng/ml), cell cycle analysis was initiated. As a first step, PC-3 cells were treated with serum-free (SFM) RPMI medium for 16 hours to achieve cell quiescence. The following morning, the 300,000 cells/well seeded in 6-well plates for 9 hours and then treated with the 50 ng/ml of nocodazole for 16 hours. Following overnight incubation of the cells with nocodazole, the cell cycle protocol was initiated. First of all, the cells' media were discarded, and the wells were washed with 500 µl of PBS; after that, 500 µl of 1 percent (w/v) trypsin-EDTA was placed into the wells, and the cells were incubated at 37°C for 5 minutes for detachment. Next, the detached cells were collected with 1 ml of complete RPMI and centrifuged at 350 *xg* for 5 minutes. The medium of the cells was discarded and cells were resuspended in 1 ml of fixation buffer (70 percent ice-cold ethanol in PBS) and placed at -20°C for 2 hours. After fixation was completed, cells were centrifuged at 350 *xg* at 4°C for 5 minutes. Once again, the supernatant was discarded, and cell pellet were resuspended with 500 µl of 0.1 percent (v/v) Triton-X 100 in PBS and incubated at room temperature for 20 minutes. The cell suspensions were then centrifuged at 350 *xg* at 4°C for 5 minutes, and cell pellet was resuspended in 200 µl of 0.05 mg/ml RNase and incubated at 37°C on heat block for 35 minutes. Next, the cell suspensions were centrifuged at 350 *xg* at 4°C for 5 minutes, and 300 µl of 0.005 mg/ml propidium iodide (PI) was added into each cell suspension, except the gating control. The cells were analyzed using Guava® easyCyte™ Flow Cytometer.

3.4.2. Lipofectamine-Based Transfection

PC-3 cells were seeded into 24-well plates (0.5×10^5 cells/well) in three replicates for three different conditions, which were lipofectamine-only (for gating), lipofectamine plus pRP-

CRISPR/Cas9 plasmid (Reporter, vehicle control cells), and lipofectamine plus pRP-CRISPR/Cas9 plasmid and donor DNA. PC-3 cells were incubated overnight in order to allow the cells to attach the plate. The next day, the complete cell culture media were replaced by SFM-RPMI and the cells were incubated overnight. 16 hours later, the media of the cells were discarded, and 50 ng/ml of nocodazole in fresh growth media was given to the cells in order to maintain the cell synchronization at G2/M phase of cell cycle. The PC-3 cells were incubated with the nocodazole-containing growth medium for 24 hours. Afterward, transfection with Lipofectamine™ 3000 Transfection Reagent was initiated according to the Invitrogen Lipofectamine™ 3000 protocol, using 1 µg/ml pRP-CRISPR/Cas9 plasmid DNA and 1µg/ml donor DNA. According to the procedure, for the gating group, 25 µl of Opti-MEM was placed into a 1.5 ml centrifuge tube, and 1.5 µl Lipofectamine 3000 Reagent was added. The sample was vortexed thoroughly for 3 seconds. The 50 µl of Opti-MEM and 2 µl of p3000 reagent were placed into fresh 1.5 ml centrifuge tube and mixed. Afterward, the p3000-containing tube and the diluted Lipofectamine 3000 reagent were mixed with the ratio 1:1 (v/v) and incubated for 15 minutes. For the Reporter PC-3 cells, 25 µl of Opti-MEM was placed into fresh 1.5 ml centrifuge tube, 1.5 µl of Lipofectamine 3000 Reagent was added, and vortexed roughly for 3 seconds. In a new 1.5 ml tube, 50 µl of Opti-MEM, 2 µl p3000 reagent, and 1 µg pRP-CRISPR/Cas9 plasmid DNA were placed and mixed. Afterward, the plasmid DNA-containing tube and the diluted Lipofectamine 3000 Reagent were mixed with the ratio 1:1 (v/v) and incubated for 15 minutes. For the construction of the targeted gene-edited HDR-mediated PC-3 cells, 1.5 µl Lipofectamine 3000 Reagent was mixed with 25 µl of Opti-MEM media in a 1.5 ml tube and mixed well by vortex for 3 seconds. Next, in a separate 1.5 ml tube, 50 µl of Opti-MEM, 2 µl of p3000 reagent, 1 µg pRP-CRISPR/Cas9 plasmid DNA, and 1 µg donor DNA were placed and mixed. Afterward, the plasmid DNA-containing tube and the diluted Lipofectamine 3000 Reagent were mixed with the ratio 1:1 (v/v) and incubated for 17 minutes. Next, the DNA-lipid complex was introduced dropwise manner to the PC-3 cells, which were then incubated with the transfection medium for 16 hours, and then the media were replaced by Opti-MEM. After 24 hours, the media of the PC-3 cells were replenished with the complete growth medium. The cells were incubated with the complete growth medium for 24 hours and sorted by FACS as described in Section 3.5.

3.5. FLUORESCENCE ACTIVATED CELL SORTING (FACS) ANALYSIS AND SINGLE CELL COLONY FORMATION

In order to sort the cells by FACS, the growth media of the cells were discarded, and then the cells were rinsed with 1X DPBS (pH 7.4). Afterward, the cells were detached and collected as described before. After the centrifugation, the cells were resuspended in 1 ml of PBS/DAPI mixture, the tubes were placed on BD FACSAria™ III Cell Sorter Flow Cytometer. The PC-3 cells that gives the GFP signals were sorted into GFP (+) cells and placed into 15 ml centrifuge tube. Afterward, in order to generate single-cell colonies, the cells were seeded into 96-well plates according to array dilution method. The method was initiated by placing 200 µl of cell suspension into the first well of a 96 well-plate, transferring 100 µl of this cell suspension vertically, and adding 100 µl of complete growth medium at each step. The transferring procedure was then repeated horizontally, which allowed a subsequent 2-fold serial dilution.

3.6. GENOTYPE ANALYSIS OF PLA2G2A RS11573156 C TO G SUBSTITUTION IN PC-3 CELL CLONES EDITED BY CRISPR/CAS9

The seeded single cells were allowed to reach confluency in 96-well plates and were then initially transferred to 24-well plates, followed by 6-well plates and T-25 cell culture flasks upon reaching to confluency. After obtaining at least five cryostock of all cell clones, cell clones were subjected to genotype analysis. For this purpose, cells were collected into 15 ml tubes for as described in Section 3.1.2. and the cell pellet was re-suspended with Lysis Buffer (10 mM Tris [pH 8.0], 100 mM EDTA (pH 8.0), 0.5 percent (w/v) sodium dodecyl Sulfate (SDS) at 37°C for 2 hours and then incubated at 50°C for 18 hours. Next, 500 µl of phenol chloroform: isoamin alcohol was added into the tubes and mixed well until a homogenous solution was obtained. Tubes were then incubated at 25°C, room temperature for 10 minutes and then centrifuged at 13,000 $\times g$ at 4°C for 5 minutes. After that, the top phase of the solution was collected and 20 µl of 5 M NaCl solution and 1 ml of absolute (100 percent (v/v)) ethanol were added. Tubes were next mixed by inverting several times until the DNA became visible and centrifuged at 13,000 $\times g$ for 5 minutes at 4°C. Finally, pellet was collected and washed with cold 70 percent ethanol as twice. The DNA pellet was air-dried at room temperature for 20 minutes. The dried pellet was then dissolved in 50 µl of TE (Tris-

EDTA) Buffer, and concentrations were measured by Nanodrop. Using the isolated DNA from the cells, a polymerase chain reaction (PCR) was done to amplify the PLA2G2A gene region as discussed in Section 3.2. To identify whether gene editing was accomplished, PCR products were sent for Sanger sequencing and results were analyzed as described previously (Section 3.2.).

3.7. DETERMINATION OF THE PLA2G2A GENE EXPRESSION LEVEL IN PC-3 CELLS

3.7.1. Total RNA isolation

Parental PC-3 cells, Reporter PC-3 cells, and HDR-mediated PC-3 cells were counted and seeded at a density of 300,000 cells/well into 6-well plates and incubated overnight. The media of the cells were replenished with 50 μ M H₂O₂ in the complete RPMI, and then cells were incubated with H₂O₂ for 4 hours. Same procedure performed with H₂O₂ was done by using 25 ng/ml interferon gamma as a separate set of experiment. After 4 hours of incubation, the cells were collected with 250 μ l of Trizol reagent into 1.5 ml fresh centrifuge tubes and 50 μ l of chloroform (4°C) was added to the tubes to be incubated at room temperature for 15 minutes. The tubes were centrifuged at 12,000 $\times g$ at 4°C for 15 minutes, and the top phase which contains RNA was collected to be mixed with equal volume of isopropanol. The samples were mixed gently by inverting the tubes four times and incubated for 10 minutes on ice. The tubes were then centrifuged at 12,000 $\times g$ at 4°C for 10 minutes, and the supernatant was discarded. The pellets were resuspended with 500 μ l of 75 percent ethanol and centrifuged at 7,500 $\times g$ at 4°C for 10 minutes. The pellet was collected and allowed to dried 25°C, room temperature, for 35 minutes. Finally, pellets were resuspended in 20 μ l of RNase-free water, and the concentration of isolated RNA samples were measured by NanoDrop. RNA samples were stored at -80°C until further applications.

3.7.2. cDNA Synthesis by Reverse Transcription PCR

The cDNA was synthesized from the isolated RNA samples using QuantiTect Reverse Transcription Kit. Initially, genomic DNA elimination was performed according to Table 3.2.

Table 3.2. cDNA Reaction mix for genomic DNA elimination

Reagents	Volume per reaction
7X gDNA Wipeout Buffer	2 μ l
Template RNA	1500 ng
RNase-free water	up to 14 μ l

After the reaction mixture was prepared for the genomic DNA elimination step, it was incubated at 42°C for 2 minutes and then placed on ice. Afterward, the reverse-transcription master mix was prepared according to Table 3.3. All components were mixed and placed into thermal cycler first at 42°C for 15 minutes and then at 95° for 3 minutes. Lastly, cDNA concentrations were measured using Nanodrop.

Table 3.3. Reaction mix for cDNA synthesis

Reagents	Volume per reaction
Quantiscript Reverse Transcriptase	1 μ l
5X Quantiscript RT Buffer	4 μ l
RT Primer Mix	1 μ l
Template RNA (After genomic DNA elimination)	14 μ l

3.7.3. Real-Time PCR (q-PCR)

To determine the gene expression levels of PLA2G2A, cDNAs of parental PC-3 cells, Reporter PC-3 cells, and HDR-mediated PC-3 cells were used to perform q-PCR. The q-PCR was done by using Applied Biosystems™ Power SYBR™ Green PCR Master Mix. cDNA was introduced to the reaction as a 1500 ng template and then the PCR master mix was prepared. Briefly, q-PCRmaster mix was prepared by adding 1.75 µl of nuclease-free water, 1.25 µl of 1 µM primer and 5 µl of SYBR Green, and q-PCR was carried out under the conditions as indicated in Table 3.4. The housekeeping gene 18S rRNA was utilized for normalization.

Table 3.4. Real-Time PCR conditions

Cycle	Temperature	Time	Phase
1	50°C	2 min	
2	95°C	2 min	Initial denaturation
3 (40 repeat)	95°C	15 sec	Denaturation
	55°C	15 sec	Annealing
	72°C	60 sec	Extension
4	60-95°C 0.5 °C increase /sec		Melt curve

3.8. STATISTICAL ANALYSIS

GraphPad Prism 8 software was utilized for the analysis of the presented data. Standard deviations were calculated from three biological replicates, expressed as means ± SD. The necessary comparisons were performed, and significance between the groups were analyzed statistically using the *t*-test, one-way ANOVA and two-way ANOVA.

4. RESULTS

4.1. GENOTYPE ANALYSIS OF PC-3 CELLS

The genotype analysis of PC-3 cells was performed by amplification of the 5' UTR region of PLA2G2A as described in Section 3.1. The chromatogram of the parental PC-3 cells obtained by Sanger sequencing. As shown in Figure 4.1, parental PC-3 cells have GC genotype with overlapping Guanine (black) and Cytosine (blue) at 116th nucleotide of the amplicon.

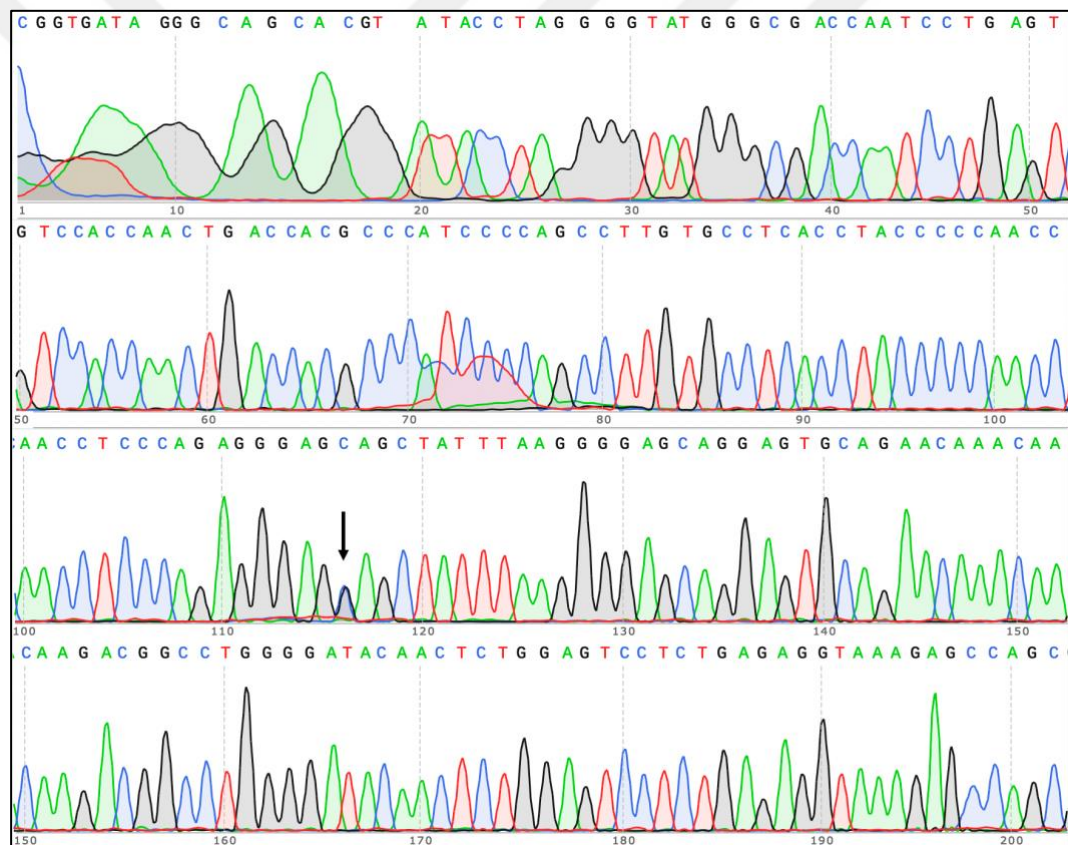


Figure 4.1. Chromatogram result of the parental PC-3 cells. The arrow represents the rs11573156 C>G polymorphism on the PLA2G2A gene region. Each nucleotide is represented as color codes: green, adenine; red, thymine; black, guanine; blue, cytosine

4.2. OPTIMIZATION OF CELL CYCLE SYNCHRONIZATION TO ENHANCE THE TRANSFECTION EFFICIENCY

4.2.1. The Effect of Nocodazole on PC-3 Cell Viability and Cell Cycle Arrest

Genetics studies suggest that nocodazole, which is an agent that quenches the cells at G2/M phase in cell cycle, enhances the HDR-efficiency from ten percent to thirty eight percent [199]. Therefore, to investigate the effect of the nocodazole on PC-3 cells, the non-toxic dose was initially selected by WST-1 cell viability and proliferation assay. Next, with respect to the selected dose, cell cycle analysis was performed.

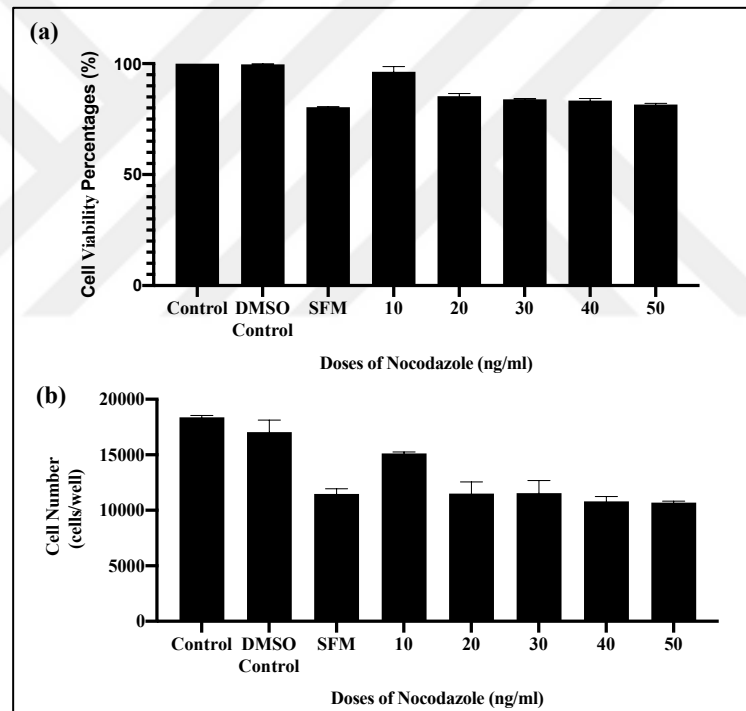


Figure 4.2. Evaluation of parental PC-3 cell viability for non-toxic dose selection of nocodazole. (a) Analysis of cell viability percentages according to applied doses and (b) analysis of cell numbers a result of nocodazole treatment. Control group represents untreated PC-3 cells, SFM refers to RPMI medium without FBS, and DMSO indicates 1 to 1000 diluted DMSO. All conditions were compared to the control group for statistically analysis. Values are shown with the mean $\pm$ SD for 3 independent experiments. * $p < 0.05$

The cell viability results of PC-3 cells in response to nocodazole were shown in Figure 4.2. The PC-3 cells were treated with SFM since it was known that medium without FBS promotes cell cycle arrest at the G0/G1 phase. Thus, SFM was used as positive control for identifying the G1 phase clearly, so that the effect of nocodazole on cell proliferation could be examined accurately. Moreover, the cytotoxic effect of DMSO (1:1000) was also examined since nocodazole was also dissolved in DMSO at a 1 to 1000 ratio for the experiment. The doses shown in Figure 4.2. (10-50 ng/ml) were selected according to the literature [199]. PC-3 cells were seeded at a density of 5,000 cells/well at the beginning of the experiment, and total cell number reached 18,347 at the end of 72 hours of incubation period. For the tested doses of nocodazole, the cell number increases around 9,000 cells, except for 10 ng/ml treatment, as a result of which the cell number reaches 15,260. For the SFM, the cells duplicated, and the cell number reached around 11,000 cells, while the number of cells in the control group increased around three-fold. For the cells incubated with DMSO-containing complete medium, the cell number and percentage viability were statistically similar to control group ($p>0.05$). Since nocodazole is an agent that induces cell cycle arrest, it can reduce the proliferation of PC-3 cells. Thus, it can be inferred that none of the doses demonstrate cytotoxic effect, but they showed a cytostatic effect on cell proliferation. When the cell numbers of the cells were evaluated, all cells treated with 10-50 ng/ml doses of nocodazole was found higher than initial seeding density which is 5,000 cells indicating no cell toxicity. All conditions were compared to the control group for statistically analysis and all the experimental groups does not significantly different from the control cell group. Therefore, it was observed that the effect of nocodazole did not cause toxicity in a dose-dependent manner (Figure 4.3.). Since the tested doses of nocodazole had similar effects on the proliferation of PC-3 cells, 50 ng/ml was selected for the cell cycle analysis to test its mitotic-arrest ability (Figure 4.4.).

SFM-treated PC-3 cell population was used to identify the G1 phase clearly. As a result, 75.64 percent of the SFM-treated PC-3 cell population was found at the G0/G1 phase, whereas 52.73 percent of the control PC-3 cells cultured in complete media and 4.22 percent of the nocodazole-treated cells were at the G0/G1 phase. According to the results, a significantly lower number of the SFM- and nocodazole-treated cells were at the G0/G1 phase, compared to the control. Besides, 13.33 percent of the control PC-3 cell population was at S phase, which was higher compared to the SFM- and nocodazole-treated PC-3 cell

groups. The cell percentages of the SFM- and nocodazole-treated populations found at the S phase were 3.25 and 2.22, respectively. In line with the literature, 93.57 percent of the nocodazole-treated cell population was significantly quenched at the G2/M phase in response to 50 ng/ml nocodazole treatment, whereas 33.26 percent of control cell population and 21.37 percent of the SFM-treated group were at the G2/M phase. Based on the statistical analyses, a significantly higher percentage of the cells in the 50 ng/ml nocodazole treatment group were found to be at the G2/M phase, compared to those in the control group ($p < 0.05$).

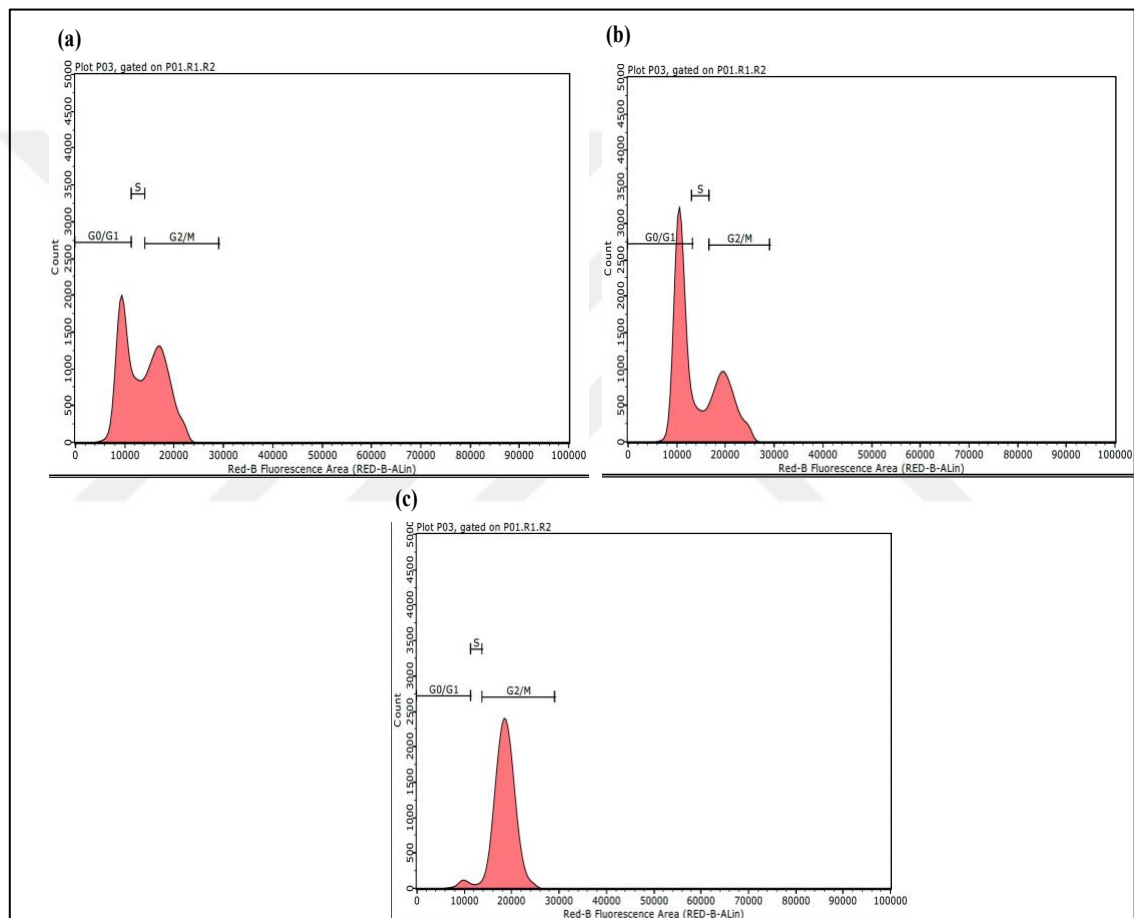


Figure 4.3. Cell cycle phase profiles of parental PC-3 cells incubated with (a) 10 percent FBS containing growth medium, (b) serum free growth medium (G0/G1 phase indicator), (c) (50 ng/ml nocodazole-in 10 percent FBS containing growth medium. Histograms were evaluated via Guave easyCyte Systems

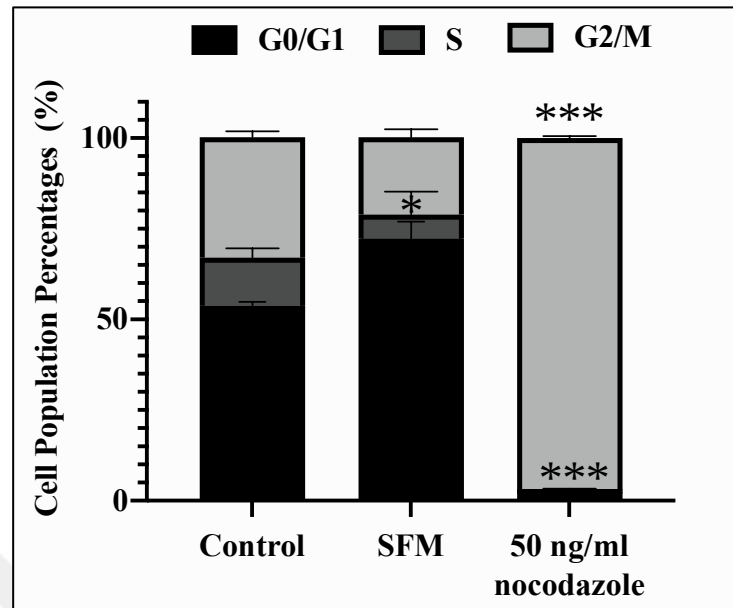


Figure 4.4. Distribution of cell cycle phases in the parental PC-3 cell population treated with 10 percent FBS containing growth medium (Control), serum free growth medium (SFM), and 50 ng/ml nocodazole-in 10 percent FBS containing growth medium. All treatment groups were compared to the untreated control group for statistical analysis. Values are shown as the mean $\pm$ SD for 3 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

4.2.2. Monitoring the Transfection Efficiency of PC-3 Cells

Based on the optimized dose of nocodazole and lipofectamine:plasmid:donor DNA ratio, transfection was initiated as described in Section 3.4.2. Following the transfection of parental PC-3 cells with the pRP-CRISPR/Cas9 plasmid and the donor DNA, GFP-positive cells were sorted by FACS (Figure 4.5.). Gating was performed using parental PC-3 cells treated with lipofectamine only. The DAPI filter was used to differentiate non-viable cells, and the FITC filter was used to identify the GFP-positive cells. Correspondingly, quadrant 3 (Q3) on the quadrant histogram shows the weakly stained cells on both filters, while Q1 and Q4 show the strongly positive cells for the respective filters and Q2 indicates strongly double-stained cells. In this regard, Q4 shows the viable and highly GFP-positive cells; i.e., the cells with a high transfection efficiency. Thus, the population in Q4 were sorted for further analysis. Although a low number of GFP-positive cells were detected in Q3, this

quadrant was excluded from the sorting procedure to include only the cells with the highest transfection efficiency.

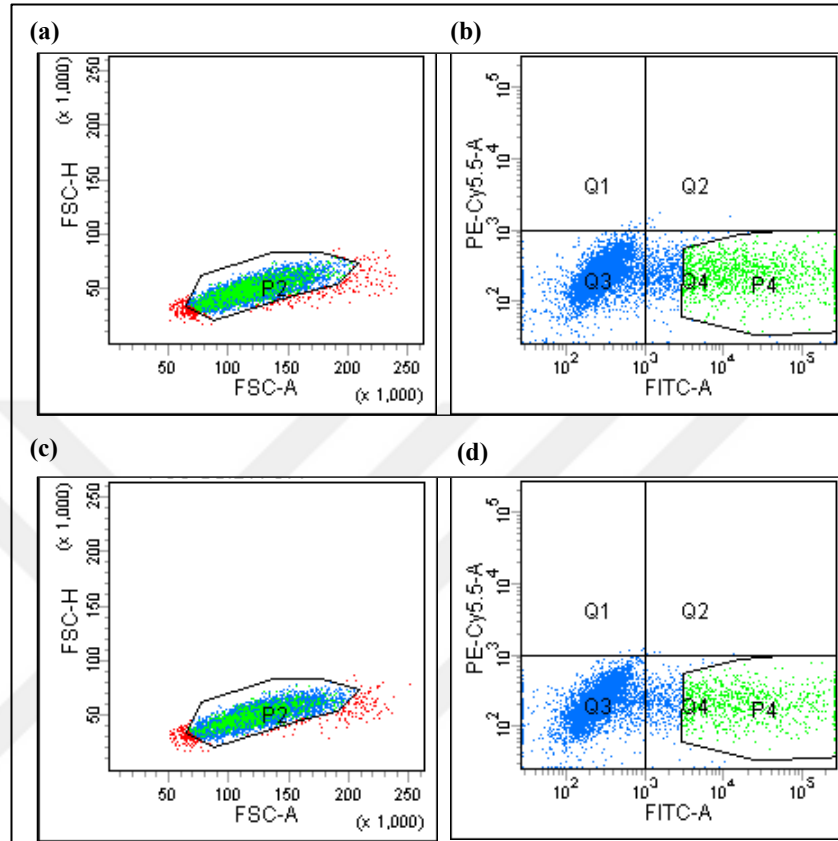


Figure 4.5. Gating of the Reporter cells and HDR-mediated PC-3 cells by FACS. (a-b) Gating of the Reporter PC-3 cells was adjusted by including the cells in the fourth quadrant (Q4) since the GFP-positive population (P4) appears in Q4. (c-d) The same adjustment was performed for HDR-mediated PC-3 cells. The low-GFP population in Q3 was excluded from the sorting to increase the selection efficiency

After the gating was adjusted, GFP (+) cells were sorted. The Reporter PC-3 cells were obtained as a result of transfection with the CRISPR plasmid. HDR-mediated PC-3 cells was obtained by co-transfection of the CRISPR plasmid and donor DNA used as the template. Analysis of sorting efficiency (Figure 4.6.) showed that 97.3 and 98.5 percent of the Reporter and HDR-mediated PC-3 cells were successfully sorted.

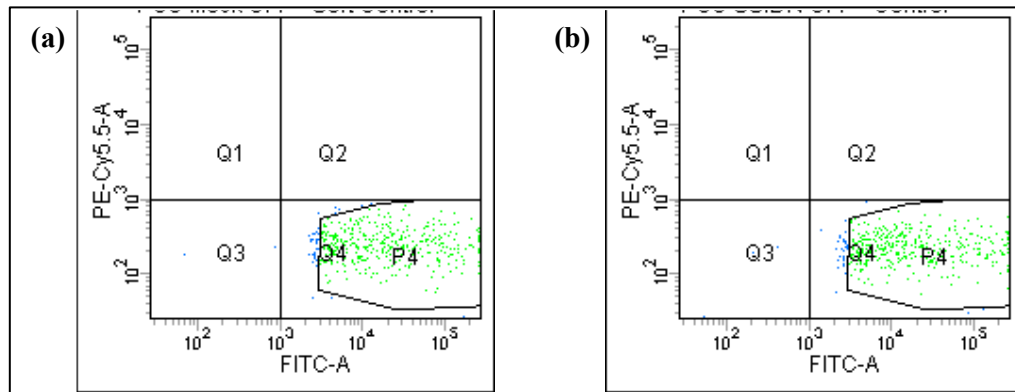


Figure 4.6. The sorting efficiency of (a) Reporter PC-3 cells and (b) HDR-mediated PC-3 cells

Since only the pRP-CRISPR/Cas9 plasmid (Vector Builder #B180618-1024xfd; Figure 3.1.) contains GFP as a reporter, GFP-positivity indicates the cells which uptake the CRISPR plasmid for both the Reporter (Figure 4.7.) and HDR-mediated PC-3 cells (Figure 4.8.). Following the sorting procedure, the GFP (+) Reporter PC-3 cells were pooled, while HDR-mediated PC-3 cells were seeded as single cells, as described in Section 3.5. GFP expression in sorted cells were visualized following 48 hours of incubation, using a fluorescent microscope, to observe the qualitative efficiency of the sorted cells.

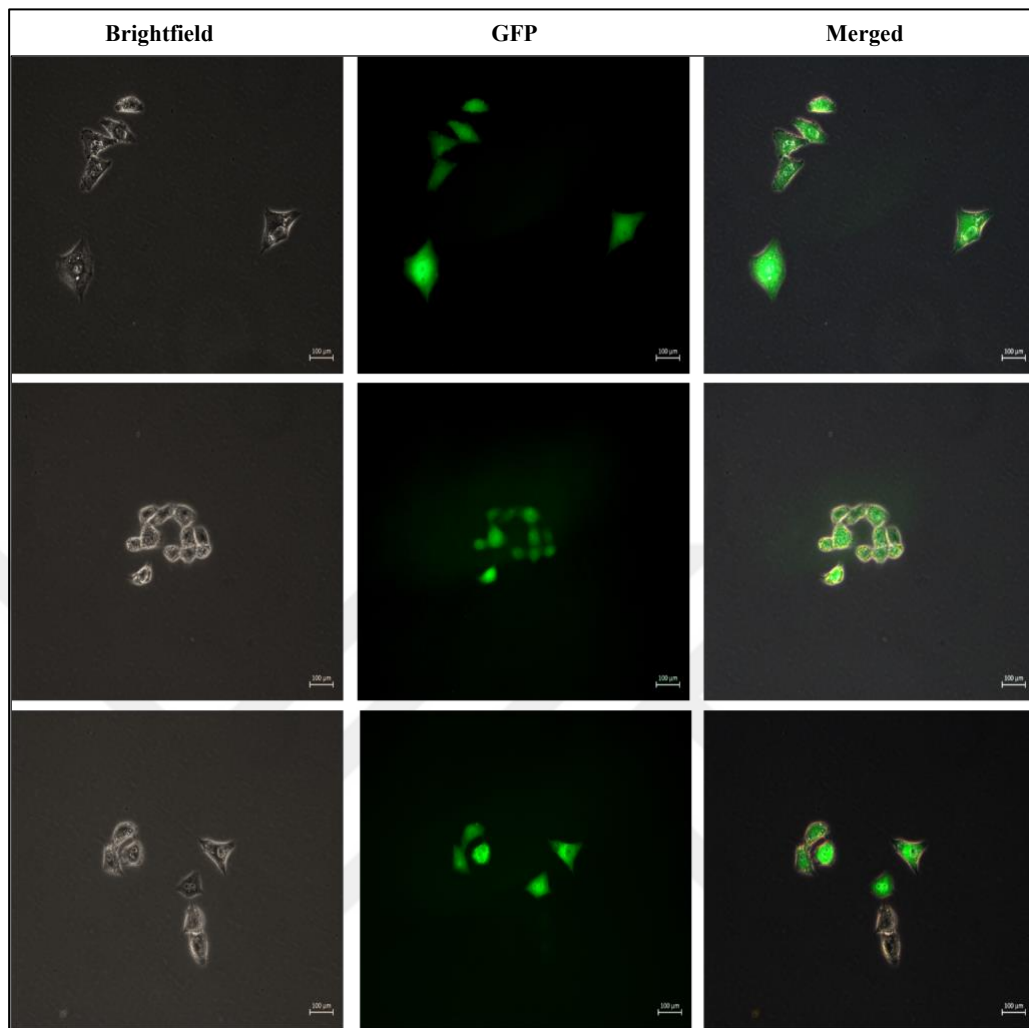


Figure 4.7. Representative images of Reporter PC-3 cells transfected with pRP-CRISPR/Cas9 plasmid. Transfected cell images were obtained by fluorescence microscopy at 10X objective at the end of 48 hours of incubation. Images were obtained from at least 3 independent regions of the well. Scale bars: 100 µm

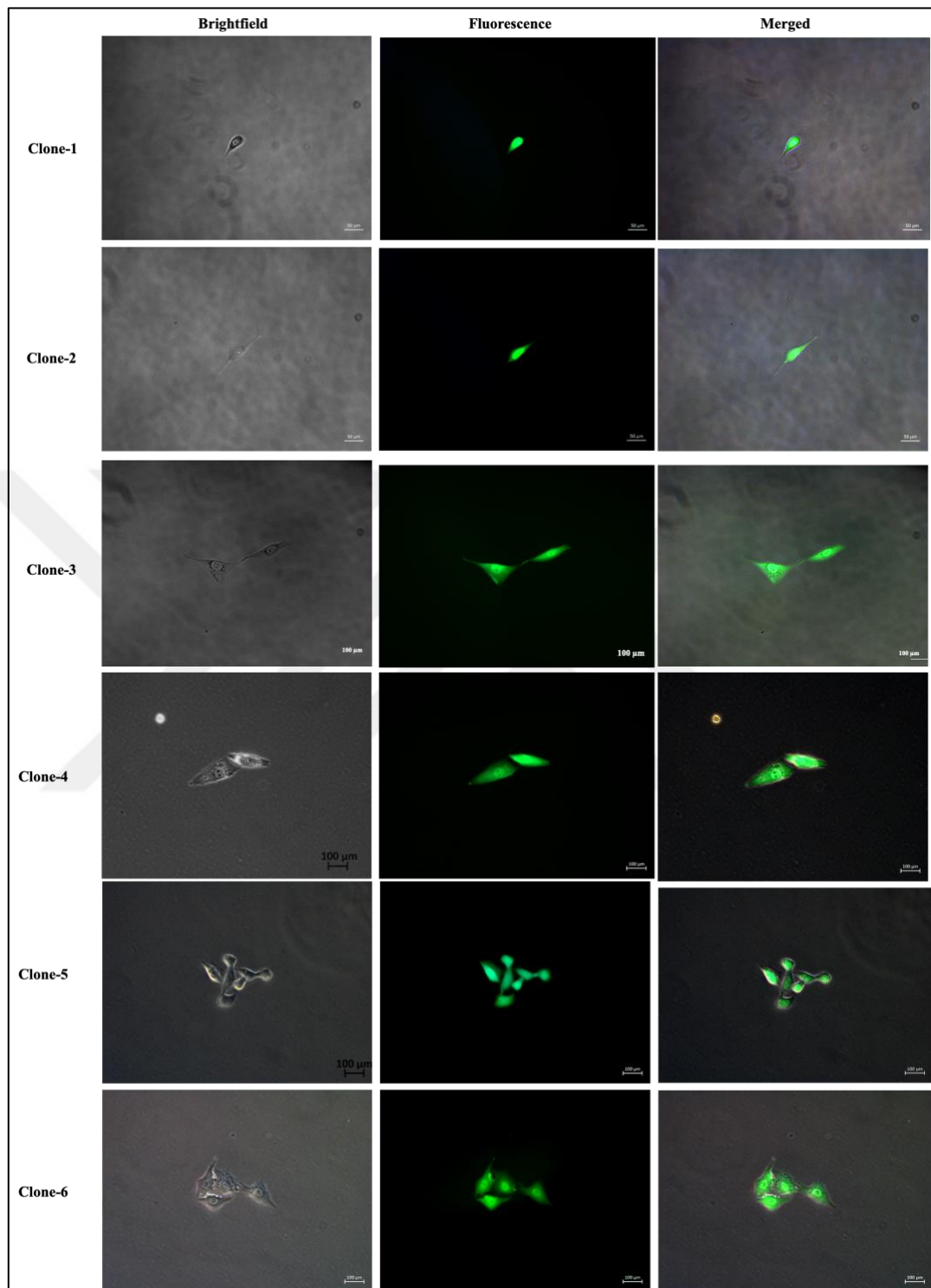


Figure 4.8. Representative images of HDR-mediated single PC-3 cells transfected with the pRP-CRISPR/Cas9 plasmid and designed donor DNA. Images were obtained from 14 wells successfully seeded with a single transfected cell. Transfected cell images were obtained by fluorescence microscopy at 10X objectives following 48 hours of incubation. Images from 6 representative wells are shown. Scale bars: 100 μ m

4.3. GENOTYPE ANALYSIS OF PLA2G2A RS11573156 C TO G SUBSTITUTION IN PC-3 CELL CLONES EDITED BY CRISPR/CAS9

As it described in the previous section, GFP (+) cells were sorted by FACS. However, GFP-positivity cannot be regarded as evidence for a successful gene-editing since it only indicates the uptake of the pRP-CRISPR/Cas9 plasmid. Therefore, genotype information of the single cell-derived cell clones was necessary to identify the successful uptake of the donor DNA for HDR-mediated repair, along with the pRP-CRISPR/Cas9 plasmid. Thus, Sanger sequencing was performed in order to determine the genotypes of the single cell-derived HDR-mediated PC-3 cells. Since Reporter PC-3 cells were not repaired by NHEJ after DSB, the GC polymorphism was not altered (Figure 4.9.). However, this cell clones were also used in further analyses to verify that the functional activities of the cells were not affected due to the transfection. As a result, it was found that while Reporter PC-3 cells still had the GC polymorphism, Clone 2 among the fourteen HDR-mediated PC-3 cells established from GFP-positive cells was successfully edited to the homozygous GG genotype (Figure 4.10.).

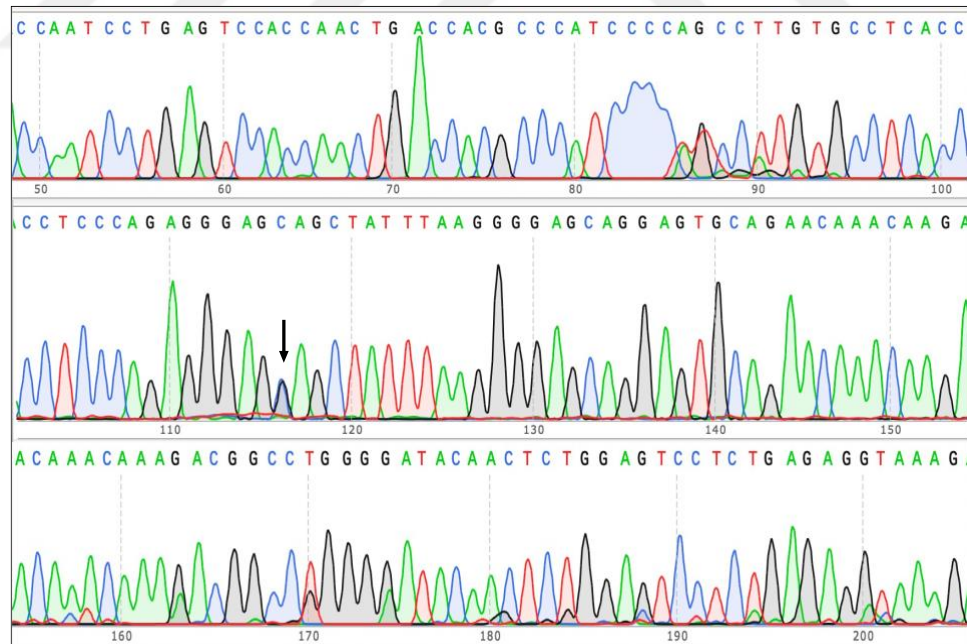


Figure 4.9. Chromatogram of Reporter PC-3 cell. The arrow represents the edited rs11573156 C>G polymorphism on the PLA2G2A gene region

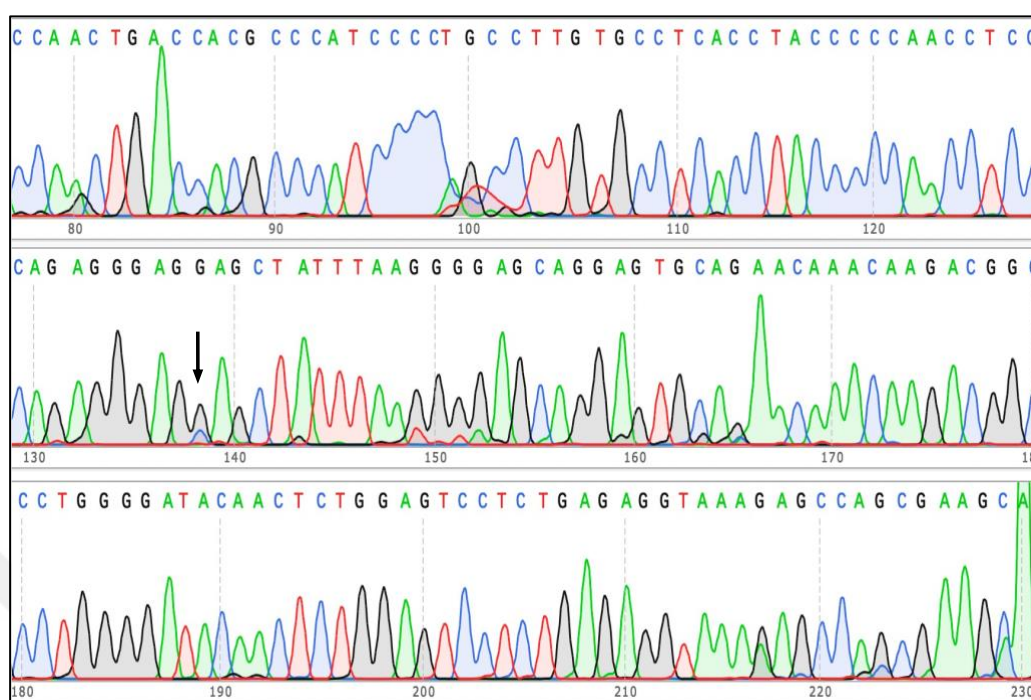


Figure 4.10. Chromatogram of HDR-mediated PC-3 cell clone number 2. The arrow represents the site of gene-editing, rs11573156 C>G polymorphism, on the PLA2G2A gene region

4.4. DETERMINATION OF PLA2G2A EXPRESSION LEVEL IN PARENTAL, REPORTER, AND HDR-MEDIATED PC-3 CELLS

4.4.1. Determination of the Non-Toxic Concentration for Hydrogen Peroxide

The level of PLA2G2A gene expression does not reach detectible levels without oxidative stress conditions and cytokine priming [200]. Thus, PLA2G2A gene expression level was quantitatively analyzed in response to hydrogen peroxide (H₂O₂) and interferon- γ treatment of the parental PC-3 cells. Since H₂O₂ is toxic, initially the selection of concentration was performed using WST-1 assay using standard curve for cell number (Section 3.4.1.). The concentration and incubation period of interferon- γ was selected according to the optimized concentration and incubation times reported for PC-3 cells in the literature [201,202].

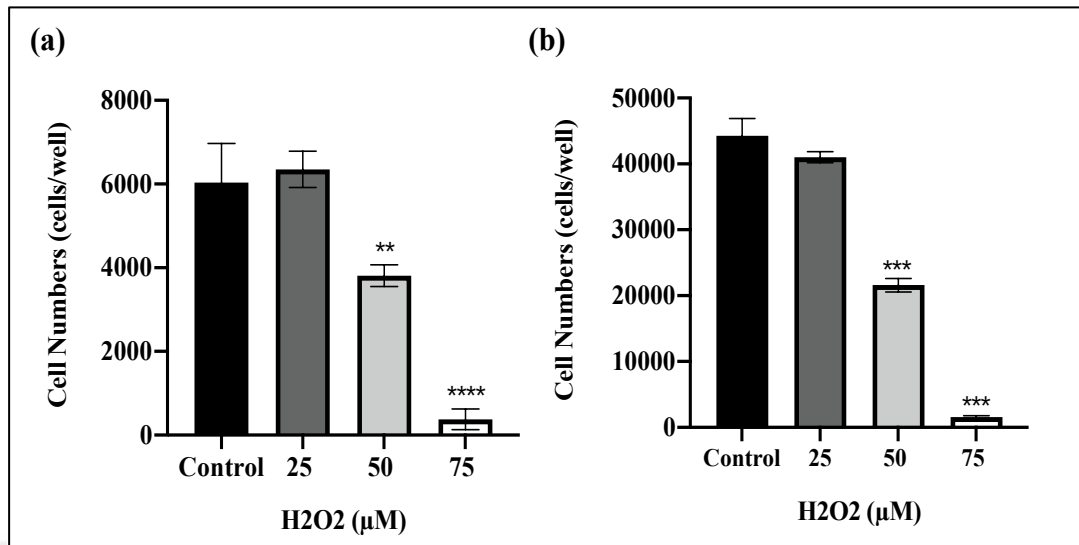


Figure 4.11. Evaluation of cell viability in response to H₂O₂. H₂O₂ was used to treat the parental PC-3 cells for 4 hours. Non-treated cells served as a control. Following the H₂O₂ treatment WST-1 assay was performed at the end of the 4-hour incubation (a) and (b) 72 hours after the replenishing of the H₂O₂ containing media with complete medium. Cell numbers were determined extrapolating the absorbance values from the standard cell plate with known cell number. Values were shown with the mean $\pm$ SD for 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Figure 4.11. shows that at the end of 4 h, 50 μM H₂O₂ treatment had a significant effect on the viability of parental PC-3 cells (3,931 viable cells) compared to the control cells (6,810 viable cells). While, 25 μM H₂O₂ treatment did not show any cytotoxic effect on parental PC-3 cells (6,030 viable cells). The 72 h-treatment with 50 μM H₂O₂ resulted in reduction in cell viability, while 75 μM treatment did exert toxicity. Therefore, 50 μM was selected as the maximum non-toxic concentration of H₂O₂ to treat parental PC-3 cell.

4.4.2. Detection of PLA2G2A Gene Expression in response to Hydrogen Peroxide and Interferon- γ treatment

In order to determine the effect of GG and GC genotype of PLA2G2A rs11573156 SNP on PLA2G2A gene expression, real-time PCR analysis of PLA2G2A mRNA level was performed. As a result of the gene editing, identifying the changes of PLA2G2A gene expression level is essential to investigate the role of PLA2G2A rs1157315 polymorphism

on PCa progression. As mentioned in Section 3.4., Reporter PC-3 cells were transfected with the pRP-CRISPR/Cas9 plasmid to analyze the transfection efficiency via the GFP reporter. HDR-mediated PC-3 cells were transfected with pRP-CRISPR/Cas9 plasmid and HDR-repair occurred via the donor DNA carrying the GG genotype on the 5'UTR region of PLA2G2A. To identify the mRNA levels of PLA2G2A, real-time PCR was performed on parental, Reporter, and HDR-mediated PC-3 cells, as described in Section 3.8.3. The expression analysis of real-time PCR showed no detectable PLA2G2A levels for any cells.

Accumulating evidence suggests that PLA2G2A is expressed in response to inflammatory stimuli in TME [61,124,129]. Therefore, the PLA2G2A expression was next determined in response to H₂O₂ or interferon- γ cytokine priming that mimics the inflammatory TME. PLA2G2A expression was found at detectable levels in PC-3 cells when parental, Reporter and HDR-mediated PC-3 cells were treated with 50 μ M of H₂O₂.

Relative comparison of PLA2G2A expression (Figure 4.12.a.) showed that parental and Reporter PC-3 cells exhibited comparable levels of PLA2G2A expression in response to H₂O₂. Similarly, PLA2G2A expression in response to interferon- γ cytokine priming showed the fold changes of expression in parental PC-3 and Reporter PC-3 cells as 1.00 and 1.061, respectively (Figure 4.12.b.).

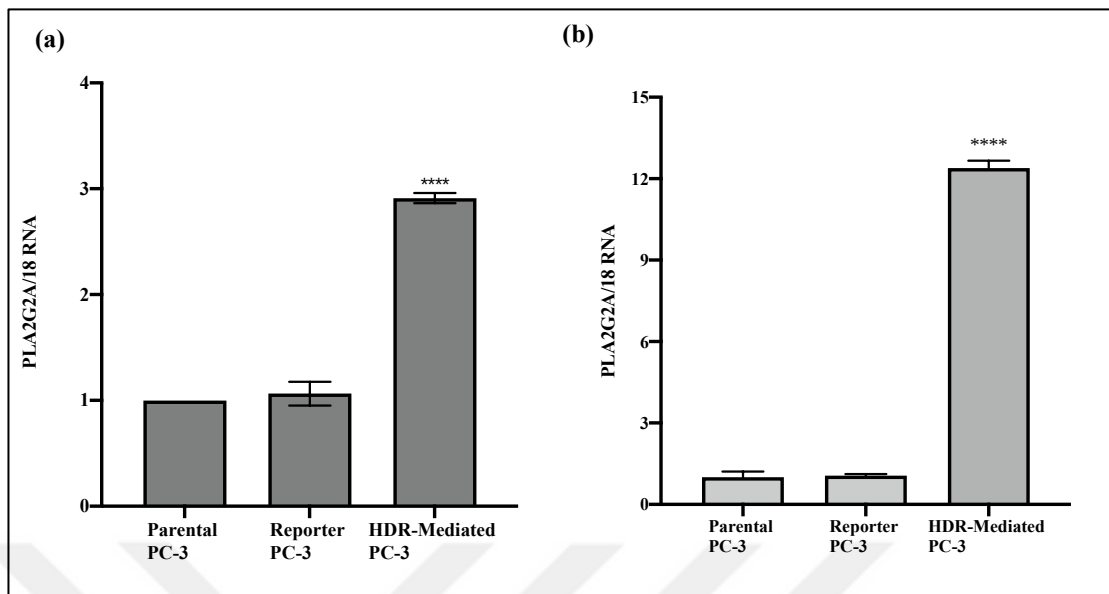


Figure 4.12. The relative PLA2G2A gene expression levels of PC-3 cells determined by real time-qPCR. PLA2G2A expression was determined in response to (a) 50 μ M H_2O_2 and (b) 25ng/ml interferon- γ cytokine priming. PLA2G2A expression at mRNA level was normalized to 18sRNA house-keeping gene. Results were expressed as fold changes in relative PLA2G2A expression (PLA2G2A/18sRNA) by considering the levels of PLA2G2A/18sRNA expression in parental PC-3 as 1. Data are expressed as the mean $\pm$ SD for 3 independent experiments. ****p < 0.0001

Editing GC genotype to the homozygous GG genotype in PLA2G2A rs11573156 SNP using the CRISPR/Cas9 technology led to increased PLA2G2A expression levels. The expression level of PLA2G2A in HDR-mediated PC-3 cells increased to 3-fold and 12-fold compared to parental PC-3 cells, in response to H_2O_2 and interferon - γ cytokine priming, respectively. In other words, the PLA2G2A expression level was significantly increased in HDR-mediated PC-3 cells compared to parental PC-3 cells as a result of gene editing.

4.5. MONITORING THE CELL PROLIFERATION AND CYCLE IN PC-3 CELLS

4.5.1. Effect of PLA2G2A rs11573156 SNP on the Proliferation Rate of PC-3 cells

Based on our previous study, the homozygous G allele might indicate a reduced risk of metastasis in PCa [197]. In order to investigate whether G allele confers a negative effect on cell survival, WST-1 assay was used to measure the rate of cell proliferation in parental, Reporter, and HDR-mediated PC-3 cells.

Cell viability profiles and proliferation rates in PC-3 cells upon increased PLA2G2A expression in response to H₂O₂ were demonstrated (Figure 4.13.). After 24 hours of incubation, the percentage cell viabilities of parental, Reporter, and HDR-mediated PC-3 cells were 100, 99, and 80 percent, respectively. Moreover, at the end of 48 hours of incubation, the percentage cell viabilities of parental, Reporter and HDR-mediated PC-3 cells were 154, 159, and 115 percent, respectively, and at the end of 72 hours, cell percentages reached to 221, 223, and 155 percent in the same respective order of the cell groups. Cell numbers at the end of 24 hours were 8,750, 8,630, and 6,960 in parental, Reporter, and HDR-mediated PC-3 cells. At the end of the 48 hours of incubation the cell numbers were respectively increased to 13,478, 14,179 and 10,145 while at the end of 72 hours, cell numbers were recorded as 19,339, 19,554, and 13,630 for parental, Reporter, and HDR-mediated PC-3 cells, respectively. Statistical analysis showed that Reporter PC-3 shows a similar proliferation pattern as parental PC-3 cells, while a significantly reduced cell proliferation rate was observed in HDR-mediated PC-3 cells. After acquisition of the cell numbers, the population doubling times were calculated using the growth trend equation obtained from the growth curves of parental, Reporter and HDR-mediated PC-3 cells. The population doubling times for parental and Reporter PC-3 cells were calculated as 36 hours while that of HDR-mediated PC-3 cells was found as 48 hours (Table 4.2), suggesting that HDR-mediated PC-3 cells show remarkably reduced cell proliferation rate when compared to parental PC-3 cells.

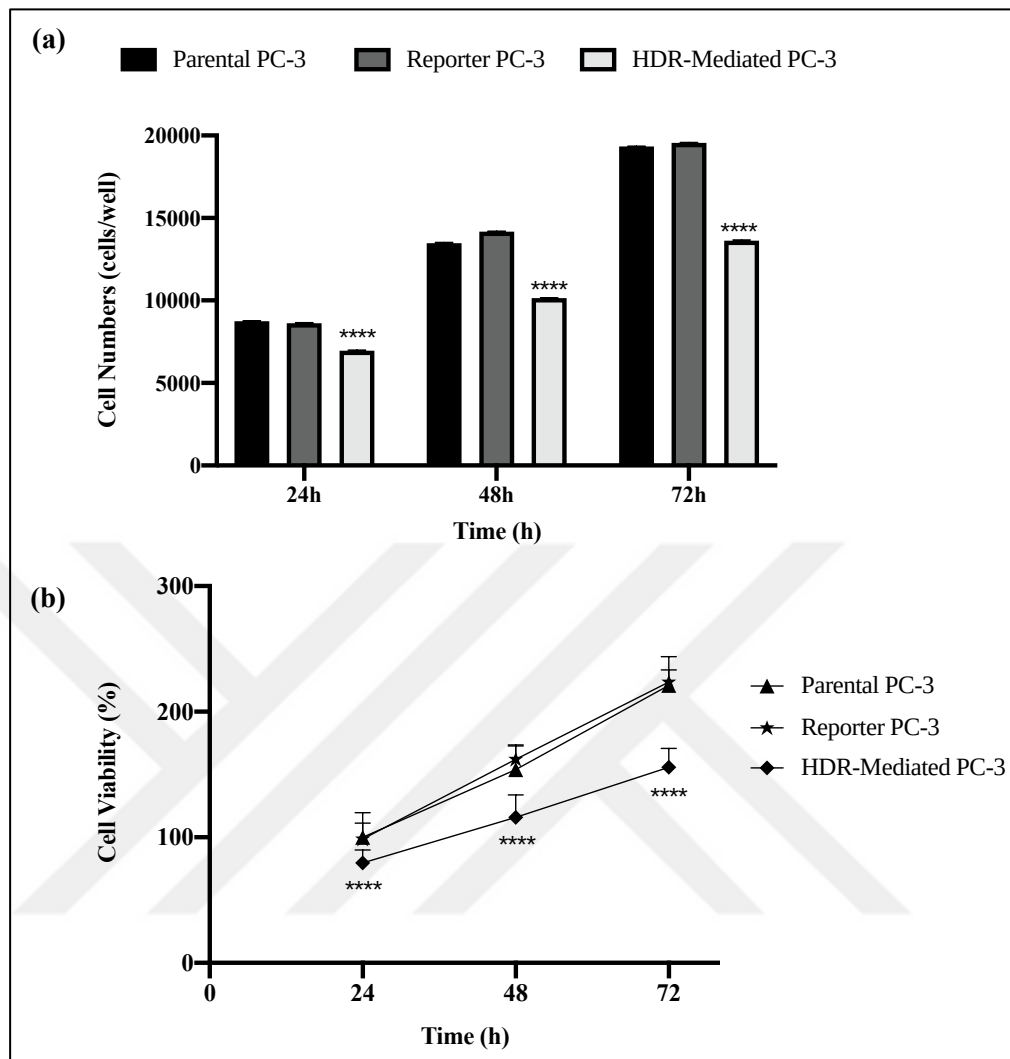


Figure 4.13. Cell proliferation patterns of parental, Reporter, and HDR-mediated PC-3 cells upon amplification of the PLA2G2A expression in response to H₂O₂. (a) Cell numbers and (b) cell viability percentages after 24, 48, and 72 hours of incubation periods. Values are shown as the mean $\pm$ SD for 3 independent experiments. ****p < 0.0001. Cell viability was assessed by measuring the change in absorbance values following WST-1 assay at 24h and 72 h intervals. The viability percentages were calculated by normalizing the absorbance values obtained from parental PC-3 cells to 100 percent

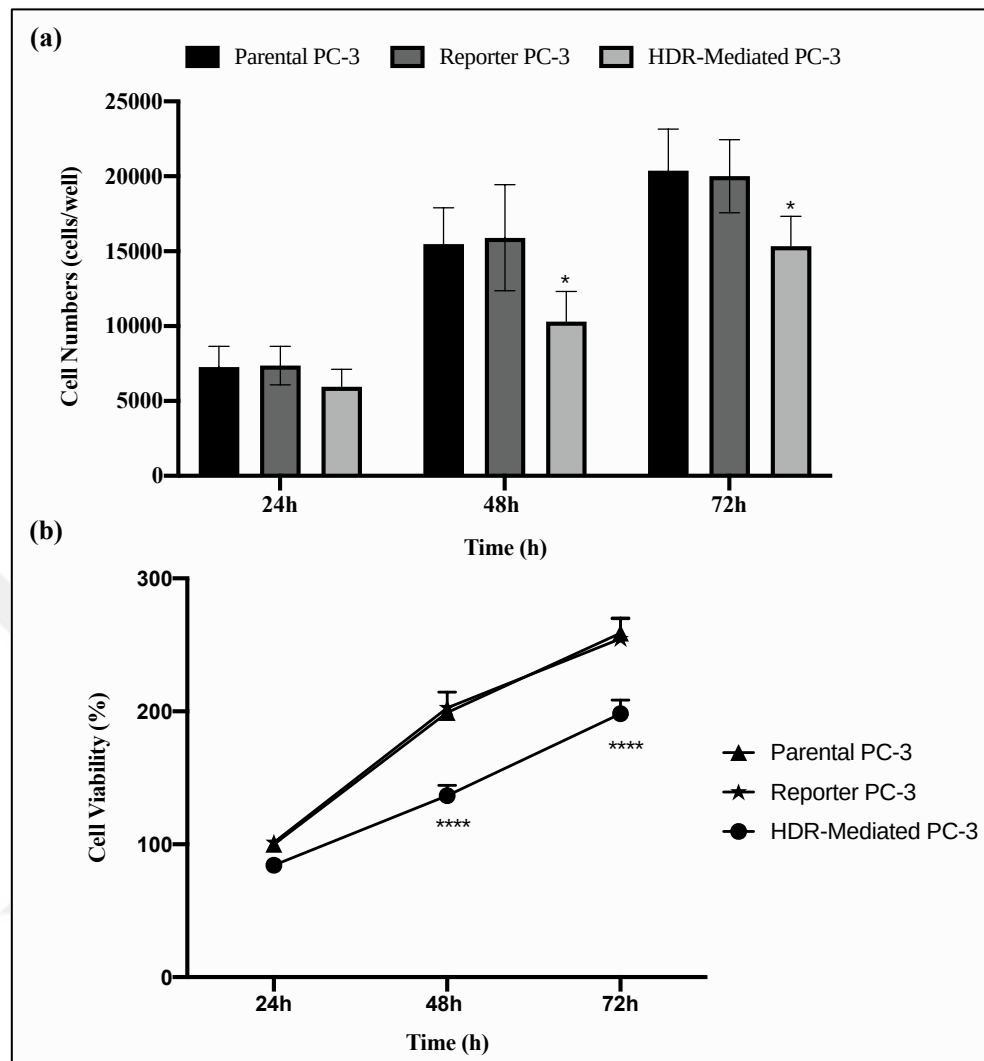


Figure 4.14. Cell proliferation patterns of parental, Reporter, and HDR-mediated PC-3 cells upon amplification of the PLA2G2A expression in response to interferon- γ cytokine priming. (a) Cell numbers and (b) cell viability percentages after 24, 48, and 72 hours of incubation periods. Values are shown as the mean $\pm$ SD for 3 independent experiments. ****p < 0.0001. Cell viability was assessed by measuring the change in absorbance values following WST-1 assay at 24h and 72 h intervals. The viability percentages were calculated by normalizing the absorbance values obtained from parental PC-3 cells to 100 percent

Table 4.1. Doubling times of the PC-3 cells calculated from the growth trend equation

	Doubling Time (h)	
	H ₂ O ₂	Interferon - γ
Parental PC-3	36	35
Reporter PC-3	36	35
HDR-Mediated PC-3	48	48

Cell viability profiles and proliferation rates in PC-3 cells upon increased PLA2G2A expression in response to interferon- γ cytokine priming were demonstrated (Figure 4.14.). After 24 hours of incubation, the percentage cell viabilities of parental, Reporter, and HDR-mediated PC-3 cells were 100, 101, and 84 percent, respectively. Moreover, at the end of 48 hours of incubation, the percentage cell viabilities of parental, Reporter and HDR-mediated PC-3 cells were 199, 200, and 136 percent, respectively, cell percentages reached to 258, 254, and 199 percent in the same respective order of the cell groups. Cell numbers at the end of 24 hours were 7,269, 7,372, and 5,956 in parental, Reporter, and HDR-mediated PC-3 cells. At the end of the 48 hours of incubation the cell numbers were respectively increased to 15,479, 15,897, and 10,304 while at the end of 72 hours, cell numbers were recorded as 20,385, 20,015, and 15,346 cells for parental, Reporter, and HDR-mediated PC-3 cells, respectively. Statistical analysis showed that Reporter PC-3 shows a similar proliferation pattern as parental PC-3 cells, while a significantly reduced cell proliferation rate was observed in HDR-mediated PC-3 cells. After the acquisition of the cell numbers, doubling times were calculated using the growth trend equation from the growth curves of parental, Reporter and HDR-mediated PC-3 cells as 35 while that of HDR-mediated PC-3 cells were found as 48 hours (Table 4.2), suggesting that HDR-mediated PC-3 cells show remarkably reduced cell proliferation rate when compared to parental PC-3 cells.

Overall, cell proliferation results of the PC-3 cells were acquired upon amplification of the PLA2G2A expression in response to H₂O₂ and interferon- γ cytokine priming exerted similar effects on cell proliferation; no statistically significant differences between the results have been found.

4.5.2. Effect of PLA2G2A rs11573156 SNP on the Cell Cycle Profiles of PC-3 Cells

Differences calculated for doubling-time among PC-3 cells, suggested that PLA2G2A rs11573156 SNP might also lead to altered cell cycle profiles in PC-3 cells following induction of PLA2G2A expression in response to H₂O₂ (Figure 4.15) and interferon- γ (Figure 4.16.). In order to investigate the effect of the homozygous G allele on the percentage distribution of PC-3 cells to G1, S and G2/M phases of cell cycle, flow cytometry analysis was used as described in Section 3.4.1.

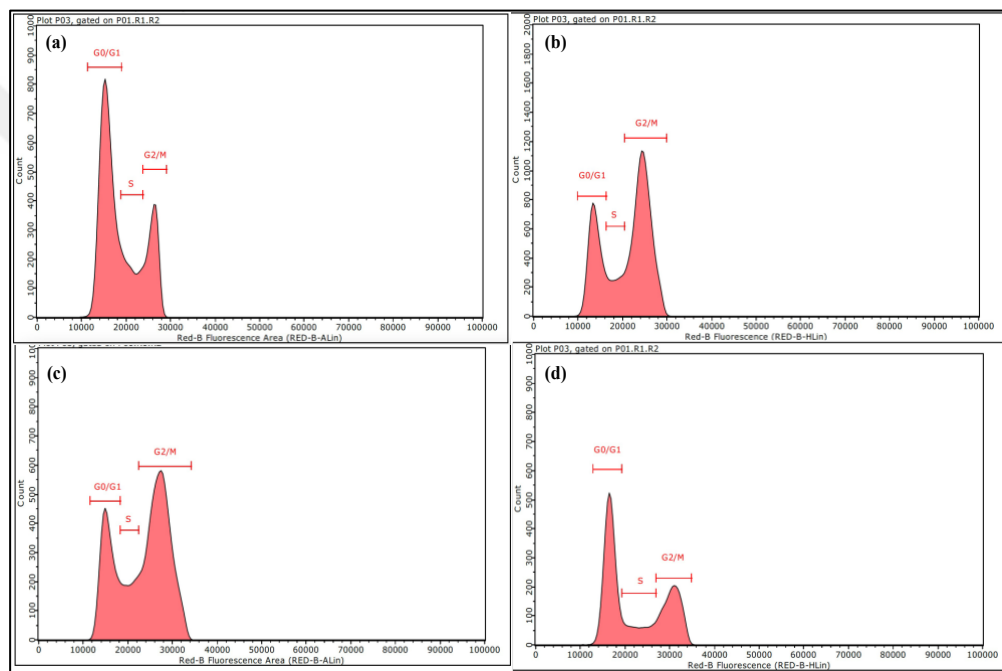


Figure 4.15. Cell cycle phase profiles of parental, Reporter, and HDR-mediated PC-3 cells upon amplification of the PLA2G2A expression in response to H₂O₂. (a) parental PC-3 cells incubated with serum free growth medium (G0/G1 phase indicator), (b) parental PC-3 cells incubated with 10 percent FBS containing growth medium, (c) Reporter PC-3 cells incubated with 10 percent FBS containing medium, and (d) HDR-mediated PC-3 cells incubated with 10 percent FBS containing medium. Histograms were evaluated via Guava easyCyte System

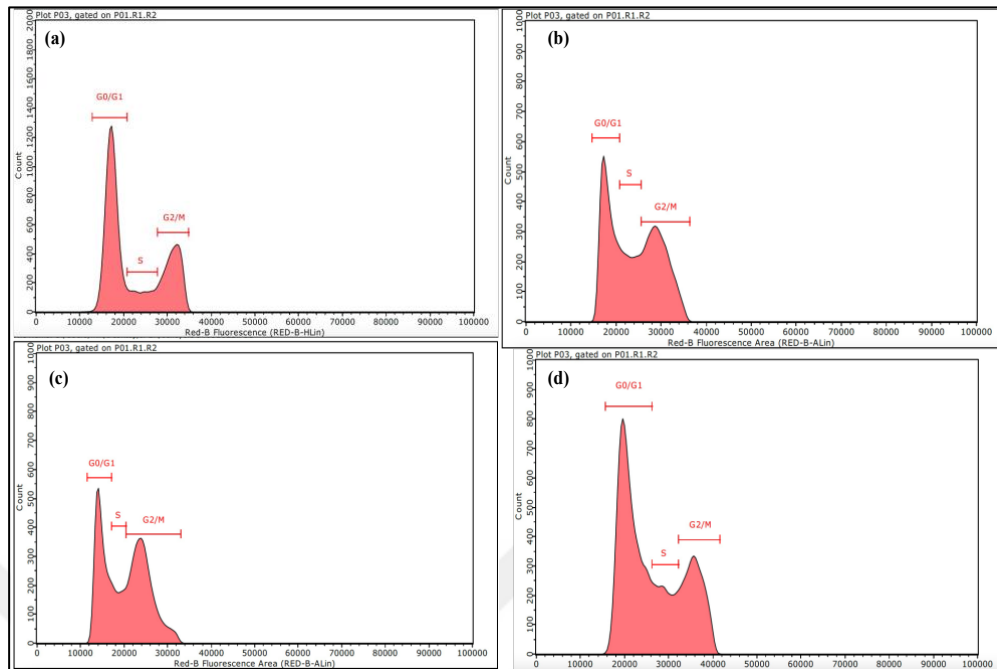


Figure 4.16. Cell cycle phase profiles of parental, Reporter, and HDR-mediated PC-3 cells upon amplification of the PLA2G2A expression in response to interferon- γ cytokine priming. (a) parental PC-3 cells incubated with serum free growth medium (G0/G1 phase indicator), (b) parental PC-3 cells incubated with 10 percent FBS containing growth medium, (c) Reporter PC-3 cells incubated with 10 percent FBS containing medium, and (d) HDR-mediated PC-3 cells incubated with 10 percent FBS containing medium.

Histograms were evaluated via Guava easyCyte Systems

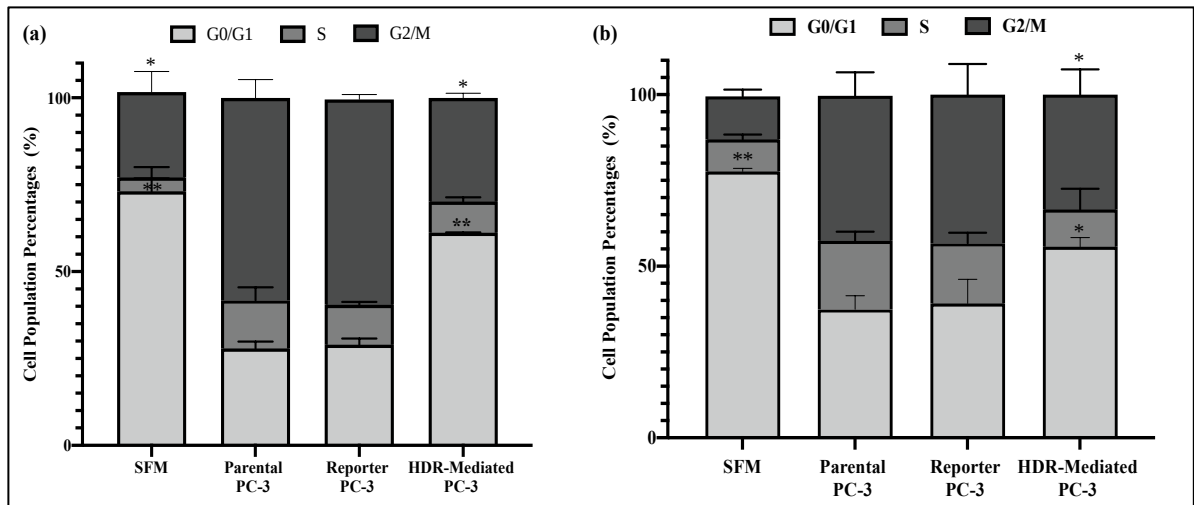


Figure 4.17. Distribution of cell cycle phases (G0/G1, S, G2/M) in parental, Reporter, and HDR-mediated PC-3 cells. Parental PC-3 cell population treated with serum free growth medium (SFM), parental PC-3 cell population treated with 10 percent growth medium (Parental PC-3), Reporter-PC-3 cell population treated with 10 percent growth medium (Reporter PC-3), HDR-mediated PC-3 cell population treated with 10 percent growth medium (HDR-mediated PC-3). All cell groups were compared to the parental PC-3 cell group for statistical analysis. Values are shown as the mean $\pm$ SD for 3 independent experiments. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001

The cell cycle analysis of parental, Reporter, and HDR-mediated PC-3 cells revealed that parental and Reporter PC-3 cells showed similar phase distributions, while the phase distribution of HDR-mediated PC-3 cells significantly differed (Figure 4.17.). SFM-treated parental PC-3 cell population was used as positive control to identify the G1 phase clearly since SFM promotes cell cycle arrest at the G0/G1 phase. As a result, 77.63 of the SFM-treated cells quenched at the G0/G1 phase, while 9.31 percent of the population was found at S phase and 12.54 percent at the G2/M phase. For parental PC-3 cells, 37.45 percent of the population was found at the G0/G1 phase, 19.95 percent at the S phase, and 42.28 percent at the G2/M phase of the cell cycle. Similarly, for Reporter PC-3 cells, 39.12 percent of the cell population was found at the G0/G1 phase, 17.58 percent at the S phase, and 43.30 percent at the G2/M phase of the cell cycle. Meanwhile, 55.69 percent of the HDR-mediated PC-3 cells was at the G0/G1 phase, 10.83 percent at the S phase, and 33.48 percent at the G2/M phase of the cell cycle. According to these results, the majority of parental (42.28 percent) and Reporter PC-3 cells (43.30 percent) were at the G2/M phase, the last checkpoint

of the cell cycle, indicating that these cells were undergoing cell division. On the other hand, 55.69 percent of HDR-mediated PC-3 cells were at the G0/G1 phase, indicating that the majority of HDR-mediated cells were not ready for division, compared to parental and Reporter PC-3 cells.



5. DISCUSSION

sPLA2 is a member of the ubiquitous phospholipase enzyme family that is responsible for the hydrolysis of membrane phospholipids into fatty acids and lipophilic molecules [92]. sPLA2 cleaves the glycerophospholipid at the sn-2 position, which results in the release of fatty acids lysophospholipids and pro-inflammatory lipid mediators. Lipid mediators such as eicosanoids are responsible in the host defense against pathogens, thereby regulate the inflammatory processes [99]. Most of the released fatty acids and lysophospholipids are important bioactive lipid molecules that have roles in cell growth, survival, cytoskeleton arrangement, and regulation of apoptosis and inflammation [203]. Therefore, various members of the sPLA2 enzyme family are associated with cancer. There is conflicting evidence on sPLA2, regarding its role as a tumor promoter or tumor suppressor. Based on the recent studies, while sPLA2 has a tumorigenic role in breast [204], lung [205], and prostate cancer [206], it has an anti-tumorigenic role in ovarian [207] and esophagus cancer [208], as evidenced by a decrease in the division rate hence metastasis of these cancer cells.

PCa is the second ranked types of cancer in worldwide. According to the recent findings, PCa was the leading cause of cancer-related death among men in 2021 [209]. Therefore, selection of a convenient therapy mode and development of new strategies against PCa is crucial. For most of the patients with PCa, ADT is the most convenient therapy [210]. However, the major concern about ADT is the development of castration-resistant PCa [211]. Therefore, in recent studies, researchers focused on the investigation of the molecular mechanisms not only to understand the progression of PCa but also to obtain the best response to the treatment [212–214].

The studies about PCa over two decades have demonstrated the importance of genome analysis. Xu *et al.* has reported that to date, more than 200 SNPs have been identified in the genomes of patients with PCa through GWAS [215]. Evaluation of SNP-based risk scores and rare pathogenic mutations as a cause of SNPs enabled the discovery of more than 100 haplotype SNPs [215], some of which affect the susceptibility to the disease [216]. Our previous study has demonstrated the association of rs11573156 C>G (5'UTR) polymorphism found in the non-coding, intron region of PLA2G2A, with PCa [197]. This polymorphism is associated with alternative splicing of the PLA2G2A transcript, and causes the skipping of exon 2 during the transcription process [198]. Exon 2 has target sites for

miRNA binding, in addition to the transcription factors, acting as a repressor. The skipping of exon 2 results in elevated PLA2G2A serum and mRNA levels [198]. Our previous findings suggested that the homozygous G allele may exert an anti-metastatic effect in patients with PCa [197].

Genome editing has been used to manipulate the genes of organisms to improve the functions or modify the acquired diseases [155]. Recently, by the discovery of the CRISPR/Cas9 technology, genome editing was revolutionized. The CRISPR/Cas9 system became a widely used genome editing technology owing to its high precision. In this study, it was aimed to investigate the molecular and functional effects of the homozygous G allele on PCa by establishing a model PCa cell line (PC-3) using the CRISPR/Cas9 gene editing system. This study is the first to establish a cell model by editing the rs11573156 C>G polymorphism by CRISPR/Cas9 gene editing system.

Base-editing by CRISPR/Cas9 system has been widely preferred for the treatment of genetic or acquired diseases [162]. However, there is a major limitation of base-editing using the CRISPR/Cas9 system, namely the off-target effects; that is, the non-intentional editing of random DNA regions other than the target DNA site [167]. One strategy used to overcome the off-target effect is employing a donor template in order to repair the DSB of DNA by HDR-mediated repair rather than NHEJ [184]. However, according to Yang *et al.*, although the precision of HDR-mediated repair is higher than that of NHEJ, the efficiency is lower [217]. According to Liu *et al.*, prior to genome editing via CRISPR/Cas9 system, using the anti-mitotic agent nocodazole for the cell cycle synchronization increases the HDR efficiency from ten percent to thirty eight percent [199]. Nocodazole quenches the cell cycle at the G2/M phase [218], and Yang *et al.* demonstrated that genome editing during the G2/M phase improves the efficiency of donor template integration [219]; therefore, this strategy was used in this study to synchronize PC-3 cells prior to gene editing. However, since nocodazole is an anti-cancer drug, it was important to select a non-toxic concentration. Previously, nanomolar concentrations of nocodazole were successfully utilized for the G2/M phase synchronization [218]; in order to identify the optimum concentration of nocodazole for PC-3 cells, several nanomolar concentrations were used in cell viability and proliferation analysis, based on the literature [218]. Of the concentrations used in experiment 50 ng/ml was selected as effective, which was in line with literature for PC-3 cells [218].

Additionally, the selection of a long homology arm on complementing the site of the DSB and using a single-stranded rather than a double-stranded template also enhances the precision of HDR. Song *et al.* demonstrated that the linear HDR template showed the highest HDR efficiency [220]. In this regard, the design of donor template and homology arm in this study were selected based on these validated notions [184]. Another solution that has been used to overcome the off-target limitation is using transient transfection. Since the Cas9 enzyme continues to cut the DNA as long as it is located in the nucleus, transient transfection has been used to eliminate the sustained expression of the pRP-CRISPR/Cas9 plasmid, thereby decreasing the off-target effect. Shen *et al.* demonstrated the minimization of the off-target effects through the transient expression of Cas9 [221]. Therefore, a transient expression system was utilized in this study.

The first step of this study was the establishment of the PCa cell model using CRISPR/Cas9-based gene-editing on PC-3 human prostate adenocarcinoma cells. As validated by sequence analysis of PLA2G2A gene, PC-3 cells carrying the homozygous G allele for rs11573156 SNP was successfully established. In order to investigate whether polymorphic GG genotype effect the PLA2G2A gene expression, HDR-mediated PC-3 cells along with Reporter and parental PC-3 cells were primed, as accumulating evidence suggests that PLA2G2A is expressed in response to inflammatory stimuli in the TME [18,61,124,129]. Therefore, the PLA2G2A expression was determined in response to H₂O₂ or interferon- γ cytokine priming that mimics the inflammatory TME leading to increased PLA2G2A gene expression [222–224]. In the TME, cancer cells secrete H₂O₂, which causes an oxidative stress on fibroblast cells resulting in their transformation to CAFs [225]. The CAFs then start to secrete higher levels of H₂O₂ than normal fibroblasts. The NF- κ B pathway is triggered as a result of this positive feedback loop involving further production of H₂O₂, leading to sPLA2-IIA biosynthesis and release in the downstream of the NF- κ B pathway [225]. In the TME, there are plenty of released cytokines causing inflammation. Interferon- γ is one of these cytokines, produced by natural killer cells and T cells, with both antitumor and protumor effects [226]. Due to the correlation between sPLA2-IIA and inflammatory conditions, cytokine release also alters the expression of PLA2G2A [226,227]. Thus, in order to mimic the induction of PLA2G2A expression by the tumor microenvironment, H₂O₂ and interferon- γ cytokine priming was utilized in this study.

Interferon- γ and H₂O₂ can be toxic above the physiological concentrations and hence affect the cell viability [228,229]. In this context, Park *et al.*, showed that at increasing concentrations H₂O₂ caused a G1-phase arrest while prolonged incubation with H₂O₂ (12 hours) led to apoptosis indicating that H₂O₂ treatment should be fine-tuned to avoid any cytostatic and/or cytotoxic effect [228]. As Oliveira-Marques *et al.* examined the optimal exposure time to H₂O₂ for triggering the NF- κ B pathway as 4 hours, this treatment-duration was chosen in our experiments [230]. In line with literature, we observed a decrease in rate of proliferation following the treatment with H₂O₂ above 50 μ M, therefore in our experimental setup PC-3 cells were treated with 50 μ M H₂O₂ for 4 hours. In agreement with Park *et al.*, treatment of parental PC-3 with H₂O₂ prolonged the doubling time from 26 hours to 36 hours without causing any toxicity.

Champelovier *et al.* demonstrated that interferon- γ upregulates the PLA2G2A expression by three-fold and inhibits the proliferation of RT112 (bladder carcinoma) cells [229]. In addition, they have shown that a high concentration of interferon- γ (>10 ng/ml) triggers apoptosis hence the treatment period should be shorter than 12 hours [229]. Another study by Menschikowski *et al.* used a respectively higher concentration for interferon- γ (25 ng/ml) to treat PC-3 cells for 24 hours and showed that PLA2G2A expression was induced in response to this treatment [130]. In order to keep similar treatment duration for the priming protocol, we have tested the effect of interferon- γ at 25 ng/ml for 4 hours on gene expression of PLA2G2A in PC-3 cells. Selection of this time point was important as it allowed the direct comparison of H₂O₂ and interferon- γ treatment on cell proliferation under the same treatment duration. The treatment of parental PC-3 with interferon- γ delayed the doubling time of the parental PC-3 cells similar to H₂O₂ to 35 hours (Section 4.5.1.). However, interferon- γ treatment induced the PLA2G2A gene expression levels more drastically when compared to H₂O₂ treatment. The expression level of PLA2G2A in HDR-mediated PC-3 cells was increased to 3-fold of parental PC-3 cells in response to H₂O₂, whereas a 12-fold increase in PLA2G2A expression level was detected in response to interferon- γ cytokine priming in HDR-mediated PC-3 cells. In support of our findings, Peilot *et al.* demonstrated that interferon- γ induce PLA2G2A expression by activating the transcription factor STAT3 [231]. Therefore, the difference between PLA2G2A gene expression level in response to H₂O₂ and interferon- γ cytokine priming can be explained by direct induction of PLA2G2A

gene by interferon- γ , while H_2O_2 indirectly induce PLA2G2A gene expression through activation of pro-inflammatory pathway [232].

As the elevated levels of PLA2G2A was found to be associated with tumor grade and anti-metastatic potential in cancers [197,233], the relative change in the proliferation rate of PC-3 cells due to heterozygous and homozygous G allele in 5' UTR region rs11573156 SNP of PLA2G2A was next explored following H_2O_2 and interferon- γ cytokine priming. Our results showed that HDR-mediated PC-3 cells that carry the homozygous G allele have a reduced rate of proliferation with respect to parental and Reporter PC-3 cells with GC genotype in response to both H_2O_2 and interferon- γ . This reduction in proliferation can be explained with the increased PLA2G2A expression detected in HDR-mediated PC-3 cells as Zhao *et al.* demonstrated that sPLA2-IIA expression inhibits the EGF signal transduction in A-431 human epidermoid carcinoma cells [234]. Thus, the observed decline in the proliferation rate of HDR-mediated PC-3 cells might in part depend on the inhibition of EGF signaling. Anti-proliferative role of sPLA2-IIA was also suggested by Schewe *et al.* who showed that sPLA2-IIA interferes Wnt signaling through Yap activation in the intestine, preventing cell proliferation and adjusting the intestinal cell differentiation and maturation [235]. On the other hand, Ganesan *et al.* showed that gene silencing of PLA2G2A results in a decrease of invasive character in advanced gastric cancer, whereas in the early stage, elevated PLA2A has a tumor suppressor role, reducing the cell proliferation rate via Wnt signaling and negatively regulating the metastatic genes S100A4 and NEDD9 [233].

Cytostatic effect of sPLA2-IIA can also be due to explained by sPLA2-IIA catalyzed production of PGE_2 as Kisslov *et al.* showed that PGE_2 is able to Akt activation to promote the localization of β -catenin in the nucleus, thus controlling the cell cycle by reducing cyclin D1 activity and arresting the cell cycle at the G0/G1 phase in gastric cancer cells [236]. In support of these findings, in our study, we investigated the proliferation patterns of parental, Reporter, and HDR-mediated PC-3 cells after detecting the PLA2G2A expression level in response to interferon - γ cytokine priming (Section 4.4.2.). Our results demonstrated that the majority of the HDR-mediated PC-3 cell population was quenched at the G0/G1 stage, while the majority of parental and Reporter PC-3 cells progressed to the G2/M phase. In other words, parental and Reporter PC-3 cells were almost ready for mitosis, whereas HDR-mediated PC-3 cells were at the initial stage of the cell cycle control point.

In summary, our results suggested that gene editing of the GC genotype (rs11573156 C>G heterozygous polymorphism) to homozygous G allele on the 5'UTR region of PLA2G2A results in reduced proliferative capacity in PC-3 cells. Xing *et al.* proposed that in gastric cancer PLA2G2A might be a potential biomarker in early phase detection [227]. Based on our results, PLA2G2A might also be potential biomarker in early stages of prostate cancer, and used by clinicians in decision making for the right drug regimen.



6. CONCLUSION AND FUTURE PERSPECTIVE

Although the roles of PLA2G2A in cancer have not been fully elucidated yet, a previous study of our group found a potential association GC genotype (rs11573156 C>G heterozygous polymorphism) on the 5'UTR region of PLA2G2A with the enhanced serum level of PLA2G2A and reduced metastatic effect [197]. Based on these findings, rs11573156 C>G nucleotide switching was performed on PC-3 prostate cancer cells using the CRISPR/Cas9 gene editing system. Notably, this is the first study to establish a PCa cell model by CRISPR/Cas9 gene editing and investigate the effects of the homozygous G allele on the 5'UTR region of the PLA2G2A gene.

In a very recent study, Overway *et al.* reported the significant effects of SNPs on enzyme activity [237]. Therefore, it can be deduced that, as a result of gene editing, enzyme activity level might be affected. To investigate whether changes in PLA2G2A expression is mirrored by sPLA2-IIa activity, sPLA2 IIA specific-assay can be performed for detecting and comparing the enzyme activity levels of HDR-mediated, Reporter and parental PC-3 cells.

Our results demonstrated that the homozygous G allele results in an elevated PLA2G2A gene expression and reduced proliferative capacity in PC-3 cells. Besides the anti-proliferative role of PLA2G2A, the secreted form sPLA2-IIA has been reported to have opposing roles in different cancer types. For instance, according to a recent study by Mehta *et al.*, sPLA2-IIA was found to be upregulated, and JNK signaling was enhanced in breast cancer; thus, cell proliferation and EMT potential were also triggered [238]. Based on this study, in the future study the possible induction of EMT may be investigated in the established PC-3 cells by detecting the expression levels of well-known EMT markers such as E-cadherin, N-cadherin, Snail1/2, Twist1/2, vimentin, and/or fibronectin 1.

As a continuation of this study, further *in vitro* and *in vivo* evidence validating the association of the polymorphism with the risk of metastasis are also required. For this purpose, functional analyses can be performed to demonstrate the metastatic ability of HDR-mediated PC-3 cells in comparison to parental PC-3 cells using techniques as scratch and scatter assays. Literature suggests that sPLA2-IIA impose anti-cancer effect through inhibition of EGFR or Wnt signaling. In line with that, EGFR and Wnt pathways may be investigated by analyzing the protein levels of phosphorylated EGFR (anti-tyr-1173-p-EGFR),

phosphorylated PI3K (p-PI3 Kinase p85 (Tyr 458)/p55 (Tyr199)), phosphorylated Akt (Phospho-Akt (Ser473)) and translocation of β -catenin using Western Blot.

In conclusion, this study examined the potential effect of rs11573156 C>G SNP on PLA2G2A gene expression and suggests that the observed difference in the proliferation rates of PC-3 cells with C allele and G allele may be due to alteration in the expression levels of PLA2G2A gene expression. This limitation can be addressed in the future studies using downregulation of PLA2G2A using siRNA against PLA2G2A in HDR-mediated PC-3 cells to investigate whether the observed decline in the proliferation rate of HDR-mediated PC-3 can be attributed to increased levels of sPLA2-IIA.



REFERENCES

1. Masumoto H, Rodríguez-Vázquez JF, Verdugo-López S, Murakami G, Matsubara A. Fetal topographical anatomy of the female urethra and descending vagina: A histological study of the early human fetal urethra. *Ann Anat.* 2011;193(6):500–8.
2. Attard G, Parker C, Eeles RA, Schröder F, Tomlins SA, Tannock I, *et al.* Prostate cancer. *The Lancet.* 2016;387(10013):70–82.
3. Fibbi B, Penna G, Morelli A, Adorini L, Maggi M. Chronic inflammation in the pathogenesis of benign prostatic hyperplasia. *Int J Androl.* 2011;33(3):475–88.
4. Denmeade DR, Isaacs JT. Cellular Organization of the Normal Prostate. *Holland-Frei Cancer Medicine.* 2003:58.
5. Abate-Shen C, Shen MM. Mouse models of prostate carcinogenesis. *Trends Genet.* 2002;18(5): 1-9.
6. Timms BG. Prostate development: a historical perspective. *Differentiation.* 2008;76(6):565–77.
7. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Grönberg H, Drake CG, *et al.* Inflammation in prostate carcinogenesis. *Nat Rev Cancer.* 2007;7(4):256–69.
8. Andriole GL, Crawford ED, Grubb RL, Buys SS, Chia D, Church TR, *et al.* Mortality results from a randomized prostate-cancer screening trial. *N Engl J Med.* 2009;360(13):1310–19.
9. Davila-Legorreta E, Rodenas-Gil EA, Navarro-Ruesga I, Santa Maria-Orozco FJ, Cantellano-Orozco M, Pacheco-Gahbler C. Progressive vision loss as the initial symptom of Prostate Cancer. *Urol Case Rep.* 2022;42:102000.
10. Chu LW, Ritchey J, Devesa SS, Quraishi SM, Zhang H, Hsing AW. Prostate Cancer Incidence Rates in Africa. *Prostate Cancer.* 2011;2011:1–6.
11. Crawford ED. Epidemiology of prostate cancer. *Urology.* 2003;62(6 Suppl 1):3–12.
12. Zorlu F, Divrik RT, Eser S, Yorukoglu K. Prostate cancer incidence in Turkey: an epidemiological study. *Asian Pac J Cancer Prev.* 2014;15(21):9125–30.

13. Mohler J, Bahnson RR, Boston B, Busby JE, D'Amico A, Eastham JA, *et al.* NCCN clinical practice guidelines in oncology: prostate cancer. *J Natl Compr Canc Netw.* 2010;8(2):162–200.
14. Stark T, Livas L, Kyprianou N. Inflammation in prostate cancer progression and therapeutic targeting. *Transl Androl and Urol.* 2015;4(4):455-63.
15. Taitt HE. Global Trends and Prostate Cancer: A Review of Incidence, Detection, and Mortality as Influenced by Race, Ethnicity, and Geographic Location. *Am J of Men's Health.* 2018;12(6):1807–23.
16. Hester J, Ventetuolo C, Lahm T. Sex, Gender, and Sex Hormones in Pulmonary Hypertension and Right Ventricular Failure. *Compr Physiol.* 2019;10(1):125-70.
17. Le H, Arnold JT, McFann KK, Blackman MR. DHT and testosterone, but not DHEA or E2, differentially modulate IGF-I, IGFBP-2, and IGFBP-3 in human prostatic stromal cells. *Am J Physiol Endocrinol and Metab.* 2006;290(5):E952-60.
18. Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, *et al.* Role of tumor microenvironment in tumorigenesis. *J Cancer.* 2017;8(5):761-73.
19. Lazebnik Y. What are the hallmarks of cancer? *Nat Rev Cancer.* 2010;10(4):232–3.
20. Hallmarks of Cancer: The Next Generation. *Cell.* 2011;144(5):646–74.
21. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol.* 2002;196(3):254–65.
22. Yoshida GJ. Regulation of heterogeneous cancer-associated fibroblasts: the molecular pathology of activated signaling pathways. *J Exp Clin Cancer Res.* 2020;16(39):112.
23. Devlin HL, Mudryj M. Progression of prostate cancer: Multiple pathways to androgen independence. *Cancer Lett.* 2009;274(2):177–86.
24. Lamont KR, Tindall DJ. Minireview: Alternative Activation Pathways for the Androgen Receptor in Prostate Cancer. *Mol Endocrinol.* 2011;25(6):897–907.

25. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer*. 2001;1(1):34–45.
26. Ashida S, Nakagawa H, Katagiri T, Furihata M, Iizumi M, Anazawa Y, *et al*. Molecular Features of the Transition from Prostatic Intraepithelial Neoplasia (PIN) to Prostate Cancer Genome-wide Gene-expression Profiles of Prostate Cancers and PINs. *Cancer Res*. 2004;64(17):5963–72.
27. Algaba F, Montironi R. Impact of Prostate Cancer Multifocality on Its Biology and Treatment. *J Endourol*. 2010;25(5):799–804.
28. Karantanos T, Corn PG, Thompson TC. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. *Oncogene*. 2013;32(49):5501–11.
29. Datta D, Aftabuddin M, Gupta DK, Raha S, Sen P. Human Prostate Cancer Hallmarks Map. *Sci Rep*. 2016;6(1):1-14.
30. Rybak, A. P., Bristow, R. G., & Kapoor, A. Prostate cancer stem cells: deciphering the origins and pathways involved in prostate tumorigenesis and aggression. *Oncotarget*. 2015;6(4):1900-19.
31. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646–74.
32. Danielsson F, Skogs M, Huss M, Rexhepaj E, O’Hurley G, Klevebring D, *et al*. Majority of differentially expressed genes are down-regulated during malignant transformation in a four-stage model. *Proc Natl Acad Sci U S A*. 2013;110(17):6853–8.
33. Tindall DJ, Scardino PT. *Recent advances in prostate cancer: Basic science discoveries and clinical advances*. World Scientific Publishing Co. 2011.
34. Langley RR, Fidler IJ. The seed and soil hypothesis revisited—The role of tumor-stroma interactions in metastasis to different organs. *Int J Cancer*. 2011;128(11):2527–35.

35. Cook LM, Shay G, Aruajo A, Lynch CC. Integrating new discoveries into the “vicious cycle” paradigm of prostate to bone metastases. *Cancer and Metastasis Rev.* 2014;33(2–3):511–25.
36. Wang G, Zhao D, Spring DJ, Depinho RA. Genetics and biology of prostate cancer. *Genes Dev.* 2018;32(17–18):1105.
37. Rybak AP, Bristow RG, Kapoor A. Prostate cancer stem cells: DeciFphering the origins and pathways involved in prostate tumorigenesis and aggression. *Oncotarget.* 2015;6(4):1900–19.
38. Benafif S, Kote-Jarai Z, Eeles RA. A Review of Prostate Cancer Genome-Wide Association Studies (GWAS). *Cancer Epidemiol Biomarkers Prev.* 2018;27(8):845–57.
39. Cicek MS, Conti D v., Curran A, Neville PJ, Paris PL, Casey G, *et al.* Association of Prostate Cancer Risk and Aggressiveness to Androgen Pathway Genes: SRD5A2, CYP17, and the AR. *Prostate.* 2004;59(1):69–76.
40. Chen C, Bhalala H v., Vessella RL, Dong JT. KLF5 is frequently deleted and down-regulated but rarely mutated in prostate cancer. *Prostate.* 2003;55(2):81–8.
41. Dong JT. Prevalent mutations in prostate cancer. *J Cell Biochem.* 2006;97(3):433–47.
42. Yan Y, Huang H. Interplay Among PI3K/AKT, PTEN/FOXO and AR Signaling in Prostate Cancer. *Adv Exp Med Biol.* 2019;1210(3):319–31.
43. Leung JK, Sadar MD. Non-Genomic Actions of the Androgen Receptor in Prostate Cancer. *Front Endocrinol (Lausanne).* 2017;8(2):17.
44. Lonergan P, Tindall D. Androgen receptor signaling in prostate cancer development and progression. *J Carcinog.* 2011;10(1):20.
45. Kwon EM, Salinas CA, Kolb S, Fu R, Feng Z, Stanford JL, *et al.* Genetic polymorphisms in inflammation pathway genes and prostate cancer risk. *Cancer Epidemiol Biomarkers Prev.* 2011;20(5):923–33.
46. Bozeman CB, Carver BS, Eastham JA, Venable DD. Treatment Of Chronic Prostatitis Lowers Serum Prostate Specific Antigen. *J Urol.* 2002;167(4):1723–6.

47. Egevad L, Granfors T, Karlberg L, Bergh A, Stattin P. Prognostic value of the Gleason score in prostate cancer. *BJU Int.* 2002;89(6):538–42.
48. Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, *et al.* Delineation of prognostic biomarkers in prostate cancer. *Nature.* 2001;412(6849):822–6.
49. Gleason DF, Mellinger GT, Ardring LJ. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *J Urol.* 1974;111(1):58–64.
50. Zetsche B, Volz SE, Zhang F. A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nat Biotechnol.* 2015;33(2):139–42.
51. Singhal N, Soni S, Bonthu S, Chattopadhyay N, Samanta P, Joshi U, *et al.* A deep learning system for prostate cancer diagnosis and grading in whole slide images of core needle biopsies. *Sci Rep.* 2022;12(1):1–11.
52. Litwin MS, Tan HJ. The Diagnosis and Treatment of Prostate Cancer: A Review. *JAMA.* 2017;317(24):2532–42.
53. Cheng L, Montironi R, Bostwick DG, Lopez-Beltran A, Berney DM. Staging of prostate cancer. *Histopathology.* 2012;60(1):87–117.
54. Alaimo A, Lorenzoni M, Ambrosino P, Bertossi A, Bisio A, Macchia A, *et al.* Calcium cytotoxicity sensitizes prostate cancer cells to standard-of-care treatments for locally advanced tumors. *Cell Death Dis.* 2020;11(12):1–17.
55. Trewartha D, Carter K. Advances in prostate cancer treatment. *Nat Rev Drug Discov.* 2013;12(11):823–4.
56. Zelefsky MJ, Kuban DA, Levy LB, Potters L, Beyer DC, Blasko JC, *et al.* Multi-institutional analysis of long-term outcome for stages T1–T2 prostate cancer treated with permanent seed implantation. *Int J of Radiat Oncol Biol Phys.* 2007;67(2):327–33.

57. Welch HG, Albertsen PC. Prostate Cancer Diagnosis and Treatment After the Introduction of Prostate-Specific Antigen Screening: 1986–2005. *J Natl Cancer Inst.* 2009;101(19):1325–29.
58. Donnelly SE, Donnelly BJ, Saliken JC, Raber EL, Vellet AD. Prostate Cancer: Gadolinium-enhanced MR Imaging at 3 Weeks Compared with Needle Biopsy at 6 Months after Cryoablation 1. *Radiology* 2004;232(3):830–3.
59. Greten FR, Grivennikov SI. Inflammation and Cancer: Triggers, Mechanisms, and Consequences. *Immunity.* 2019;51(1):27–41.
60. Coussens LM, Werb Z. Inflammation and cancer. *Nature.* 2002;420(6917):860–7.
61. Florian R. Greten, Sergei I. Grivennikov. *Immunity.* 2019; 51(1): 27–41.
62. Zhao H, Wu L, Yan G, Chen Y, Zhou M, Wu Y, *et al.* Inflammation and tumor progression: signaling pathways and targeted intervention. *Signal Transduct Targeted Ther.* 2021;6(1):1–46.
63. Kirschenbaum A, Klausner AP, Lee R, Unger P, Yao S, Liu XH, *et al.* Expression of cyclooxygenase-1 and cyclooxygenase-2 in the human prostate. *Urology.* 2000;56(4):671–6.
64. Kim BH, Kim C il, Chang HS, Choe MS, Jung HR, Kim DY, *et al.* Cyclooxygenase-2 Overexpression in Chronic Inflammation Associated with Benign Prostatic Hyperplasia: Is It Related to Apoptosis and Angiogenesis of Prostate Cancer? *Korean J Urol.* 2011;52(4):253.
65. Partin AW. Cyclooxygenase-2 as a Marker for Prostate Cancer. *Rev Urol.* 2001;3(2):107.
66. C.S. Foster,D.G. Bostwick,H. Bonkhoff,J.-E. Damber,T. van der Kwast,R. Montironi &W.A. Sakr. Cellular and molecular pathology of prostate cancer precursors. *Scand J Urol Nephrol Suppl.* 2009;34(205):19-43.
67. Janiczek M, Szyłberg Ł, Szyłberg Ł, Antosik P, Kasperska A, Marszałek A, *et al.* Expression Levels of IL-17A, IL-17F, IL-17RA, and IL-17RC in Prostate Cancer with Taking into Account the Histological Grade according to Gleason Scale in

- Comparison to Benign Prostatic Hyperplasia: In Search of New Therapeutic Options. *J Immunol Res.* 2020;2020:1-7.
68. Netti GS, Franzin R, Stasi A, Spadaccino F, dello Strologo A, Infante B, *et al.* Role of Complement in Regulating Inflammation Processes in Renal and Prostate Cancers. *Cells.* 2021;10(9):2426.
 69. Moradi S, Issah A, Mohammadi H, Mirzaei K. Associations between dietary inflammatory index and incidence of breast and prostate cancer: a systematic review and meta-analysis. *Nutrition.* 2018;55(56):168–78.
 70. Duloy AMS, Calhoun EA, Clemens JQ. Economic impact of chronic prostatitis. *Curr Urol Rep.* 2008;8(4):336–9.
 71. Sfanos KS, de Marzo AM. Prostate cancer and inflammation: the evidence. *Histopathology.* 2012;60(1):199.
 72. Komura K, Sweeney CJ, Inamoto T, Ibuki N, Azuma H, Kantoff PW. Current treatment strategies for advanced prostate cancer. *Int J Urol.* 2018;25(3):220–31.
 73. Davies AH, Zoubeidi A. Targeting androgen receptor signaling: a historical perspective. *Endocr Relat Cancer.* 2021;28(8):11–8.
 74. Rannamäe E, Saarma U, Bläuer A. Cultural influences on the castration age of cattle in the northern Baltic Sea region during the medieval and post-medieval periods. *Journal of Archaeological Science.* 2022:137.
 75. CHUTE R. Radical Retropubic Prostatectomy for Cancer. *J Urol.* 1954;71(3):347–72.
 76. Bagshaw MA. Modification of the Radiosensitivity of Hamster Cells in vitro. *Nature.* 1962;193(4813):389–91.
 77. Denmeade SR, Isaacs JT. A history of prostate cancer treatment. *Nat Rev Cancer.* 2002;2(5):389–96.
 78. Albertsen PC, Fryback DG, Storer BE, Kolon TF, Fine J. The Impact of Co-morbidity on Life Expectancy Among Men with Localized Prostate Cancer. *J Urol.* 1996;156(1):127–32.

79. Tamanoi F, Matsumoto K, Doan TLH, Shiro A, Saitoh H. Convergence of the Study on Monochromatic X-rays and the Research on Nanoparticles Opens Up a Possibility to Develop a New Type of Radiation Therapy. *Preprints*. 2020.
80. Connor MJ, Gorin MA, Ahmed HU, Nigam R. Focal therapy for localized prostate cancer in the era of routine multi-parametric MRI. *Prostate Cancer Prostatic Dis*. 2020;23(2):232–43.
81. Singla N, Ghandour RA, Raj G v. Investigational luteinizing hormone releasing hormone (LHRH) agonists and other hormonal agents in early-stage clinical trials for prostate cancer. *Expert Opin Investig Drugs*. 2019;28(3):249–59.
82. Brawer MK. Hormonal Therapy for Prostate Cancer. *Rev Urol*. 2006;8(Suppl.2):S35–37.
83. Vandekerkhove G, Struss WJ, Annala M, Kallio HML, Khalaf D, Warner EW, *et al*. Circulating Tumor DNA Abundance and Potential Utility in De Novo Metastatic Prostate Cancer. *Eur Urol*. 2019;75(4):667–75.
84. Wang F, Li Z, Feng X, Yang D, Lin M. Advances in PSMA-targeted therapy for prostate cancer. *Prostate Cancer Prostatic Dis*. 2021;25(1):11–26.
85. Petrioli R, Francini E, Fiaschi AI, Laera L, Roviello G. Targeted Therapies for Prostate Cancer. *Cancer Invest*. 2015;33(7):276–85.
86. Merseburger AS, Waldron N, Ribal MJ, Heidenreich A, Perner S, Fizazi K, *et al*. Genomic Testing in Patients with Metastatic Castration-resistant Prostate Cancer: A Pragmatic Guide for Clinicians. *Eur Urol*. 2021;79(4):519–29.
87. Nader R, el Amm J, Aragon-Ching J. Role of chemotherapy in prostate cancer. *Asian J Androl*. 2018;20(3):221.
88. Tanaka N, Nishimura K, Okajima E, Ina K, Ogawa O, Nagata H, *et al*. Docetaxel-based chemotherapy combined with dexamethasone 1 mg daily oral administration for castration-resistant prostate cancer: Long-term outcomes. *Int J Urol*. 2019;26(8):797–803.

89. Cevik O, Acidereli H, Turut FA, Yildirim S, Acilan C. Cabazitaxel exhibits more favorable molecular changes compared to other taxanes in androgen-independent prostate cancer cells. *J Biochem Mol Toxicol*. 2020;34(9):e22542.
90. Alexandra R. Harris, Matthew J. Perez, Jennifer M. Munson. Docetaxel facilitates lymphatic-tumor crosstalk to promote lymphangiogenesis and cancer progression. *BMC Cancer*. 2018;18(1):718.
91. Fay EK, Graff JN. Immunotherapy in Prostate Cancer. *Cancers (Basel)*. 2020;12(7):1–17.
92. Aloulou A, Rahier R, Arhab Y, Noiriel A, Abousalham A. Phospholipases: An Overview. *Methods Mol Biol*. 2018:69–105.
93. Waite M. Chapter 8 Phospholipases. *New Comprehensive Biochemistry*. 1991:269–295.
94. Guo Z, Vikbjerg AF, Xu X. Enzymatic modification of phospholipids for functional applications and human nutrition. *Biotechnol Adv*. 2005;23(3):203–59.
95. Bill CA, Vines CM. Phospholipase C. *Adv Exp Med Biol*. 2020;1131:215–42.
96. Titball RW, Basak AK. Membrane-damaging and cytotoxic phospholipases. *The Comprehensive Sourcebook of Bacterial Protein Toxins*. 2006:516–34.
97. Bamji-Mirza M, Yoa Z. Phospholipases. *AOCS Lipid Library*. 2011:12-15.
98. Casale J, Kacimi SEO, Varacallo M. *Biochemistry, Phospholipase A2*. StatPearls. 2021.
99. Murakami M, Sato H, Taketomi Y. Updating phospholipase a2 biology. *Biomolecules*. 2020;10(10):1–33.
100. Murakami M, Taketomi Y, Girard C, Yamamoto K, Lambeau G. Emerging roles of secreted phospholipase A2 enzymes: Lessons from transgenic and knockout mice. *Biochimie*. 2010;92(6):561–82.
101. Murakami M. Novel functions of phospholipase A2s: Overview. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2019;1864(6):763–5.

102. Dennis EA, Cao J, Hsu YH, Magrioti V, Kokotos G. Phospholipase A2 Enzymes: Physical Structure, Biological Function, Disease Implication, Chemical Inhibition, and Therapeutic Intervention. *Chem Rev.* 2011;111(10):6130.
103. Kudo I, Murakami M. Phospholipase A2 enzymes. *Prostaglandins & Other Lipid Mediators.* 2002;3–58.
104. Valentin E, Ghomashchi F, Gelb MH, Lazdunski M, Lambeau G. On the diversity of secreted phospholipases A(2). Cloning, tissue distribution, and functional expression of two novel mouse group II enzymes. *J Biol Chem.* 1999;274(44):31195–202.
105. Underwood KW, Song C, Kriz RW, Chang XJ, Knopf JL, Lin LL. A novel calcium-independent phospholipase A2, cPLA2-gamma, that is prenylated and contains homology to cPLA2. *J Biol Chem.* 1998;273(34):21926–32.
106. Schievella AR, Regier MK, Smith WL, Lin LL. Calcium-mediated translocation of cytosolic phospholipase A2 to the nuclear envelope and endoplasmic reticulum. *J Biol Chem.* 1995;270(51):30749–54.
107. Clark JD, Lin LL, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, *et al.* A novel arachidonic acid-selective cytosolic PLA2 contains a Ca²⁺-dependent translocation domain with homology to PKC and GAP. *Cell.* 1991;65(6):1043–51.
108. Dennis EA. The growing phospholipase A2 superfamily of signal transduction enzymes. *Trends Biochem Sci.* 1997;22(1):1–2.
109. Arai H, Koizumi H, Aoki J, Inoue K. Platelet-activating factor acetylhydrolase (PAF-AH). *J Biochem.* 2002;131(5):635–40.
110. Hattori K, Adachi H, Matsuzawa A, Yamamoto K, Tsujimoto M, Aoki J, *et al.* cDNA cloning and expression of intracellular platelet-activating factor (PAF) acetylhydrolase II. Its homology with plasma PAF acetylhydrolase. *J Biol Chem.* 1996;271(51):33032–8.
111. Tjoelker LW, Wilder C, Eberhardt C, Stafforini DM, Dietsch G, Schimpf B, *et al.* Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. *Nature.* 1995;374(6522):549–53.

112. Mitsios J v., Vini MP, Stengel D, Ninio E, Tselepis AD. Human platelets secrete the plasma type of platelet-activating factor acetylhydrolase primarily associated with microparticles. *Arterioscler Thromb Vasc Biol.* 2006;26(8):1907–13.
113. Shayman JA, Tesmer JJG. Lysosomal phospholipase A2. *Biochim Biophys Acta Mol Cell Biol Lipids.* 2019;1864(6):932–40.
114. Duncan RE, Sarkadi-Nagy E, Jaworski K, Ahmadian M, Hei SS. Identification and functional characterization of adipose-specific phospholipase A2 (AdPLA). *J Biol Chem.* 2008;283(37):25428–36.
115. Quach ND, Arnold RD, Cummings BS. Secretory phospholipase A2 enzymes as pharmacological targets for treatment of disease. *Biochem Pharmacol.* 2014;90(4):338–48.
116. Murakami M. Lipoquality control by phospholipase A 2 enzymes. *Proc Jpn Acad Ser B Phys Biol Sci.* 2017;93(9):677–702.
117. Bahrami A, Taheri M, Arabestani MR, Soleimani M, Mohammadi M, Golabchi F, *et al.* Harnessing the Natural Toxic Metabolites in COVID-19. *Evid Based Complement Alternat Med.* 2022;2022:1–7.
118. Lambeau G, Gelb MH. Biochemistry and Physiology of Mammalian Secreted Phospholipases A2. *Annu Rev Biochem.* 2008;77(3):495-520.
119. Weinrauch Y, Abad C, Liang NS, Lowry SF, Weiss J. Mobilization of potent plasma bactericidal activity during systemic bacterial challenge. Role of group IIA phospholipase A2. *J Clin Invest.* 1998;102(3):633–8.
120. Laine VJO, Grass DS, Nevalainen TJ. Resistance of transgenic mice expressing human group II phospholipase A2 to Escherichia coli infection. *Infect Immun.* 2000;68(1):87–92.
121. Han WK, Sapirstein A, Hung CC, Alessandrini A, Bonventre J v. Cross-talk between cytosolic phospholipase A2 alpha (cPLA2 alpha) and secretory phospholipase A2 (sPLA2) in hydrogen peroxide-induced arachidonic acid release in murine mesangial cells: sPLA2 regulates cPLA2 alpha activity that is responsible for arachidonic acid release. *J Biol Chem.* 2003;278(26):24153–63.

122. Boyanovsky BB, Webb NR. Biology of Secretory Phospholipase A₂. *Cardiovasc Drugs Ther.* 2009;23(1):61.
123. Dore E, Boilard E. Roles of secreted phospholipase A₂ group IIA in inflammation and host defense. *Biochim Biophys Acta Mol Cell Biol Lipids.* 2019;1864(6):789–802.
124. Boudreau LH, Duchez AC, Cloutier N, Soulet D, Martin N, Bollinger J, *et al.* Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A₂ to promote inflammation. *Blood.* 2014;124(14):2173–83.
125. Duchez AC, Boudreau LH, Bollinger J, Belleannée C, Cloutier N, Laffont B, *et al.* Platelet microparticles are internalized in neutrophils via the concerted activity of 12-lipoxygenase and secreted phospholipase A₂-IIA. *Proc Natl Acad Sci U S A.* 2015;112(27):E3564–73.
126. Schewe M, Franken PF, Sacchetti A, Schmitt M, Joosten R, Böttcher R, *et al.* Secreted Phospholipases A₂ Are Intestinal Stem Cell Niche Factors with Distinct Roles in Homeostasis, Inflammation, and Cancer. *Cell Stem Cell.* 2016;19(1):38–51.
127. Rouault M, le Calvez C, Boilard E, Surrel F, Singer A, Ghomashchi F, *et al.* Recombinant production and properties of binding of the full set of mouse secreted phospholipases A₂ to the mouse M-type receptor. *Biochemistry.* 2007;46(6):1647–62.
128. Van den Broeck T, Joniau S, Clinckemalie L, Helsen C, Prekovic S, Spans L, *et al.* The Role of Single Nucleotide Polymorphisms in Predicting Prostate Cancer Risk and Therapeutic Decision Making. *Biomed Res Int.* 2014;2014(1):3-13.
129. Yoshimura A. Signal transduction of inflammatory cytokines and tumor development. *Cancer Sci.* 2006;97(6):439–47.
130. Menschikowski M, Hagelgans A, Gussakovsky E, Kostka H, Paley EL, Siegert G. Differential Expression of Secretory Phospholipases A₂ in Normal and Malignant Prostate Cell Lines: Regulation by Cytokines, Cell Signaling Pathways, and Epigenetic Mechanisms. *Neoplasia.* 2008;10(3):279–86.
131. Oleksowicz L, Liu Y, Bracken RB, Gaitonde K, Burke B, *et al.* Abstract 3631: Secretory phospholipase A₂-IIa is a target gene of the HER/HER2-elicited pathway

- and a potential plasma biomarker for poor prognosis of prostate cancer. *Prostate*. 2012;72(10):1140-9.
132. Davis JN, Kucuk O, Sarkar FH. Genistein Inhibits NF- κ B Activation in Prostate Cancer Cells. *Nutr Cancer*. 1999;35(2):167–74.
 133. Mirtti T, Laine VJO, Hiekkänen H, Hurme S, Rowe O, Nevalainen TJ, *et al*. Group IIA phospholipase A2 as a prognostic marker in prostate cancer: relevance to clinicopathological variables and disease-specific mortality. *APMIS*. 2009;117(3):151–61.
 134. Dong Z, Liu Y, Scott KF, Levin L, Gaitonde K, Bracken RB, *et al*. Secretory phospholipase A2-IIa is involved in prostate cancer progression and may potentially serve as a biomarker for prostate cancer. *Carcinogenesis*. 2010;31(11):1948–55.
 135. Lu S, Zhongyun D. Overexpression of secretory phospholipase A2-IIa supports cancer stem cell phenotype via HER/ERBB-elicited signaling in lung and prostate cancer cells. *Int J Oncol*. 2017;50(6):2113–22.
 136. Chen D, Wei Y, Li X, Epstein S, Wolosin JM, Asbell P. sPLA2-IIa is an inflammatory mediator when the ocular surface is compromised. *Experimental Eye Research*. 2009;88(5):880–8.
 137. Dacheux M, Chaouch S, Joy A, Labat A, Payré C, Petit-Paitel A, *et al*. Role of human group IIA secreted phospholipase A2 in malaria pathophysiology: Insights from a transgenic mouse model. *Biochimie*. 2021;189:120–36.
 138. Juffrie M, Vo Meer GM, Hack CE, Haasnoot K, Sutaryo, Veerman AJP, *et al*. Inflammatory mediators in dengue virus infection in children: interleukin-6 and its relation to C-reactive protein and secretory phospholipase A2. *Am J Trop Med Hyg*. 2001;65(1):70–5.
 139. Chisini LA, Cademartori MG, Conde MCM, Costa F dos S, Salvi LC, Tovo-Rodrigues L, *et al*. Single nucleotide polymorphisms of taste genes and caries: a systematic review and meta-analysis. *Acta Odontol Scand*. 2021;79(2):147–55.
 140. Sim JJ, Chan FM, Chen S, Meng Tan BH, Mi Aung KM. Achieving GWAS with homomorphic encryption. *BMC Med Genomics*. 2020;13(Suppl 7):90.

141. Keegan NP, Fletcher S. A spotter's guide to SNPtic exons: The common splice variants underlying some SNP–phenotype correlations. *Mol Genet Genomic Med.* 2022;10(1):e1840.
142. Schork NJ, Fallin D, Lanchbury JS. Single nucleotide polymorphisms and the future of genetic epidemiology. *Clin Genet.* 2000;58(4):250–64.
143. Hemminki K, Försti A, Bermejo JL. Single nucleotide polymorphisms (SNPs) are inherited from parents and they measure heritable events. *Journal of Carcinogenesis.* 2005;4:2.
144. Edwards D, Forster JW, Chagné D, Batley J. What Are SNPs? *Association Mapping in Plants.* 2007;41–52.
145. Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Hum Genet.* 1980;32(3):314–31.
146. Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum Genet.* 1992;90(1–2):41–54.
147. Drazen JM, Yandava CN, Dubé L, Szczerback N, Hippensteel R, Pillari A, *et al.* Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. *Nat Genet.* 1999;22(2):168–70.
148. Tatiana Popova VB, Barillot MHS. Analysis of Somatic Alterations in Cancer Genome: From SNP Arrays to Next Generation Sequencing. *Genomics I Humans, Animals and Plants.* 2013.
149. Ge J, Budowle B. Forensic investigation approaches of searching relatives in DNA databases. *J Forensic Sci.* 2021;66(2):430–43.
150. Radanova M, Levkova M, Manev R, Levkova M, Mihaylova G, Maneva M, *et al.* Single Nucleotide Polymorphisms in microRNA Genes and Colorectal Cancer Risk and Prognosis. *Biomedicines.* 2022;10(1):156.

151. Farashi S, Kryza T, Clements J, Batra J. Post-GWAS in prostate cancer: from genetic association to biological contribution. *Nat Rev Cancer*. 2019;19(1):46–59.
152. Kraemer RJ, Coffey DS. The interaction of natural and synthetic polyanions with mammalian nuclei: I. DNA synthesis. *Biochim Biophys Acta*. 1970;224(2):553–67.
153. Reddy DS, Reddy T. Pharmaceutical and Biotechnological Applications of Transgenic Technology. *International Journal of Pharmaceutical Sciences and Nanotechnology*. 2018;11(4):4145–53.
154. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*. 2013;8(11):2281–308.
155. Hillary VE, Ceasar SA, Ignacimuthu S. Genome engineering in insects: focus on the CRISPR/Cas9 system. *Prog Mol Biol Transl Sci*. 2021;179:197–223.
156. Marraffini LA. CRISPR-Cas immunity in prokaryotes. *Nature*. 2015;526(7571):55–61.
157. Russo E. The birth of biotechnology. *Nature*. 2003;421(6921):456–7.
158. Rath D, Amlinger L, Rath A, Lundgren M. The CRISPR-Cas immune system: Biology, mechanisms and applications. *Biochimie*. 2015;117:119–28.
159. Rodios P. Genome editing with CRISPR Cas9 system. Master Thesis, KTH – Royal Institute of Technology, 1827.
160. Bakr Shabbir MA, Hao H, Shabbir MZ, Hussain HI, Iqbal Z, Ahmed S, *et al*. Survival and evolution of CRISPR-Cas system in prokaryotes and its applications. *Front Immunol*. 2016;7:375.
161. Pickar-Oliver A, Gersbach CA. The next generation of CRISPR–Cas technologies and applications. *Nat Rev Mol Cell Biol*. 2019;20(8):490–507.
162. Hille F, Charpentier E. CRISPR-Cas: biology, mechanisms and relevance. *Philos Trans R Soc Lond B Biol Sci*. 2016;371(1707):20150496.
163. Hua K, Tao X, Han P, Wang R, Zhu JK. Genome Engineering in Rice Using Cas9 Variants that Recognize NG PAM Sequences. *Mol Plant*. 2019;12(7):1003–14.

164. Moon SB, Kim DY, Ko JH, Kim YS. Recent advances in the CRISPR genome editing tool set. *Exp Mol Med*. 2019;51(11):1–11.
165. Fagerlund RD, Staals RHJ, Fineran PC. The Cpf1 CRISPR-Cas protein expands genome-editing tools. *Genome Biol*. 2015;16:251.
166. Scollan ME. CRISPR-Cas orthologs and variants. *CRISPR Genome Surgery in Stem Cells and Disease Tissues*. 2022;7–38.
167. Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Mol Ther Nucleic Acids*. 2015;4(11):e264.
168. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, *et al*. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature*. 2015;523(7561):481–5.
169. DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res*. 2013;41(7):4336–43.
170. Karvelis T, Gasiunas G, Siksnys V. Methods for decoding Cas9 protospacer adjacent motif (PAM) sequences: A brief overview. *Methods*. 2017;121–122:3–8.
171. Sharma A, Toepfer CN, Ward T, Wasson L, Agarwal R, Conner DA, *et al*. CRISPR/Cas9 Mediated Fluorescent Tagging of Endogenous Proteins in Human Pluripotent Stem Cells. *Curr Protoc Hum Genet*. 2018;96:21.11.1–20.
172. Waters CA, Strande NT, Pryor JM, Strom CN, Mieczkowski P, Burkhalter MD, *et al*. The fidelity of the ligation step determines how ends are resolved during nonhomologous end joining. *Nat Commun*. 2014;5:4286.
173. Bétermier M, Bertrand P, Lopez BS. Is Non-Homologous End-Joining Really an Inherently Error-Prone Process? *PLoS Genet*. 2014;10(1):e1004086.
174. Ochi T, Blackford AN, Coates J, Jhujh S, Mehmood S, Tamura N, *et al*. DNA repair. PAXX, a paralog of XRCC4 and XLF, interacts with Ku to promote DNA double-strand break repair. *Science*. 2015;347(6218):185–8.

175. Li H, Yang Y, Hong W, Huang M, Wu M, Zhao X. Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. *Signal Transduct Target Ther*. 2020;5(1):1.
176. Sung P, Klein H. Mechanism of homologous recombination: Mediators and helicases take on regulatory functions. *Nat Rev Mol Cell Biol*. 2006;7(10):739–50.
177. Pawelczak KS, Gavande NS, VanderVere-Carozza PS, Turchi JJ. Modulating DNA Repair Pathways to Improve Precision Genome Engineering. *ACS Chem Biol*. 2018;13(2):389–96.
178. Lambert S, Lopez BS. Characterization of mammalian RAD51 double strand break repair using non-lethal dominant-negative forms. *EMBO J*. 2000;19(12):3090–9.
179. Mehta A, Haber JE. Sources of DNA Double-Strand Breaks and Models of Recombinational DNA Repair. *Cold Spring Harb Perspect Biol*. 2014;6(9):a016428.
180. Jasin M, Rothstein R. Repair of strand breaks by homologous recombination. *Cold Spring Harb Perspect Biol*. 2013;5(11):a012740.
181. Pardo B, Gómez-González B, Aguilera A. DNA repair in mammalian cells: DNA double-strand break repair: how to fix a broken relationship. *Cell Mol Life Sci*. 2009;66(6):1039–56.
182. Li K, Wang G, Andersen T, Zhou P, Pu WT. Optimization of Genome Engineering Approaches with the CRISPR/Cas9 System. *PLoS One*. 2014;9(8):e105779.
183. Bennardo N, Gunn A, Cheng A, Hasty P, Stark JM. Limiting the persistence of a chromosome break diminishes its mutagenic potential. *PLoS Genet*. 2009;5(10):e1000683.
184. Liang X, Potter J, Kumar S, Ravinder N, Chesnut JD. Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA. *J Biotechnol*. 2017;241:136–46.
185. Singh P, Schimenti JC, Bolcun-Filas E. A mouse geneticist's practical guide to CRISPR applications. *Genetics*. 2015;199(1):1–15.

186. Liu Z, Liao Z, Chen Y, Han L, Yin Q, Xiao H. Application of Various Delivery Methods for CRISPR/dCas9. *Mol Biotechnol*. 2020;62(8):355–63.
187. Li Z, Zhang D, Xiong X, Yan B, Xie W, Sheen J, *et al*. A potent Cas9-derived gene activator for plant and mammalian cells. *Nat Plants*. 2017;3(12):930–6.
188. Sun X, Hu Z, Chen R, Jiang Q, Song G, Zhang H, *et al*. Targeted mutagenesis in soybean using the CRISPR-Cas9 system. *Sci Rep*. 2015;5:10342.
189. Glass Z, Lee M, Li Y, Xu Q. Engineering the Delivery System for CRISPR-based Genome Editing. *Trends Biotechnol*. 2018;36(2):173–85.
190. Bamford RA, Zhao ZY, Hotchin NA, Styles IB, Nash GB, Tucker JHR, *et al*. Electroporation and Microinjection Successfully Deliver Single-Stranded and Duplex DNA into Live Cells as Detected by FRET Measurements. *PLoS One*. 2014;9(4):e95097.
191. Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, *et al*. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat Biotechnol*. 2014;32(12):1262–7.
192. Chuang CK, Chen CH, Huang CL, Su YH, Peng SH, Lin TY, *et al*. Generation of GGTA1 Mutant Pigs by Direct Pronuclear Microinjection of CRISPR/Cas9 Plasmid Vectors. *Anim Biotechnol*. 2017;28(3):174–81.
193. Wang H, la Russa M, Qi LS. CRISPR/Cas9 in Genome Editing and Beyond. *Annu Rev Biochem*. 2016;85:227–64.
194. Bayly GR. Lipids and disorders of lipoprotein metabolism. *Clinical Biochemistry: Metabolic and Clinical Aspects*. 2014;702–36.
195. Wilton DC. Phospholipases. *Biochemistry of Lipids, Lipoproteins and Membranes*. 2008;305–29.
196. Leung SY, Chen X, Chu KM, Yuen ST, Mathy J, Ji J, *et al*. Phospholipase A2 group IIA expression in gastric adenocarcinoma is associated with prolonged survival and less frequent metastasis. *Proc Natl Acad Sci U S A*. 2002;99(25):16203–8.

197. Ozturk K, Onal MS, Efiloglu O, Nikerel E, Yildirim A, Telci D. Association of 5'UTR polymorphism of secretory phospholipase A2 group IIA (PLA2G2A) gene with prostate cancer metastasis. *Gene*. 2020;742:144589.
198. Exeter HJ, Folkersen L, Palmen J, Franco-Cereceda A, Cooper JA, Kalea AZ, *et al*. Functional analysis of two PLA2G2A variants associated with secretory phospholipase A2-IIA levels. *PLoS One*. 2012;7(7):e41139.
199. Liu M, Rehman S, Tang X, Gu K, Fan Q, Chen D, *et al*. Methodologies for improving HDR efficiency. *Front Genet*. 2019;9:691.
200. Menschikowski M, Hagelgans A, Kostka H, Eisenhofer G, Siegert G. Involvement of Epigenetic Mechanisms in the Regulation of Secreted Phospholipase A2 Expressions in Jurkat Leukemia Cells. *Neoplasia*. 2008;10(11):1195–203.
201. Thelen P, Wuttke W, Jarry H, Grzmil M, Ringert RH. Inhibition of telomerase activity and secretion of prostate specific antigen by silibinin in prostate cancer cells. *J Urol*. 2004;171(5):1934–8.
202. Kim W, Son B, Lee S, Do H, Youn B. Targeting the enzymes involved in arachidonic acid metabolism to improve radiotherapy. *Cancer Metastasis Rev*. 2018;37(2-3):213-225.
203. Peng Z, Chang Y, Fan J, Ji W, Su C. Phospholipase A2 superfamily in cancer. *Cancer Lett*. 2021;497:165–77.
204. Yamashita SI, Yamashita JI, Ogawa M. Overexpression of group II phospholipase A2 in human breast cancer tissues is closely associated with their malignant potency. *Br J Cancer*. 1994;69(6):1166–70.
205. Bennett DT, Deng XS, Yu JA, Bell MT, Mauchley DC, Meng X, *et al*. Cancer stem cell phenotype is supported by secretory phospholipase A2 in human lung cancer cells. *Ann Thorac Surg*. 2014;98(2):439–46.
206. Jiang J, Neubauer BL, Graff JR, Chedid M, Thomas JE, Roehm NW, *et al*. Expression of group IIA secretory phospholipase A2 is elevated in prostatic intraepithelial neoplasia and adenocarcinoma. *Am J Pathol*. 2002;160(2):667–71.

207. Ren J, Xiao YJ, Singh LS, Zhao X, Zhao Z, Feng L, *et al.* Lysophosphatidic acid is constitutively produced by human peritoneal mesothelial cells and enhances adhesion, migration, and invasion of ovarian cancer cells. *Cancer Res.* 2006;66(6):3006–14.
208. Mauchley D, Meng X, Johnson T, Fullerton DA, Weyant MJ. Modulation of growth in human esophageal adenocarcinoma cells by group IIa secretory phospholipase A2. *J Thorac Cardiovasc Surg.* 2010;139(3):591–9.
209. Ashrafizadeh M, Paskeh MDA, Mirzaei S, Gholami MH, Zarrabi A, Hashemi F, *et al.* Targeting autophagy in prostate cancer: preclinical and clinical evidence for therapeutic response. *J Exp Clin Cancer Res.* 2022;41(1):105.
210. Lorenc T, Klimczyk K, Michalczywska I, Słomka M, Kubiak-Tomaszewska G, Olejarsz W. Exosomes in Prostate Cancer Diagnosis, Prognosis and Therapy. *Int J Mol Sci.* 2020;21(6):2118.
211. Kretschmer A, Tilki D. Biomarkers in prostate cancer – Current clinical utility and future perspectives. *Crit Rev Oncol Hematol.* 2017;120:180–93.
212. Ding Y, Lee MJ, Gao Y, Bu P, Coarfa C, Miles B, *et al.* Neuropeptide Y nerve paracrine regulation of prostate cancer oncogenesis and therapy resistance. *Prostate.* 2021;81(1):58–71.
213. Sikka S, Chen L, Sethi G, Kumar AP. Targeting PPAR γ signaling cascade for the prevention and treatment of prostate cancer. *PPAR Res.* 2012; 2012:968040.
214. Lee JH, Kim C, Baek SH, Ko JH, Lee SG, Yang WM, *et al.* Capsazepine inhibits JAK/STAT3 signaling, tumor growth, and cell survival in prostate cancer. *Oncotarget.* 2017;8(11):17700–11.
215. Xu J, Resurreccion WK, Shi Z, Wei J, Wang CH, Zheng SL, *et al.* Inherited risk assessment and its clinical utility for predicting prostate cancer from diagnostic prostate biopsies. *Prostate Cancer Prostatic Dis.* 2022.
216. Xu J, Zheng SL, Isaacs SD, Wiley KE, Wiklund F, Sun J, *et al.* Inherited genetic variant predisposes to aggressive but not indolent prostate cancer. *Proc Natl Acad Sci U S A.* 2010;107(5):2136–40.

217. Yang H, Ren S, Yu S, Pan H, Li T, Ge S, *et al.* Methods Favoring Homology-Directed Repair Choice in Response to CRISPR/Cas9 Induced-Double Strand Breaks. *Int J Mol Sci.* 2020;21(18):6461.
218. Vasquez RJ, Howell B, Yvon AMC, Wadsworth P, Cassimeris L. Nanomolar concentrations of nocodazole alter microtubule dynamic instability in vivo and in vitro. *Mol Biol Cell.* 1997;8(6):973–85.
219. Yang D, Scavuzzo MA, Chmielowiec J, Sharp R, Bajic A, Borowiak M. Enrichment of G2/M cell cycle phase in human pluripotent stem cells enhances HDR-mediated gene repair with customizable endonucleases. *Sci Rep.* 2016;6:21264.
220. Song F, Stieger K. Optimizing the DNA Donor Template for Homology-Directed Repair of Double-Strand Breaks. *Mol Ther Nucleic Acids.* 2017;7:53–60.
221. Shen CC, Hsu MN, Chang CW, Lin MW, Hwu JR, Tu Y, *et al.* Synthetic switch to minimize CRISPR off-target effects by self-restricting Cas9 transcription and translation. 2019;47(3):e13.
222. Vecchi L, Araújo TG, Azevedo FVP de V, Mota STS, Ávila V de MR, Ribeiro MA, *et al.* Phospholipase A2 Drives Tumorigenesis and Cancer Aggressiveness through Its Interaction with Annexin A1. *Cells.* 2021;10(6):1472.
223. Aksoylar HI, Patsoukis N. Treatment with Exogenously Added Catalase Alters CD8 T Cell Memory Differentiation and Function. *Adv Biol.* 2022;2101320.
224. Casey SC, Vaccari M, Al-Mulla F, Al-Temaimi R, Amedei A, Barcellos-Hoff MH, *et al.* The effect of environmental chemicals on the tumor microenvironment. *Carcinogenesis.* 2015;36 Suppl 1(Suppl 1):S160–83.
225. Avagliano A, Granato G, Ruocco MR, Romano V, Belviso I, Carfora A, *et al.* Metabolic Reprogramming of Cancer Associated Fibroblasts: The Slavery of Stromal Fibroblasts. *Biomed Res Int.* 2018;2018:6075403.
226. Gocher AM, Workman CJ, Vignali DAA. Interferon- γ : teammate or opponent in the tumour microenvironment? *Nat Rev Immunol.* 2021;22(3):158–72.

227. Xing XF, Li H, Zhong XY, Zhang LH, Wang XH, Liu YQ, *et al.* Phospholipase A2 group IIA expression correlates with prolonged survival in gastric cancer. *Histopathology*. 2011;59(2):198–206.
228. Park WH. Hydrogen peroxide inhibits the growth of lung cancer cells via the induction of cell death and G1-phase arrest. *Oncol Rep*. 2018;40(3):1787–94.
229. Champelovier P, Atifi ME, Mantel F, Michallat S, Simon A, Rostaing B, *et al.* Effects of Tumor Necrosis Factor- α (TNF α) and Interferon- γ (IFN γ) on Gene Expression Profiles in Bladder Carcinoma Cells Using Oligonucleotide Microarray Analysis. *Cancer Genomics Proteomics*. 2004;1(5–6):455–64.
230. De Oliveira-Marques V, Cyrne L, Marinho HS, Antunes F. A quantitative study of NF-kappaB activation by H₂O₂: relevance in inflammation and synergy with TNF-alpha. *J Immunol*. 2007;178(6):3893–902.
231. Peilot H, Rosengren B, Bondjers G, Hurt-Camejo E. Interferon- γ Induces Secretory Group IIA Phospholipase A2 in Human Arterial Smooth Muscle Cells: Involvement of Cell Differentiation, STAT-3 Activation, and Modulation by Other Cytokines. *J Biol Chem*. 2000;275(30):22895–904.
232. Fijneman RJA, Peham JR, van de Wiel MA, Meijer GA, Matise I, Velcich A, *et al.* Expression of Pla2g2a prevents carcinogenesis in Muc2-deficient mice. *Cancer Sci*. 2008;99(11):2113–9.
233. Ganesan K, Ivanova T, Wu Y, Rajasegaran V, Wu J, Ming HL, *et al.* Inhibition of gastric cancer invasion and metastasis by PLA2G2A, a novel beta-catenin/TCF target gene. *Cancer Res*. 2008;68(11):4277–86.
234. Zhao S, Du XY, Chen JS, Zhou YC, Song JG. Secretory Phospholipase A2 Inhibits Epidermal Growth Factor-Induced Receptor Activation. *Exp Cell Res*. 2002;279(2):354–64.
235. Schewe M, Franken PF, Sacchetti A, Schmitt M, Joosten R, Böttcher R, *et al.* Secreted Phospholipases A2 Are Intestinal Stem Cell Niche Factors with Distinct Roles in Homeostasis, Inflammation, and Cancer. *Cell Stem Cell*. 2016;19(1):38–51.

236. Kisslov L, Hadad N, Rosengraten M, Levy R. HT-29 human colon cancer cell proliferation is regulated by cytosolic phospholipase A(2) α dependent PGE(2) via both PKA and PKB pathways. *Biochim Biophys Acta*. 2012;1821(9):1224–34.
237. Overway EM, Bosma KJ, Claxton DP, Oeser JK, Singh K, Breidenbach LB, *et al*. Nonsynonymous single-nucleotide polymorphisms in the G6PC2 gene affect protein expression, enzyme activity, and fasting blood glucose. *J Biol Chem*. 2022;298(2):101534.
238. Mehta D, Roy S, Joshi P, Parab M, Waghmare SK. Secretory phospholipase sPLA2-IIA loss impairs tumorigenic and metastatic potential in breast cancer cells. *Biochem Biophys Res Commun*. 2022;597:102–8.