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INTRINSIC PROGRAMMED DEATH LIGAND 1 (PD-L1) ROLE IN PROMOTING TRIPLE NEGATIVE BREAST CANCER (TNBC) PROGRESSION

Duaa Salem Alkaabi
United Arab Emirates University

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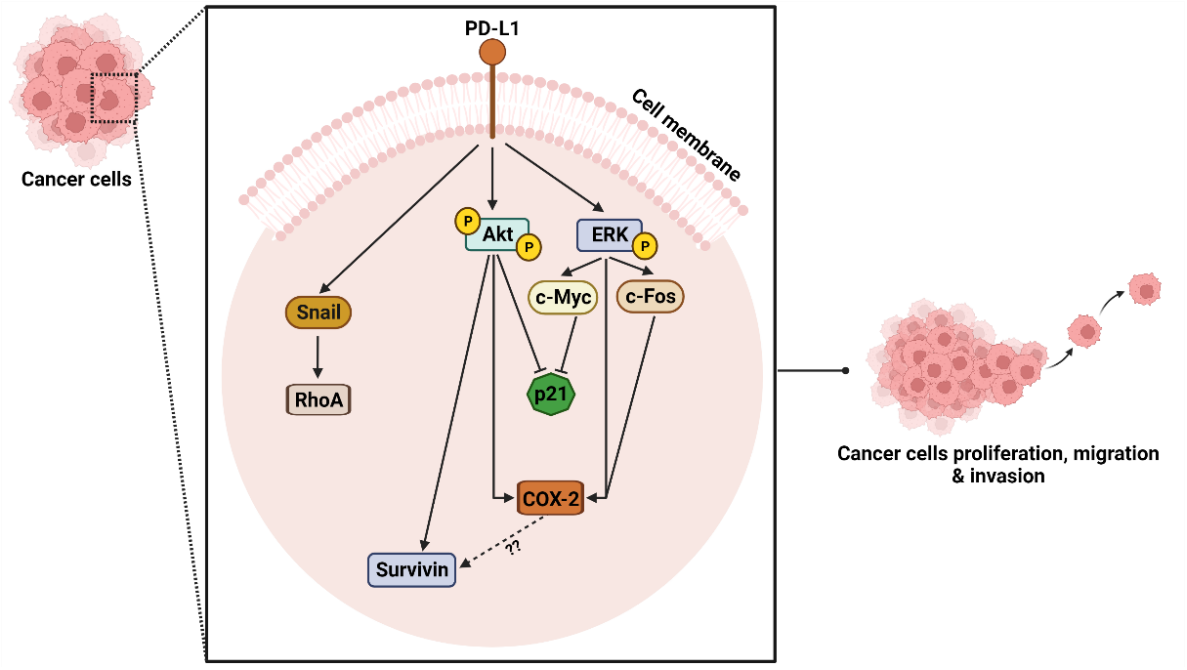

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Duaa Salem Saleh Suliman Alkaabi

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Pharmacology and Toxicology)

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Cover: The potential downstream signaling of PD-L1 in TNBC.
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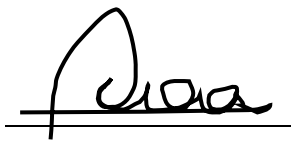
Declaration of Original Work

I, Duaa Salem Alkaabi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Intrinsic Programmed Death Ligand 1 (Pd-L1) Role in Promoting Triple Negative Breast Cancer (Tnbc) Progression*", hereby, solemnly declare that this is the original research work done by me under the supervision of Prof. Samir Attoub, in the College of Medicine and Health Sciences at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature: 

Date: 29/3/2023

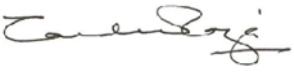
Approval of the Master Thesis

This Master Thesis is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Prof. Samir Attoub
   Title: Professor
   Department of Pharmacology and Therapeutics
   College of Medicine and Health Sciences

   Signature  Date 29/3/2023

2) Member: Prof. Haider Raza
   Title: Professor
   Department of Biochemistry and Molecular Biology
   College of Medicine and Health Sciences

   Signature  Date 29/3/2023

3) Member (External Examiner): Prof. Rafat El-Awady
   Title: Professor & Dean of the College of Graduate Studies and Scientific Research
   Department of Pharmacy Practice and Pharmacotherapeutics
   Institution: University of Sharjah, UAE

   Signature  Date 29/3/2023

This Master Thesis is accepted by:

Acting Dean of the College of Medicine and Health Sciences: Professor Juma AlKaabi

Signature 

Date 11/05/2023

Dean of the College of Graduate Studies: Professor Ali Al-Marzouqi

Signature 

Date 11/05/2023

Abstract

This thesis is concerned with Triple Negative Breast Cancer (TNBC), which is characterized by lack of estrogen, progesterone, and HER2 receptors expression, making targeted drugs ineffective. TNBC has a high proliferative rate, which results in a poor prognosis. Chemotherapy, the primary treatment for metastatic breast cancer, is not an ideal choice due to its toxicity towards normal cells. Therefore, Cancer-targeted therapy has been developed to improve the specificity and strength of the immune system against cancer cells. PD-L1 is an immunosuppressive protein that inactivates T cells by binding to the inhibitory receptor PD-1. The clinical use of PD-L1 blockade agents has progressed beyond basic mechanistic studies. However, the lack of knowledge on how PD-L1 regulates cancer hallmarks can lead to missed therapeutic opportunities. The main objective of this research is to identify the phenotypic changes in TNBC cells after PD-L1 gene knockout and pinpoint the PD-L1 related intrinsic cellular signaling pathways responsible for promoting cancer growth and migration. The study investigates PD-L1 role in the progression of TNBC MDA-MB-231 cancer by using three designs of CRISPR-Cas9 lentiviral particles to knock out the PD-L1. Our results revealed that PD-L1 knockout significantly inhibited MDA-MB-231 cell proliferation and colony formation in vitro and tumor growth in the chick embryo chorioallantoic membrane (CAM) model in vivo. PD-L1 knockout also decreased the migration and invasion of MDA-MB-231 cells in vitro. We demonstrate a potential mechanism by which PD-L1 promotes MDA-MB-231 cells' malignancy through the regulation of PI3K/AKT and MAPK/ERK pathways. We have also reported that PD-L1 regulate the expression of p21, RhoA, c-Fos, c-Myc, Survivin, COX-2, in TNBC cells. Our findings provide insights into new potential cancer therapeutic strategies and suggest the development of anti-PD-L1 combination therapies for effective TNBC treatment.

Keywords: PD-L1, TNBC, proliferation, migration, invasion, CAM, AKT, ERK.

Title and Abstract (in Arabic)

دور المُبرمج للوفاة الرابط 1 (PD-L1) في تعزيز تقدم سرطان الثدي السلبي الثلاثي(TNBC)

الملخص

تتناول هذه الأطروحة سرطان الثدي الثلاثي السلبي (TNBC) الذي يتميز بنقص في مستقبلات هرمون الاستروجين والبروجسترون و HER2 مما يجعل الأدوية المستهدفة غير فعالة .يرتبط TNBC بمعدل تكاثر مرتفع يؤدي إلى سرعة تدهور الحالة الطبية .على الرغم من أن العلاج الكيميائي هو النهج الرئيسي لعلاج سرطان الثدي النقيلي، إلا أنه يظل خيارًا غير فعال بسبب سميّته تجاه الخلايا الطبيعية. لذلك، تم تطوير العلاج الموجه للخلايا السرطانية بهدف تصميم علاجات فعالة لتحسين انتقائية وقوة جهاز المناعة ضد الخلايا السرطانية. PD-L1 هو بروتين مثبط للمناعة يعمل على تثبيط الخلايا التائية عن طريق الارتباط بالمستقبل المثبطPD-1 .الاستخدام السريري للأدوية الموجهة لقمع مستقبلات PD-L1 أحرزت تقدمًا استباقي عن التقدم في الدراسات الأساسية الميكانيكية لهذه المستقبلات. يمكن أن يؤدي نقص المعرفة حول كيفية تأثير PD-L1 للسمات المميزة للسرطان إلى ضياع العديد من الفرص العلاجية .الهدف الرئيسي من هذه الأطروحة هو تحديد التغيرات المظهرية في خلايا TNBC بعد حذف جين PD-L1وتحديد مسارات الإشارات الخلوية الداخلية المرتبطة بـ PD-L1 المسؤولة عن تعزيز تكاثر الخلايا السرطانية والقدرة على الهجرة. بحثت الدراسة في دورPD-L1 في تطور سرطان TNBC MDA-MB-231 عن طريق استخدام تقنية التعديل الوراثي CRISPR-Cas9 بثلاثة تصاميم مختلفة من جزيئات lentiviral موجهة لاقتطاع جين PD-L1 . كشفت نتائجنا أن غياب البروتين PD-L1 منع بشكل كبير تكاثر خلايا MDA-MB-231 وقابلية تكوين مستعمرات خلوية في البيئة المختبرية ومنع نمو الورم السرطاني في نموذج الغشاء المشيمي لجنين الدجاج (CAM) في بيئة الجسم الحي. نوضح هنا آلية محتملة يقوم من خلالها PD-L1 بتعزيز خباثة الخلايا-MDA MB-231من خلال تنظيم مسارات PI3K / AKT و .MAPK / ERK . لقد ذكرنا أيضا أن PD-L1 يمكنه تنظيم البروتينات المسرطنة لـ p21 و Rho-A و c-Fos و c-Myc و Survivin و COX-2 في خلايا TNBC. نطمح أن تساعد هذه النتائج في توفير استراتيجيات علاجية جديدة للسرطان في المستقبل تطوير علاجات تركيبة مضادة لـ PD-L1 لعلاج TNBC بشكل فعال.

مفاهيم البحث الرئيسية: PD-L1, TNBC, الانتشار, الهجرة, غزو, CAM, AKT, ERK.

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Finally, my biggest thanks and gratitude to my parents, brothers and husband for the endless support, prayers and encouragement in the past few years.

Dedication

To all scholars "Never give up to gain knowledge and keep on study"

To my beloved parents and family

Table of Contents

List of Figures

List of Abbreviations

AC	Adriamycin and Cyclophosphamide
ADCs	Antibody Drug Conjugates
Akt	Serine/Threonine Kinase
AR	Androgen Receptor
BL1	Basal-Like 1
BL2	Basal-Like 2
CAF	Cyclophosphamide, Adriamycin, and Fluorouracil
CAM	Chorioallantoic Membrane
CCRCC	Clear Cell Renal Cell Carcinoma
CEF-T	Cyclophosphamide, Epirubicin, Fluorouracil and Taxel
CMF	Cyclophosphamide, Methotrexate, and Fluorouracil
cPDL1	Cytoplasmic PD-L1
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSC-like	Cancer Stem Cells-Like
DFS	Disease-Free Survival
MMR	DNA Mismatch Repair
DSBs	DNA Double-Strand Breaks
EMT	Epithelial Mesenchymal Transition
EMT-TFs	Epithelial Mesenchymal Transition Transcription Factors
ER	Estrogen Receptor
ERK	Extracellular Signal-Regulated Kinase
FBS	Fetal Bovine Serum
FDA	U.S. Food and Drug Administration
gBRCAm	Germline BRCA mutation
HCC	Hepatocellular Carcinoma

HER2	Human Epidermal Growth Factor Receptor 2
HL	Hodgkin's Lymphoma
HR	Hormone Receptor
HRD	Homologous Recombination Deficiency
IAPs	Inhibitors of Apoptosis
ICIs	Immune Checkpoint Inhibitors
KO	Knockout
LAR	Luminal Androgen Receptor
mAbs	Monoclonal Antibodies
MAPK	Mitogen-Activated Protein Kinase
mPD-L1	Membrane PD-L1
MSI-H	Microsatellite Instability-High
NCCN	National Comprehensive Cancer Network
nPD-L1	Nuclear PD-L1
NSCLC	Non-Small Cell Lung Cancer
OS	Overall Survival
PARP	Poly (ADP-ribose) Polymerase
PARPi	PARP Inhibitors
pCR	Pathological Complete Response
PD-1	Programmed Death 1
PD-L1	Programmed Death Ligand 1
PI3K	Phosphatidylinositol 3-Kinase
PKB	Protein Kinase B
PR	Progesterone Receptor
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SG	Sacituzumab Govitecan

sgRNA	Single Guided RNA
shRNA	Short Hairpin RNA
siRNA	Short Interfering RNA
sPD-L1	Serum PD-L1
SSBs	DNA Single Strand Breaks
TAC	Taxel, Adriamycin, and Cyclophosphamide
TC	Docetaxel and Cyclophosphamide
TILs	Tumor-Infiltrating Lymphocytes
TMB	Tumor Mutational Burden
TNBC	Triple Negative Breast Cancer
Trop-2	Trophoblast Cell-Surface Antigen 2
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

Chapter 1: Introduction

1.1 Overview

Cancer is a major health issue and is one of the leading causes of morbidity and mortality worldwide. Triple negative breast cancer (TNBC) is the most challenging type of breast cancers to treat. TNBC does not express the most common therapy targets for breast cancer. Therefore, TNBC treatment is considered a hurdle due to its resistance to standard breast cancer treatments (Yin et al., 2020). Despite the emergence of new biological and targeted agents, chemotherapeutic agents are still the primary treatment option for patients with TNBC (Bou Zerdan et al., 2022; Yin et al., 2020). However, neoadjuvant chemotherapy's main limitation is the remaining micro-metastatic tumors that increase the risk of tumor recurrence and drug resistance which is associated with restricted postoperative adjuvant chemotherapy options (Bou Zerdan et al., 2022). Therefore, developing new therapeutic options to overcome the disadvantages is highly required.

Cancer immunotherapy aims to create effective treatments that enhance the specificity and potency of the immune system in fighting cancer. Over the past few years, an increasing number of studies reported that PD-1/PD-L1 immune checkpoint inhibitors are showing promising results and significant clinical response for TNBC patients (Lyons, 2019). The use of monoclonal antibodies, such as Atezolizumab, to target programmed cell death-ligand 1 (PD-L1) has transformed the treatment of different cancer types (Wu et al., 2019). PD-L1 serves two major pro-oncogenic functions. Firstly, it acts as an immunosuppressive protein by binding to the inhibitory receptor PD-1 and deactivating T cells response. Secondly, it conveys intrinsic intracellular signals that mediate cancer cell survival, regulate stress responses, and resist pro-apoptotic stimuli (Escors et al., 2018).

It is far from reality to presume that an adequate amount of knowledge is known about PD-L1 mechanisms of action and how T cells and cancer cells responses are regulated. The PD-L1 expression in cancer cells is modulated by several extrinsic and intrinsic factors, leading to the various significances of PD-L1 positivity (Han et al., 2020;

Ritprajak & Azuma, 2015). However, the clinical use of PD-L1 blockade agents is advancing far past basic mechanistic studies. The lack of knowledge on how PD-L1 regulates cancer hallmarks can lead to several missed opportunities for identifying potential therapeutic targets that will open the possibilities for targeted combinatorial interventions (Dong et al., 2018). Agents regulating PD-L1 expression can be possible adjuvant therapies for the current immune checkpoint inhibitors (J. Chen et al., 2016). As well understanding PD-L1 regulation mechanisms will support appropriate patient selection and improve the treatment effect (Yi et al., 2021).

This research work will look for the phenotypic changes in TNBC cells after gene knockout to identify PD-L1 intrinsic role associated with promoting TNBC progression. The study will shed light on the PD-L1 related intrinsic intracellular signaling pathway responsible for promoting cancer hallmarks.

1.2 Relevant Literature

1.2.1 Breast Cancer Statistics

Breast cancer is considered to be the most frequently diagnosed malignant tumor in females across the world. In 2020, new female breast cancer cases were approximately 2.26 million, representing 11.7% of total cancer cases worldwide (Sung et al., 2021). Most patients with advanced breast cancer have severe systemic metastasis, that is associated with a high recurrence rate (Barzaman et al., 2020). Indeed, breast cancer is the first major cause of cancer-related death in females. It accounts for 1 in 6 cancer deaths among women, with an estimated 684,996 deaths worldwide in 2020 (Sung et al., 2021). However, remarkable progress has been made in breast cancer treatments over the past recent years. Despite these advances, breast cancer mortality rate is still high, which is mainly due to metastatic spread (Liang et al., 2020). The 5 years overall survival rate of breast cancer patients without metastasis is greater than 86%. However, distant metastasis can lead to a dramatic reduction of this rate to only 29% (The American Cancer Society medical and editorial content team, 2022).

1.2.2 Breast Cancer Subtypes and Classification

Breast cancer is considered a heterogeneous disease, thus viewed as a family of diseases occurring in a single organ. It presents with variable histopathological, biological characteristics, and different clinical outcomes (Bou Zerdan et al., 2022). Classification and subgrouping are made according to histological grade, types, tumor stage, and biological markers, which can provide prognosis information and help make therapeutic decisions and predict outcomes (Joseph et al., 2018). The estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) are the current markers that help in making therapeutic decisions and predicting responses to hormone and receptor targeted therapies (Bou Zerdan et al., 2022; Joseph et al., 2018; Yin et al., 2020). Classifying according to molecular subtypes is based on global gene expression; these include distinct molecular classes luminal A, luminal B, basal-like, HER2-enriched, and normal-like breast cancer. Luminal breast cancer constitutes 60% of all breast tumors and is either ER-positive, PR-positive, or both; generally, it presents with good prognostic characteristics (Joseph et al., 2018). The HER2-positive class makes up to 12% of all breast tumors, and it is associated with more aggressive disease and a worse prognosis. The basal subtype is characterized by a lack of expression of all ER, PR, and HER2 receptors called triple-negative breast cancer (TNBC) (Yin et al., 2020).

1.2.3 Triple-Negative Breast Cancer (TNBC): an Overview

TNBC represents an average of 15–20% of all breast cancers worldwide and generally behave more aggressively than other subtypes of breast cancers (Yin et al., 2020). Patients with TNBC have a higher likelihood to experience an earlier onset of metastasis and rapidly progressing disease with shorter response durations to current therapies (Lyons, 2019). Patients diagnosed with TNBC experienced worse breast cancer-specific survival, worse overall survival, and a dramatic increase in death within two years of diagnosis compared with patients with hormone receptor (HR)-positive breast cancer (X. Li et al., 2017). One of the major challenges is the limited targeted therapies available to treat TNBC, unlike ER, PR patients, or HER2-directed therapy for HER2 patients (Kwapisz, 2021). In addition, management of TNBC is challenging due

to its molecular heterogeneity. A single-cell genomic analyses by RNA-sequencing indicate that the four subtypes of TNBC which are, basal- like 1 (BL1), basal-like 2 (BL2), luminal androgen receptor (LAR) and mesenchymal (M) can co-occur within given TNBC tumors (Karaayvaz et al., 2018). Thus, TNBC intra-tumoral heterogenicity highlight the need for personalized patient management (Lee & Djamgoz, 2018). Several literature evidence imply that past and current research has enormously improved breast cancer's clinical outcome. This has been attributed to the progress made in the realm of screening, diagnosis, and therapeutic strategies engaged in breast cancer management. Nevertheless, TNBC's dismal prognosis and resistance to drugs remain significant obstacles that pose current challenges in controlling the disease (Fahad Ullah, 2019).

1.2.4 Triple-Negative Breast Cancer therapeutic approaches

Along with surgery and radiation, chemotherapy has been the backbone therapy for advanced TNBC for many years up to date; since TNBC prognosis is often worse which necessitates more aggressive treatment (Kwapisz, 2021). The effectiveness of chemotherapy as a therapeutic option for neoadjuvant, adjuvant, and metastatic triple-negative breast cancer (TNBC) has been well established (Bou Zerdan et al., 2022). Initially TNBC chemosensitivity is more than hormone receptor-positive breast cancers but, the risk of recurrence in patients not achieving pathological complete response (pCR) is high (Carey et al., 2007). Currently the primary strategy available for metastatic or recurrent TNBC in clinical practice is to rechallenge with systemic chemotherapy. The National Comprehensive Cancer Network (NCCN) recommends several systemic chemotherapy treatment regimens for triple-negative breast cancer (TNBC), including Docetaxel and Cyclophosphamide (TC), Taxel, Adriamycin, and Cyclophosphamide (TAC), Adriamycin and Cyclophosphamide (AC), Cyclophosphamide, Methotrexate, and Fluorouracil (CMF), Cyclophosphamide, Adriamycin, and Fluorouracil (CAF), and Cyclophosphamide, Epirubicin, Fluorouracil, and Paclitaxel/Docetaxel (CEF-T) (Bou Zerdan et al., 2022). For preoperative and adjuvant therapy dose-dense Doxorubicin and Cyclophosphamide followed by Paclitaxel or Docetaxel and Cyclophosphamide are recommended regiments (Kaplan, 2021). For neoadjuvant chemotherapy regimen Adriamycin, cyclophosphamide, and paclitaxel combinations are considered to be standard against TNBC and result in pCR rates of 35–45% (Biswas et al., 2017). TNBC

patients with residual disease after preoperative therapy with taxane-, alkylator-, and anthracycline-based chemotherapy can receive capecitabin (Kaplan, 2021). Unfortunately, systemic chemotherapy remains an ineffective approach due to toxicity toward normal cells, poor response, and eventual multidrug resistance (Liang et al., 2020; Lee & Djamgoz, 2018). Advances in early diagnosis and comprehensive treatment strategies lead to improvement in breast cancer patient's prognosis. Moreover, the development of new agents targeting breast cancer is essential to reduce the mortality caused by this malignant disease (Liang et al., 2020). However, we are now entering a new era in TNBC management, with a number of novel therapeutic agents driving the field forward (Kwapisz, 2021). Metastatic TNBC is characterized by angiogenesis and an immunogenic microenvironment. Therefore, targeting both the cancer cell and the microenvironment represents a great treatment opportunity (Capici et al., 2022).

Although there is no standard care approach for TNBC guided by tumor biology, advancements in molecular classification and genome sequencing have led to the identification of several potential molecular targets in TNBC (Kwapisz, 2021; Lyons, 2019). The identification of new biomarkers for TNBC such as PD-L1, androgen receptor (AR), PI3K/AKT/mTOR and HER2-low expression led the way for development of new potential therapeutic approaches (Capici et al., 2022). Recent advances in TNBC treatment approaches include immunotherapy, Poly (ADP-ribose) polymerase-1 (PARP) inhibition, molecular targeted therapies, and antibody-drug conjugates (ADCs) (Capici et al., 2022; Lyons, 2019). In the clinical practice, immune checkpoint inhibitors (ICIs), such as Atezolizumab in combination with nab-paclitaxel or Pembrolizumab in combination with chemotherapy, are recommended for patients with PD-L1-positive metastatic TNBC (Chehade et al., 2021; Kaplan, 2021). Checkpoint inhibition with Atezolizumab is the first approved immunotherapy agent in programmed death-ligand 1 (PD-L1+) TNBC in clinical practice along with the Poly (ADP-ribose) polymerase (PARP) inhibitors Olaparib and Talazoparib for germline BRCA mutation associated metastatic TNBCs (gBRCAm-BC) (Lyons, 2019). In addition, antibody drug conjugate (ADC), sacituzumab govitecan, have recently been approved by the FDA for patients with metastatic TNBC who received at least two prior therapies (Chehade et al., 2021). As we have seen, three new targeted therapies that hold great promise for TNBC

in clinical practice. While immunotherapy and PARP inhibitors are already established treatments for triple-negative breast cancer (TNBC), drugs like Sacituzumab Govitecan and other antibody-drug conjugates (ADCs) have only recently emerged as potential treatments for TNBC (Capici et al., 2022). The field of targeted therapy in TNBC, driven by biomarkers, is undergoing rapid change. Currently, multiple ongoing clinical trials aims to personalize the standard treatment for this heterogeneous disease (Chehade et al., 2021).

Immunotherapy approach stimulate the host's immune system to overcome tumor immunosuppression (Capici et al., 2022). Many studies are evaluating checkpoint inhibitor's role in breast cancer, with the most encouraging results to date being in TNBC (Liu et al., 2018; Lyons, 2019). TNBC has an immunogenic microenvironment that contains tumor-infiltrating lymphocytes (TILs) including CD20+ B cells, CD4 and CD8+ T cells. A meta-analysis by Gao, et al., reported that high level of TILs in TNBC patients have better pCR, Disease-Free-Survival (DFS) and overall survival (OS) rates (Gao et al., 2020). TNBC is known to have a higher mutational burden than other breast cancers subtypes (Liu et al., 2018; Lyons, 2019). Biomarkers such as PD-1/PD-L1, tumor mutational burden (TMB), defects in DNA mismatch repair proteins (dMMR) and a subsequent microsatellite instability-high (MSI-H) can predict responses to immunotherapy in metastatic TNBC (Capici et al., 2022). Fortunately, the high mutational burden tumors exhibit superior responses and outcomes to checkpoint inhibition immunotherapy (Lyons, 2019).

TNBC is commonly linked with pathogenic gene mutations of the tumor suppressor genes BRCA1 and BRCA2, that are involved in the preservation of genome integrity. It is estimated that 80% of breast cancer patients with BRCA1 mutations are of TNBC type (Isakoff et al., 2015). BRCA1/2 are vital for the repair machinery of double stranded DNA breaks (DSBs). Mutations produce homologous recombination deficiency (HRD) which make these tumors more sensitive to DNA crosslink agents or PARP inhibitors (Geenen et al., 2018). The PARP polymerase enzyme is a nuclear protein that control genetic stability by repairing the single stranded DNA-Breaks (SSBs) using DNA base excision pathway (Y. Li et al., 2022). When PARP is inhibited in BRCA1/2

deficient tumor cells, the SSBs will accumulate ultimately leading to DSBs. Inhibition of PARP induce severe and highly selective toxicity to BRCA1/2 mutated tumor cells; a process called synthetic lethality (Y. Li et al., 2022). This is the rationale behind the use of PARP inhibitors in the treatment of TNBC patients with BRCA1/2 mutant. The current PARPi olaparib, talazoparib, and veliparib have been FDA approved for the treatment of locally advanced or metastatic breast cancer in 2018 (Y. Li et al., 2022). Olaparib and Talazoparib are now approved by the FDA and the EMA, for triple-negative metastatic breast cancer patients with BRCA 1 or 2 mutations that have been previously treated with chemotherapy before or after surgery (Capici et al., 2022). Talazoparib, which is derived from a by-product, is known as the most potent PARPi. (Y. Li et al., 2022)

A subset of TNBC tumors classified as luminal androgen receptor (LAR) present active hormone-mediated signaling via androgen receptors (Pauls et al., 2022). Meta-analyses noted that approximately 28% of TNBC patients expressed AR (Xu et al., 2020). It is reported that AR plays a role as an oncogene in TNBC, mediating LAR tumor cell growth, contrary to its anti-estrogenic and growth-inhibitory influence in ER+ breast cancer (Hickey et al., 2012). In addition, LAR cells subset response to traditional chemotherapy is often limited compared to other TNBC subtypes. Rationally AR receptor can be a potential therapeutic target in androgen expressing TNBC (Pauls et al., 2022). Enzalutamide, an AR antagonist investigated in advanced AR+ TNBC patients, showed a median OS at 17.6 m suggesting that enzalutamide is effective in the treatment of advanced AR+ TNBC (Traina et al., 2018).

The phosphatidylinositol 3-kinase (PI3K) pathway is a key regulator of survival, growth, angiogenesis, migration, and invasion. The three key intracellular signal regulatory proteins are PI3K, AKT, and mTOR. In cancer cells PI3K/AKT pathway overactive due to gain of function mutations of *PIK3CA,* loss of function of the tumor suppressor *PTEN*, signaling deregulation, amplification and mutations of receptor tyrosine kinase (Fruman & Rommel, 2014). These alterations occur in approximately 35% of TNBC (Cocco et al., 2020). The occurrence of PIK3CA mutation is more frequent in residual TNBC and AR-positive TNBC, whereas amplification of AKT3 and deletion of

PTEN are more commonly observed in the basal subtype (Y. Li et al., 2022). The deregulation of PI3K/AKT/mTOR pathway offer an emerging ‘multi-target’ for drugs at early stages of clinical development (Lee & Djamgoz, 2018). Several trials are evaluating drugs targeting the PI3K/AKT/mTOR pathway in TNBC. Currently, Inhibitors of this pathway evaluated for their antitumor response in clinical trials are Buparlisib (PI3K inhibitor), Ipatasertib, Capivasertib (AKT inhibitors), Everolimus (mTOR inhibitor) (Y. Li et al., 2022).

Vascular endothelial growth factor (VEGF) is highly expressed in 30–60% of TNBC. Both VEGF and the tyrosine kinase receptor VEGFR play principal role in breast cancer invasiveness. VEGF promotes endothelial cell angiogenesis by promoting proliferation and migration and support of newly formed blood vessels (Capici et al., 2022). Overall, VEGF-targeted therapies clinical use in TNBC require the presence of biomarkers such as serum VEGFR2 to achieve response rates. Serum VEGFR2 was shown to positively correlate with response to both Bevacizumab and Apatinib (Lee & Djamgoz, 2018). Bevacizumab, an anti-angiogenic monoclonal antibody that targets VEGF-A, was approved by the FDA for the treatment of metastatic breast cancer in 2008. However, subsequent data did not demonstrate its ability to improve overall survival or quality of life, and as a result, its approval was revoked by the FDA in 2011 (da Silva et al., 2020). Bevacizumab combination with chemotherapy was demonstrated to improve pCR in stage II to III TNBC (Y. Li et al., 2022). Several small-molecule tyrosine kinase inhibitors targeting VEGF/VEGFR such as Apatinib, Afatinib and Erlotinib are currently in clinical trials (Y. Li et al., 2022).

Antibody drug conjugates are new class for cancer treatment showing a promising result in advanced TNBC. ADCs consist of three main components: an antibody designed to target the surface antigen on the tumor cell, a linker, and a cytotoxic payload (Pauls et al., 2022). ADCs are monoclonal antibodies that enable targeted delivery of a potent cytotoxic payload to cancer cells. This is achieved through the specific binding of the ADC to a cancer cell surface antigen. Once bound, the ADC is internalized in the cancer cell and releases the cytotoxic component inside the cell (Lyons, 2019). Over 60 ADCs targeting molecular targets such as TROP-2, LIV-1, HER2, HER3, and ROR2 are

currently at various stages of development for breast cancer treatment, including trials for TNBC (Pauls et al., 2022). In epithelial cells, trophoblast cell-surface antigen 2 (Trop-2) is a commonly expressed molecule that is linked to poor prognosis and lower survival rates. Therefore, Sacituzumab Govitecan (SG) was developed as a novel ADC agent that attach to Trop-2 on the TNBC cells surface and delivers the SN-38 (topoisomerase I inhibitor) payload drug to the cancer cells with a hydrolysable linker (Bardia et al., 2021). SN-38, which is the active metabolite of irinotecan, binds to the topoisomerase 1 cleavage complex on DNA in a reversible manner. This binding results in the disruption of DNA replication in cancer cells and leads to S-phase-specific cell death (Bardia et al., 2021; Pauls et al., 2022). Currently, SG is the first and only ADC approved for use in patients with refractory metastatic TNBC (Pauls et al., 2022).

1.2.5 Programmed Death-Ligand 1 (PD-L1) Role in Cancer

Programmed death-ligand 1 (PD-L1) is a type 1 transmembrane protein also known as CD274 or B7-H1. PD-L1 is a 40-kDa protein encoded by the gene CD274 that adopts a V-like and C-like immunoglobulin structure in its extracellular domains. It is a ligand for the inhibitory checkpoint molecule PD-1 which, through their interaction, enable tumor cells to evade the immune system (Escors et al., 2018). Under normal circumstances, the immune system responds to foreign trigger antigens accumulated in the lymph nodes or spleen and promotes antigen-specific T cell proliferation. Programmed cell death protein 1 (PD-1) on the surface of T cells binds to PD-L1 and can transmit inhibition signals to block T cell proliferation and promote T cell depletion (Yin et al., 2020). Thus, blocking the immune checkpoint system is a promising treatment strategy for achieving effective antitumor immunity.

PD-L1 and PD-L2 (B7-DC, CD273) are structurally related ligands for PD-1, exhibiting different binding properties and expression patterns (Zheng et al., 2019). PD-1 and PD-L1 proteins are vital in maintaining immune homeostasis. The PD-1/PD-L1 axis restrains the hyperactivation of immune cells and suppresses the adaptive immune system during pregnancy, tissue allografts, and prevents autoimmune diseases(Bardhan et al., 2016). PD-L1 also has an affinity for CD80 (B7-1); they interact in the cis

configuration on the surface of antigen-presenting cells, which disrupt PD-L1/PD-1 binding and limit the PD-1 co-inhibitory signal (Sugiura et al., 2019).

The typical expression of PD-L1 is by immune cells like activated T cells, B cells, macrophages, and antigen presenting cells predominantly under inflammatory conditions (Han, et al., 2020). While PD-L1 expression can be detected in other non-immune cells such as mesenchymal stem cells, epithelial cells, vascular endothelium, bone marrow-derived mast cells, and tumor cells. The PD-L1 expression by tumor cells acts as an "adaptive immune mechanism" where tumor cells can escape recognition and destruction by the host immune CD8 T cells (Ritprajak & Azuma, 2015; Qin et al., 2019).

Several types of cancers express PD-L1, including breast cancer, lung cancer, ovarian cancer, renal carcinoma, and melanoma (Zheng et al., 2019). PD-L1 expression has been detected in approximately 20–30% of TNBC cases (Saleh et al., 2019). Investigational cancer cell lines that express high PD-L1 protein includes breast cancer cell lines MDA-MB-231, JIMT-1, Ovarian cancer cell line ES-2, lung cancer cell line H441, prostate cancer cell line PC-3, Pancreatic cancer cell line ASPC-1, and melanoma cell lines B16F0 (Grenga et al., 2014; Wu et al., 2019;Rom-Jurek et al., 2018). In addition, high mRNA expression with low surface PD-L1 protein were reported in SKOV3 ovarian cancer cell (Qu et al., 2018).

PD-L1 has a broad distribution in different cellular compartments (Figure 1).

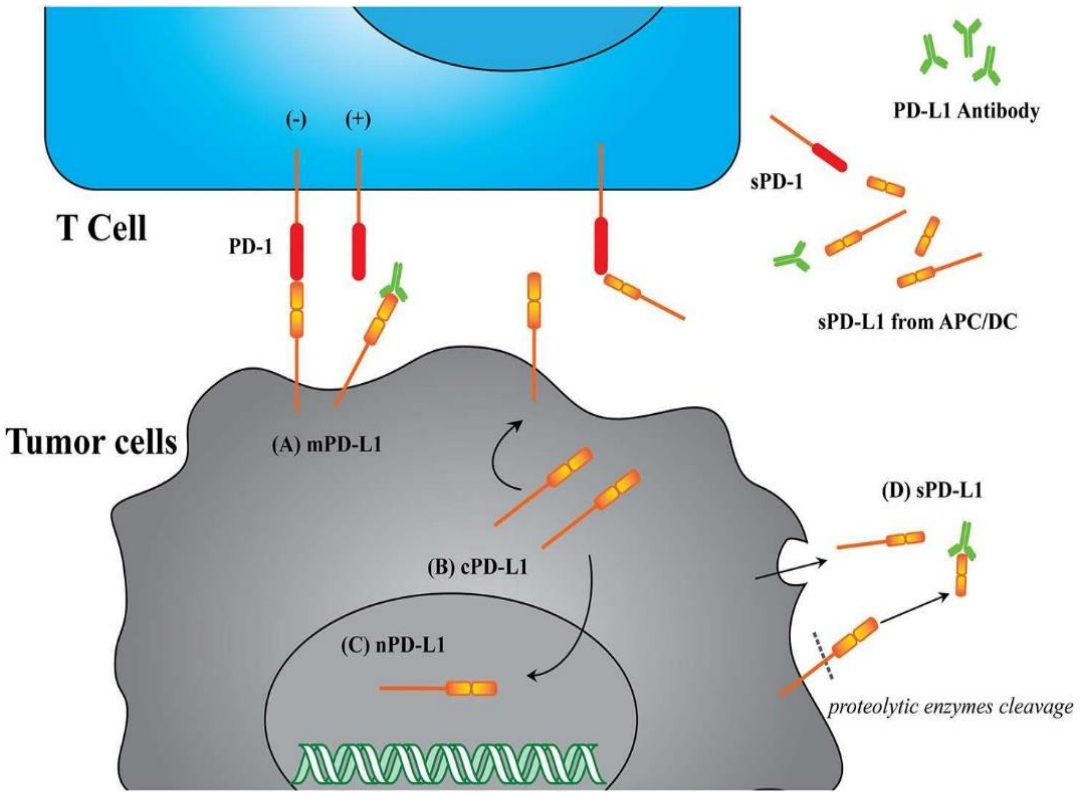

Figure 1: The different formats of PD-L1 in cellular compartments. (A) membrane PD-L1 (mPD-L1) is located on the tumor cell membrane and able to bind with PD-1 on T cells. (B) Cytoplasmic PD-L1 (cPD-L1) is located in the cytoplasm and promoted to transfer to mPD-L1. (C) Nuclear PD-L1 (nPD-L1) is located in the nuclei. (D) Serum PD-L1 (sPD-L1) is the soluble format of PD-L1 found in the serum

The PD-L1 format and structural integrity affects its anticancer immunity and functions (Escors et al., 2018; Wu et al., 2019). The known PD-L1 formats include membrane PD-L1 (mPD-L1) which binds with PD-1 for immune regulation and holds the structural integrity. Cytoplasmic PD-L1 (cPDL1) serves as a reservoir and mediate cell growth, migration and promotes MTORC1 signaling that inhibits autophagy (Clark et al., 2017)). Nuclear PD-L1 (nPD-L1) enhance chemo-resistance. Serum PDL1 (sPD-L1) is secreted from cancer cells and matured APCs into the plasma and binds with PD-1 (Wu et al., 2019). Exosomal PD-L1 carry the same membrane topology as the cell surface PD-L1. It promotes tumor growth and inhibits antitumor responses (Xie et al., 2019). The structures of these PD-L1 proteins are versatile; some cPDL1 and sPD-L1 lack the transmembrane motifs and lack the potential of glycosylated modification or dimerization (Wu et al., 2019).

PD-L1 blockade is achieved via three methods, antibody blockade, gene silencing, or small-molecule pathway inhibition. So far, there are three PD-L1 antibodies approved by FDA; Atezolizumab for urothelial carcinoma, NSCLC, TNBC, hepatocellular carcinoma (HCC); Avelumab for Merkel-cell carcinoma; Durvalumab for Urothelial carcinoma. These immunotherapies show a relatively low rate of high-grade treatment-related adverse events compared to conventional chemotherapies (Wu et al., 2019).

1.2.6 PD-L1 Expression and Intracellular Signals Regulation

Five different mechanisms regulate PD-L1 expression, first, by genomic alterations such as amplification or translocation. Second, epigenetic modifications such as methylation and acetylation of histone. Third, transcriptional regulation by oncogenic signals and inflammatory stimuli. Fourth, post-transcriptional regulation with miRNA, RAS, and Angiotensin II. Fifth, post-translational modification includes ubiquitination, phosphorylation, and glycosylation (Yi et al., 2021).

The PD-L1 receptor transmits internal cellular signals that regulate stress responses, boost the survival of cancer cells, and confer apoptosis resistance. However, due to the absence of traditional signaling motifs in the intracellular cytoplasmic portion of PD-L1, it has been difficult to systematically study its functions (Escors et al., 2018). A study presented that PD-L1 intracytoplasmic conserved regions RMLDVEKC, DTSSK, and QFEET, transduces survival signals most likely mediated by RMLDVEKC and DTSSK motifs (Gato-Cañas et al., 2017) (Figure 2).

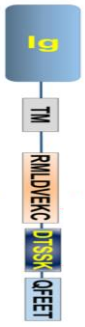

Figure 2: The Domain Structure of PD-L1. PD-L1 is a transmembrane protein consists of Ig, the extracellular immunoglobulin part; TM, the transmembrane part; RMLDVEKC, DTSSK and QFEET, the intracytoplasmic parts

Interfering with the PD-L1 signaling motif RMLDVEKC negatively regulates and sensitizes cancer cells to IFNβ cytotoxicity. Mutations in the "DTSSK" regulatory motif were hyperactive in blocking the negative regulator, enhancing resistance to IFN-mediated cytotoxicity. Intracellularly PD-L1 interferes with the IFN pathway by inhibiting STAT3 phosphorylation and preventing STAT3 upregulation of Caspase-7, thus stopping the signal cascade. Direct blockade of PD-L1 with an anti-PD-L1 antibody sensitized murine and human cancer cells to IFNβ *in vitro* (Gato-Cañas et al., 2017).

PD-L1 expression is regulated by several transcription factors HIF-1α, NF-κB, AP-1, and STATs, that binds to the gene promoter. The extracellular signals (hypoxia, cytokines, and EGF signals) are transduced via different pathways, mainly through MAPK or PI3K/AKT, to regulate PD-L1 expression on the transcriptional level (Figure 3). The regulatory effect of MAPK, JAK-STAT, NF-κB, and WNT signaling pathways promote PD-L1 expression, whereas PTEN inhibits PD-L1 transcription. Inflammatory stimuli such as IFN- α, IFN-β, IFN-γ, and IL-6 induce PD-L1 expression. In prostate cancer, the IL-6-JAK-STAT3 pathway promoted PD-L1 expression and led to immune cells resistance (Yi et al., 2021). All these pathways provide a mechanistic explanation for the abundant expression levels of PD-L1 associated with inflamed tissues and highly infiltrated tumors.

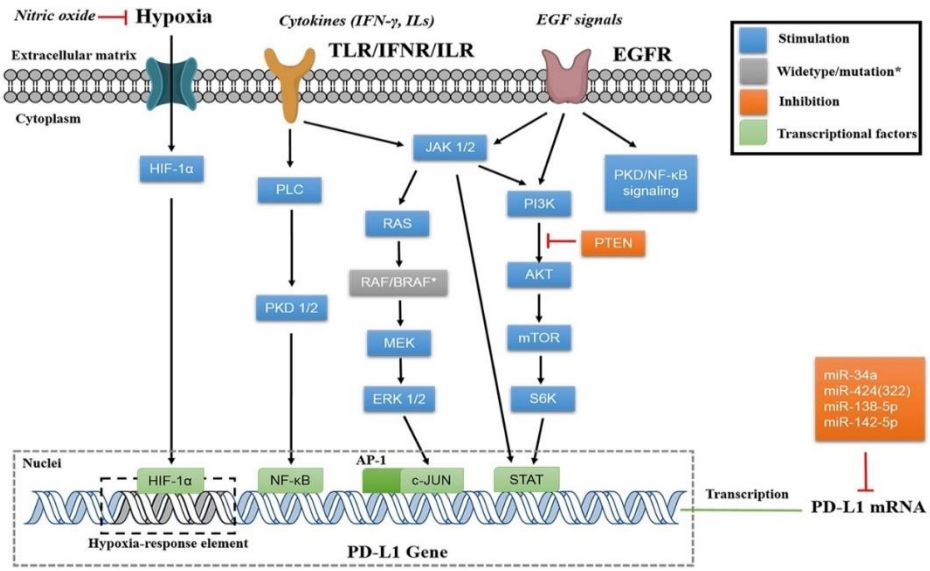

Figure 3: Signaling Pathways of PD-L1 Regulation. The extracellular signals (hypoxia, cytokines, and EGF) regulate PD-L1 expression through different pathways such as: MAPK or PI3K/AKT. HIF-1α, NF-κB, AP-1 and STATs are transcriptional factors regulate PD-L1 expression by binding to the gene promoter (Wu et al., 2019).

Various signals can modulate PD-L1 in cancer cells, which exert a critical role in tumorigenesis. PI3K/AKT pathway activation promotes PD-L1 expression through increased extrinsic signaling or decreased expression of negative regulators, such as PTEN. PTEN, a well-studied tumor suppressor, is believed to be a vital regulator of the oncogenic signaling pathway PI3K/AKT (Han et al., 2020). The loss of PTEN and the activation of PI3K have been detected in various cancer types, including hepatocellular carcinoma, prostate cancer, and breast cancer. Nearly half of PD-L1 positive TNBC samples detected are PTEN deficient (Han et al., 2020; Yi et al., 2021). Knocking down of PTEN caused PI3K/AKT activation and facilitated the transcription rise of PD-L1 (Yi et al., 2021). (R. Q. Chen et al., 2019) studied glioblastoma, a highly aggressive cancer, and reported that PD-L1 promotes glioma cell migration through Akt activation and membrane translocation. In U251 glioma cells, researchers identified that PD-L1 preferentially binds to Akt protein at the 112-480aa fragment of Akt and 128-237aa fragment of PD-L1. PD-L1 binding to Akt facilitated its membrane-translocation, thus preserved frontier glioma cells cytoskeleton after starvation.

The mitogen-activated protein kinase (MAPK) pathway role in PD-L1 regulation is dependent on the cancer cell type (Yi et al., 2021). MAPK pathway regulates cell proliferation, differentiation, invasion, metastasis, and death through phosphorylation activation (Braicu et al., 2019). In lung adenocarcinoma cells, activating EGF-MAPK signaling increased PD-L1 mRNA and protein levels (Stutvoet et al., 2019). In addition, Trametinib (a MEK inhibitor) suppressed MAPK signaling and potentiated the IFN-γ stimulated upregulation of PD-L1 in murine breast cancer cells. However, MAPK inhibitor didn’t cause any significant dysregulation on PD-L1 in cancer cells, such as: YSE30, TE-1, MKN7, PC-9, SNU-475, OE19, and BT-549 (Yi et al., 2021). Jalali and colleagues reported a connection between PD-L1 and the MAPK signalling pathway in Hodgkin's lymphoma (HL) cells. The study found that antagonistic anti-PD-L1 antibody attenuated the expression of both p-P38 and p-ERK in all HL cell lines HL-428, HL-1236, HL-HDLM2, and HL-KMH2. As there is an inverse correlation between cell survival and phospho-MAPK levels, thus the results indicate that PD-L1 employs the MAPK pathway (Jalali et al., 2019).

The HL cells stimulated with PD-L1 agonistic antibody for 48 hours under starved conditions showed increased cell survival, proliferation, and reduced apoptosis. The study utilized an array-based analysis to identify the proteins involved in promoting HL-428 HL cell line survival. The data identified several downregulated apoptotic proteins, including Bcl-x, pro-caspase 3, XIAP, survivin, and phospho-p53 (Jalali et al., 2019).

In a study of clear cell renal cell carcinoma, PD-L1 protein expression correlated positively with vascular endothelial growth factor (VEGF) expression. VEGF is a promoter of angiogenesis which sustains tumor cell growth and metastasis. VEGF upregulates PD-L1 expression and contributes to an immunosuppressive tumor microenvironment. The inhibition of VEGF creates an inflamed tumor microenvironment optimized for PD-L1 inhibitors efficacy (Xue et al., 2017). A study on microvascular endothelial cell line (MS1) suggested that PD-L1 and CD80 have negative regulatory effects on angiogenesis (Jin et al., 2011). In the MS1 cell line, PD-L1 and its receptor CD80, not PD-1, are highly expressed on mRNA and protein levels. The inhibition of PD-L1 or CD80 mRNA upregulated VEGFR2 expression and cell proliferation. PD-L1 regulation of angiogenesis is not principally through suppression of inflammation but rather an interaction between PD-L1 with CD80 in an autocrine or paracrine manner. The study results suggest that PD-L1 in inflamed tissue sets a higher threshold for angiogenic responses but not in the normal uninflamed state. Therefore, it is imperative to observe this signaling network in order to improve the presently available understanding (Jin et al., 2011).

Silencing PD-L1 in ovarian murine cell line ID8agg and melanoma cell line B16 mitigated in vitro proliferation rate. Moreover, PD-L1 deficient cells formed tumors relatively slower in immunocompetent and immunodeficient NSG mice than in control. In support of the functional role, a transcriptomic analysis, on the PD-L1 silenced ovarian cancer cell line ID8agg, identified multiple genes regulated by PD-L1. LC3-I and LC3-II genes involved in autophagy and the mTOR signaling pathway are regulated by PD-L1 expression. Though authors of the study did not address the mechanisms by which PD-L1 exerts the effects. The study proved that PD-L1 attenuation augmented

autophagy and hindered the ability of autophagy inhibitors to limit proliferation in vitro and in vivo in NSG mice (Clark et al., 2016).

Autophagy is a cellular mechanism that maintains cellular energy homeostasis upon stress. The autophagy process is highly regulated by conserved autophagic influx signaling involving PI3K/Akt/mTOR, p62, Beclin-1, and LC3 (R. Q. Chen et al., 2019). Researchers utilized CRISPR/Cas9 technique to knockout PD-L1 and study endogenous PD-L1 effects on autophagy in the U251 glioma cell line. The Western blot results demonstrated that under standard culture conditions, PD-L1 knockout did not affect p-Akt or p-mTOR. Whereas Upon 6 and 12 hours of starvation, the autophagy markers LC3II, LC3 were significantly elevated in the absence of PD-L1 (R. Q. Chen et al., 2019). The PD-L1 regulatory capacity over the mTOR pathway has been validated in murine B16 melanoma and ID8agg ovarian cancer cell lines. PD-L1 functions via activation of the mTOR-AKT pathway involved in autophagy (Escors et al., 2018).

PD-L1 induced epithelial mesenchymal transition (EMT) and stem cell-like phenotypes in metastatic clear cell renal cell carcinoma (CCRCC) which imply a presence of intrinsic PD-L1 pathway that stimulates kidney cancer progression (Nunes-Xavier et al., 2019). The bidirectional regulation between PD-L1 and EMT presents a key role in cancer immune escape (Jiang & Zhan, 2020). In addition to epithelial-mesenchymal transition transcription factors (EMT-TFs) pivotal role in regulation of EMT and cancer cell motility and dissemination they play role in promoting cancer cell plasticity, malignant transformation and maintain cancer stemness properties (Brabletz et al., 2018; Jiang & Zhan, 2020). Tumors with high expression levels of EMT-TFs such as Twist, Snail ZEB1, and Slug are enriched in stem cell-like features (Brabletz et al., 2018). In skin squamous cell carcinoma, Twist is reportedly upregulated by PD- L1 to promote EMT (Cao et al., 2011). In pulmonary adenocarcinoma, the EMT markers Snail and Vimentin expression levels correlate positively with PD-L1 expression. Whereas the epithelial marker E-cadherin expression level correlates negatively with PD-L1 expression (Kim et al., 2016). In a study of PD-L1 oncogenic role in Glioblastoma multiforme, an aggressive brain tumor, researchers demonstrated that PD-L1 activates the EMT process by upregulating N-cadherin, Vimentin, and the transcriptional factors

Slug, β-catenin. As well, PD-L1 and H-Ras's binding downregulate E-cadherin through activating the downstream MEK-Erk-dependent signaling pathway (Qiu et al., 2018).

Mansour et al. (2020) studied PD-L1 expression level in breast cancer stem-like (CSC-like) cells. CSCs are generated via the EMT process, where epithelial cells lose cell-to-cell junctions and gain mesenchymal features. The study showed that in TNBC cell line MDA-MB-23, the CSC-like cells overexpressed PD-L1 up to three folds compared to differentiated-like cells. PD-L1 expression in CSCs depends on activation of Notch/mTOR and PI3K/AKT pathway. They identified Notch3 protein as the mediator for PD-L1 overexpression (Mansour et al., 2020). These findings may indicate that patients with mesenchymal phenotypes are more likely to benefit from PD-1/ PD-L1 immunotherapy.

1.2.7 Atezolizumab Role in TNBC Regulation

Atezolizumab (trade name Tecentriq) is the first PD-L1 inhibitor approved by the FDA in 2016 for treatment of metastatic urothelial carcinoma and NSCLC (U.S. Food and Drug Administration, 2016). Atezolizumab is a fully humanized IgG1 isotype monoclonal antibody against PD-L1 (Figure 4). In 2019, Atezolizumab in combination

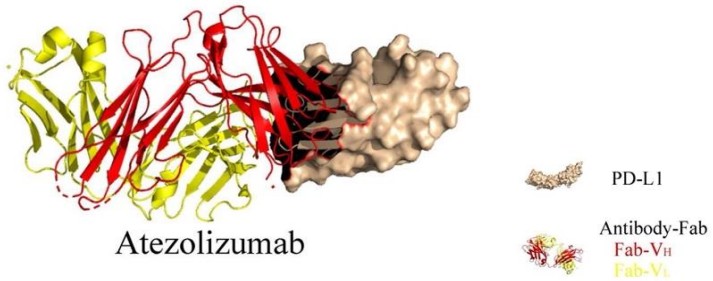

Figure 4: Overall Structure of the PD-L1 and Atezolizumab-Fab Complex (Qin et al., 2019)

with nab-paclitaxel received accelerated approval based on progression-free survival for treatment of patients with metastatic TNBC whose tumors have ≥ 1% PD-L1 expression (U.S. Food and Drug Administration, 2019). According to a study, Atezolizumab treatment induced a structural alteration in PD-L1 in the MDA-MB-231 human TNBC cell line by inducing a transition from a random coil and α-helical structure to β-sheet

conformation (Ali et al., 2019). Consequently, Atezolizumab therapy may have limited influence on intracellular cPD-L1 and nPD-L1, influencing treatment efficacy (Wu et al., 2019). As well, exosomal PD-L1 can mediate resistance to Atezolizumab immunotherapy by directly binding and neutralizing the anti-PD-L1 antibodies in the cancer microenvironment (Xie et al., 2019).

Treatment of PD-L1-positive SK-BR-3 and MDA-MB-231 breast cancer cells with Atezolizumab led to a significant decrease in cell proliferation (20%-30%). This finding suggests that PD-L1 could serve as a cancer marker/target, irrespective of the immune system's involvement. Moreover, Atezolizumab in SK-BR-3 cells showed inhibitory effects on p-Erk and p-P38 levels but no effect on the level of p-JNK. Thus, the study presented for the first time an intracellular pathway downstream PD-L1 that can be inhibited by anti-PD-L1 mAbs, while further studies are required to clarify the mechanisms of action (Passariello et al., 2019).

An investigational study by Saleh, et al., 2019 to analyze the mechanisms by which Atezolizumab affects the cancer cell line MDA-MB-231 in the absence of immune cells showed that, Atezolizumab decreased the surface expression of PD-L1 without affecting the mRNA and Protein level. Based on the transcriptomic analysis, Atezolizumab upregulated 388 genes, linked to apoptotic and DNA repair pathways, and downregulated 362 genes, linked to cell growth and differentiation, antiapoptotic pathway, cell migration and metastasis, NF-kB, PI3K/Akt/mTOR, MAPK, and CD40 signalling pathways. Functional assays showed a reduction in the proliferation and viability of MDA-MB-231 and an increase in apoptosis upon Atezolizumab treatment (Saleh et al., 2019).

1.3 Statement of the Problem

In the field of oncology, PD-L1 and PD-1 are crucial therapeutic targets. The clinical utilization of PD-L1/PD-1 blockers has advanced more rapidly than the exploration of the basic mechanisms governing this immunoregulatory interaction. Recently, few researchers suggested that independently of its interaction with PD-1, PD-L1 exerts intrinsic intracellular signals that regulate cancer cell survival, apoptosis resistance and stress responses.

While the majority of studies have concentrated on the interactions between PD-L1/PD-1 and T cell functions, only a limited number have examined the intrinsic signaling of PD-L1 molecules in tumor cells expressing PD-L1. The aim of this research is to shed light on the role of the PD-L1 intrinsic signaling pathway in cancer, with the goal of developing innovative anticancer therapeutic strategies in the future. Gaining a deeper comprehension of how PD-L1 shields cancer cells from both genotoxic damage and immune system attack will enable more informed decision-making regarding treatment options.

1.4 Research Objectives

The study's main objectives are first to generate a stable PD-L1 knockout cell line from TNBC model cells MDA-MB-231. Second to investigate the impact of PD-L1 knockout on cell proliferation and colony formation in vitro in addition to tumor growth in CAM model in vivo. Third, to explore the role of PD-L1 in TNBC migration and invasion through cell-based assays. Forth, to identify the intracellular pathways regulated by PD-L1 that are associated with tumor growth and metastasis advancement in TNBC.

Chapter 2: Materials and Methods

2.1 Cell Culture and Antibodies

MDA-MB-231 cells, human triple-negative breast cancer cells, were cultured in DMEM (Hyclone, Cramlington, UK) supplemented with antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) and 10% fetal bovine serum (Hyclone, Cramlington, UK). The cells were maintained at a temperature of 37°C under a 5% CO_2 atmosphere. The cells were passaged once a week when they reached 95% confluence, and the culture medium was changed every three days. Cell viability measured using trypan blue dye exclusion was higher than 99% in all experiments. All in vitro experiments were repeated at least three times. Antibodies to PD-L1, Akt, phospho-Akt, phospho-ERK, Snail, p21 and β-tubulin were obtained from Cell Signaling Technology (Cell Signaling, Beverly, MA, USA). Antibodies to ERK, Survivin, RhoA, Rac1, Cdc42, c-Fos, c-Myc and β-actin HRP were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). COX2 antibody was obtained from Abcam (Cambridge, UK).

2.2 Establishment of Stable PD-L1 Knockout Clones in Breast Cancer Cells

CRISPR/Cas9 technology was used to Knockout PD-L1 by introducing a double-stranded DNA break (DSB) at specific sequences in the CD274 gene. An actively dividing MDA-MB-231 cell line was seeded at a density of 4,000 cells / well into 96-well plates in the presence of serum and allowed to attach for 24 h to be 30-40% confluent at the time of transduction. Cells were transduced in serum-free medium for 24 h with three different designs of All-in-one lentiviral particles containing a single gRNA targeting PD-L1 and Cas9 in each vector or CRISPR negative control particles (Transomic technologies, Huntsville, AB, USA). Then, cells were incubated for 72 h in a complete medium. Each transduction was transferred into three 96-well plates at a density of 40 cells per plate to have one cell/well. Multiple pure GFP positive clones were expanded, harvested, and prepared for western blot analyses of PD-L1 knockout. The subclones generated from the MDA-MB-231 cell line were PD-L1.1 (pool of pure clones 2,3,8), PD-L1.2 (pool of pure clones 2,5,6), PD-L1.3 (pool of pure clones 2,19,21), and the negative control is PD-L1 control (pool of pure clones B, C, F).

2.3 In vitro Cells Proliferation Assay

The assay investigates PD-L1 knockout effect on MDA-MB-231 cells ability to divide. The cells were seeded into 6-well plastic culture plates at a density of 50,000 cells/ well supplemented with 10% FBS. At the indicated time, cells were trypsinized, suspended in 1 mL of medium, and counted daily for four consecutive days using a cell counter at an appropriate dilution (Scepter™, MerckMillipore, Darmstadt, Germany).

2.4 Colony Formation Assay

Control- and PD-L1-transduced cells were seeded into six-well plates at a density of 150 cells per well. The cells were incubated in 37°C, 5% CO_2 incubator for three weeks to form colonies. Following that, colonies underwent a series of procedures including washing three times with PBS, fixation, and then staining with 0.25% crystal violet solution dissolved in a mixture of distilled water and methanol (v/v) for 2 hours. Afterward, the colonies were washed three times with PBS, photographed, and counted. The percentage of colonies containing over 50 cells was then determined in the PD-L1-transduced colonies and compared to the control-transduced colonies assumed to be 100%.

2.5 Chick Embryo CAM Tumor Growth Assay

Eggs of the White Leghorn breed, which had been fertilized, underwent incubation under conditions of 37.5°C temperature and 50% humidity. On the third day of embryonic development (E03), the chorioallantoic membrane (CAM) was dropped by drilling a small hole through the eggshell into the air sac. Additionally, a window measuring 1 square centimeter was created on the eggshell above the CAM. At embryonic day 9 (E09), control and PD-L1 knockout MDA-MB-231 cells were trypsinized, washed with complete medium, and suspended in 80% Matrigel® Matrix (Corning, Bedford, UK). A 100-µl inoculum of 1×10^6 cells was added onto each egg CAM for a total of 15 to 16 eggs per condition. At embryonic day (E17), the upper portions of the CAM were removed, cleansed with PBS, and the tumors were cautiously excised from the healthy CAM tissues. Subsequently, the tumors were weighed to assess the impact of PD-L1 knockout on tumor growth. Chick embryos were humanely

euthanized by a topical addition of 10–30 µL pentobarbitone sodium (300 mg/mL, Jurox, Auckland, New Zealand). The viability of the embryos was determined by examining their voluntary motions, along with the integrity and pulsations of their blood vessels. Although the eggs were allocated randomly, the researchers were aware of the identity of each group and thus the experiment was not conducted in a blinded manner.

2.6 Impact on cellular migration using wound healing assay

The wound healing migration assay was utilized to monitor the ability of the MDA-MB-231 PD-L1 knockout cells to migrate and fill the wound. Control- compared to PD-L1- knockout cells (0.75 x 10^6 cells) were grown in twelve-well tissue culture dishes for 24 h. On the following day, a plastic 200 µl pipette tip was utilized to make a scratch through the confluent monolayer. Subsequently, the plates were washed twice and placed in fresh medium containing 10% FBS and incubated at 37°C. Three random locations were designated at the top of each dish where the width of the wound was assessed utilizing an inverted microscope with an objective of 4x (Olympus, Tokyo, Japan). The cells migration measurements were determined in µm at time intervals of 0, 2, 6 and 24 h. Migration was expressed as the mean ± SEM of the difference between the measurements at time zero and the subsequent time intervals up to 24 hours.

2.7 Boyden Chamber Matrigel invasion assay

The invasiveness of control compared to PD-L1 knockout cells was tested using Corning BioCoat™ Matrigel Invasion Chamber (8-µm pore size) (Corning, Bedford, MA, USA) according to manufacturer's protocol. Briefly, 100,000 cells/ 0.5 mL serum-free medium of MDA-MB-231 were seeded into the upper chamber of the system. While the lower chamber was loaded with medium having 10% FBS as chemo-attractant. The plate was incubated at 37°C for 24 h. The non-invasive cells were softly eliminated from the upper surface of the filter insert using a cotton swab. The cells that penetrated the Matrigel and passed through the 8 µm pores of the insert were identified using the CellTiter-Glo® Luminescent Cell Viability assay (Promega Corporation, Madison, USA). The luminescent signal was measured using GLOMAX Luminometer system. Data were presented as a proportional invasion (%) of PD-L1 Knockout cells in comparison with the control cells. The impact of PD-L1 knockout on cellular invasion

was presented as a percentage (%) by comparing the invasiveness of PD-L1- knockout cells to the control cells.

2.8 The Oris™ Matrigel Cell Invasion Assay

To further investigate the impact of PD-L1 depletion on MDA-MB-231 cells invasiveness, The Oris™ Cell Invasion Assay (platypus technologies, Madison, USA) was used within a 3-dimensional extracellular matrix comprised of Matrigel (Corning, Bedford, UK). Cells were seeded at a density of 100,000 cells per well into 96-well plate having stoppers and coated with Matrigel of a concentration 3.5 mg/ml. After 24 h, the silicone stoppers were removed from the confluent cells' monolayer. The wells were washed with PBS and then covered with 40 µl of Matrigel at a concentration of 5.5-6 mg/ml. The plate was incubated at 37°C for 45 minutes after which a complete medium was added to each well and the plate was kept back in the incubator for 24 and 48 h allowing the cells to invade the Matrigel matrix center. The invasiveness of the MDA-MB-231 cells was assessed using an Olympus fluorescence microscope (Olympus, Tokyo, Japan) in the presence of the detection mask. Representative images taken at 0, 24 and 48 h were converted to black and white. Using Image J, the density of the invasive cells at 48 h was measured and compared to the control cells density, the invasiveness of which is assumed to be 100%.

2.9 Western Blotting Analysis

MDA-MB-231 cells were trypsinized, collected with complete medium, centrifuged and suspended in PBS. Then the total cellular proteins of the cell's pallet were isolated using RIPA buffer (25 mM Tris-HCl, pH 7.6, 1% nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.5% protease inhibitors cocktail (Sigma, Steinheim, Germany), 1% PMSF, 1% phosphatase inhibitor cocktail (Thermo Scientific, Rockford, USA)). Following centrifugation at 14,000 rpm for 20 minutes at 4°C to eliminate insoluble material, the whole cell lysates were gathered and the protein concentration of the lysate was determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of total proteins (20µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE gel) then electro-transferred onto nitrocellulose membranes (Merck Millipore, Cork, Ireland); blocked for

1 h at room temperature with 5% non-fat milk in TBST (TBS and 0.05% Tween 20). The blots were probed with primary, β-tubulin, and β-actin antibodies overnight at 4°C. After that, the blots were washed and incubated with secondary antibodies. Immunoreactive bands of PD-L1, Akt, p-Akt (Ser473), p-Akt (Thr308), ERK, p-ERK, p21, Survivin, COX-2, c-Fos, c-Myc, Rho A, Snail, Rac1, Cdc42, β-tubulin and β-actin were detected using ECL chemiluminescent substrate (Thermo Fisher Scientific, Waltham, Massachusetts, US), and chemiluminescence was visualized using the LiCOR C-DiGit blot scanner (LI-COR Biotechnology, US). Densitometry analysis was performed using Image Studio Digits software (LI-COR Biotechnology, US). The intensities of the bands were normalized to the intensities of the corresponding β-actin or β-tubulin bands.

2.10 Statistics

All in vitro experiments were repeated at least three times, and results are expressed as means ± SEM of the specified data. Statistical analysis was carried out with SPSS version 26 software (San Diego, CA, USA). Statistical significance between experimental and control values was assessed by one-way ANOVA followed by Dunnett's post-hoc multiple comparisons test. *P <0.05, **P <0.01. ***P <0.001. ****P <0.0001 indicate a significant difference.

Chapter 3: Results and Discussions

3.1 PD-L1 Knockout in TNBC MDA-MB-231 Cell Line

In the present study, we first investigated PD-L1 expression level in four different breast cell lines. The TNBC cells, MDA-MB-231, showed a very high PD-L1 expression with almost no expression in two human hormone-dependent breast cancer cell lines, T47D and MCF7. In addition, no expression of PD-L1 was detected in non-tumorigenic breast epithelial cell line MCF 10A (Figure 5A). To explore the role of PD-L1 on major tumor progression hallmarks in TNBC cells, MDA-MB-231 cells were stably transduced with three different designs of all-in-one lentiviral particles containing in each vector a single gRNA targeting PD-L1 and Cas9 or CRISPR negative control non-targeting particles. GFP-positive cells were expanded in 96-well plates to generate pure clones (15 to 20 for each design). These clones were analyzed by western blotting to confirm PD-L1 knockout. As expected, the control CRISPR has no impact on PD-L1 expression in MDA-MB-231 cells (Figure 5B). Three clones from each design showed a complete knockout of PD-L1 (Figure 5C-E). The three pure clones were pooled together for each PD-L1 sgRNA design or negative control to obtain a better representative pool of pure clones. The generated pools of clones were named PD-L1.1 (pool of clones 2, 3, 8), PD-L1.2 (pool of clones 2, 5, 6), PD-L1.3 (pool of clones 2, 19, 21), and the negative control is named PD-L1 control (pool of clones B, C, F) (Figure 5F).

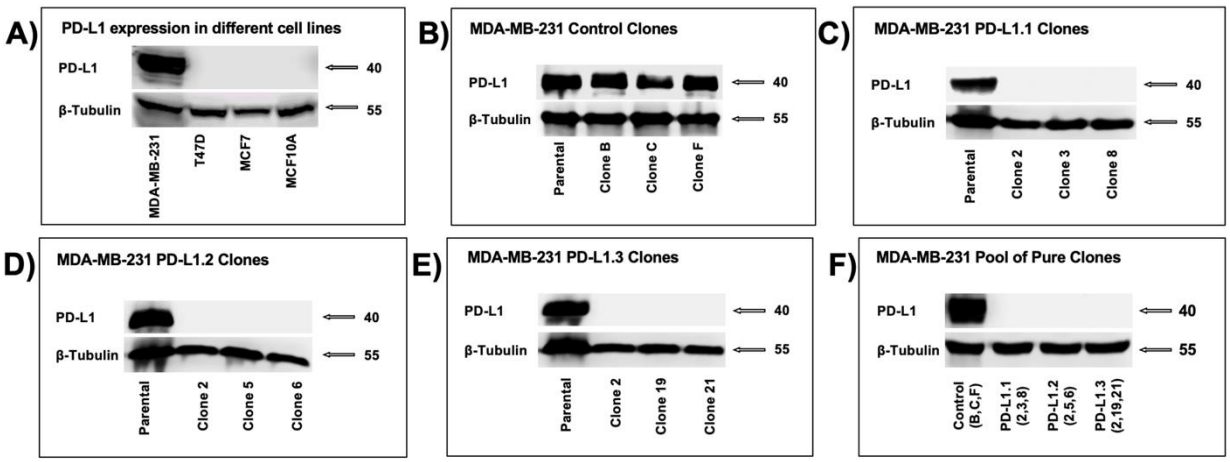

Figure 5: PD-L1 knockout in MDA-MB-231 cell line using CRISPR/Cas9. (A) Western blot of PD-L1 level in different breast cell lines. (B, C, D, E) Western blot of PD-L1 level in the selected pure clones of the control, PD-L1.1, PD-L1.2 and PD-L1.3. (F) Western blot of PD-L1 level in the pool of the three selected pure clones from the control and each design of gRNA targeting PD-L1.

3.2 PD-L1 Knockout Decreases MDA-MB-231 Cells Proliferation, Colony Formation *In Vitro* and Tumor Growth *In Vivo*

A high proliferation rate is a prominent hallmark of breast cancer, and it is significantly associated with the level of PD-L1 expression in breast cancer cells (Ghebeh et al., 2007). Therefore, MDA-MB-231 cell proliferation rates were determined up to 4 days using a cell counter (Scepter™, MerckMillipore, Darmstadt, Germany). The results showed significantly decreased proliferation rates in PD-L1 knockout cells PD-L1.1, PD-L1.2 and PD-L1.3 compared to the control cells after incubation for 1, 2, 3 and 4 days (Figure 6A). Consequently, the MDA-MB-231 PD-L1 knockout cells' ability to form colonies were investigated. PD-L1.1, PD-L1.2 and PD-L1.3 cells were seeded into six-well plates at a density of 150 cells per well for three weeks to form colonies. The results showed a significantly decreased number of colonies in PD-L1.1, PD-L1.2 and PD-L1.3 compared to the control (Figure 6B, C). These findings imply that PD-L1 absence substantially limits the growth of breast cancer.

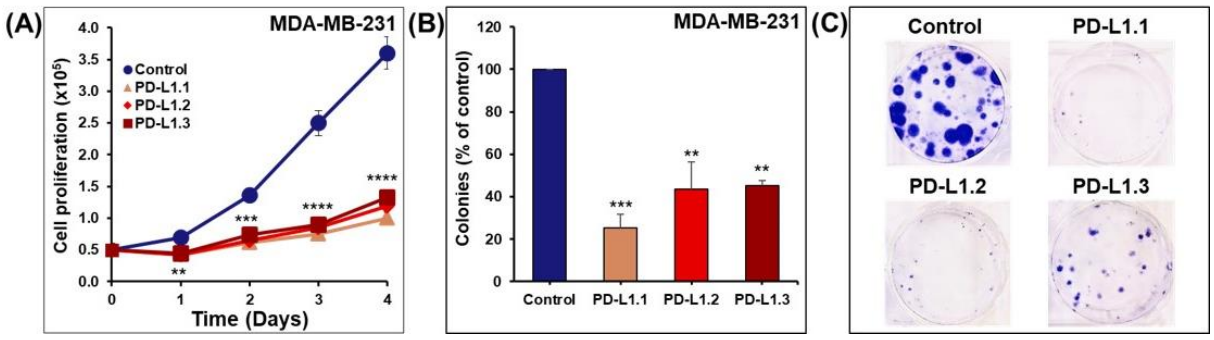

Figure 6: PD-L1 knockout decreased proliferation rate and colony growth of MDA-MB-231 in vitro. (A) Control- and PD-L1 knockout cells were seeded into six-well plates for 1, 2, 3 and 4 days. The cells proliferation was determined using a cell counter as described in the Materials and Methods. (B, C) The control and knockout cells were seeded into six-well plate and left to form colonies for 3 weeks after which the colonies were fixed, stained, and counted. Experiments were repeated three times. Shapes/Columns represent mean; bars represent S.E.M. **P < 0.01, ***P < 0.001, ****P < 0.0001.

To confirm the relevance of our in vitro data, the anticancer impact of PD-L1 knockout was investigated *in vivo* using the chick embryo CAM tumor growth assay. Cells grafted on the CAM at E09 were left to form tumors. At E17, tumors were retrieved from the upper CAM and weighed. In line with our in vitro findings, we found that PD-L1.1, PD-L1.2 and PD-L1.3 tumor xenografts were significantly smaller than control tumors by 70%, 69%, and 61%, respectively (Figure 7A, B). The impact of

MDA-MB-231 tumour xenografts on chick embryos survival was evaluated by assessing the number of alive embryos in control and PD-L1 Knockout groups. There was no difference in the number of surviving embryos between all the groups at the end of the experiment (Figure 7C).

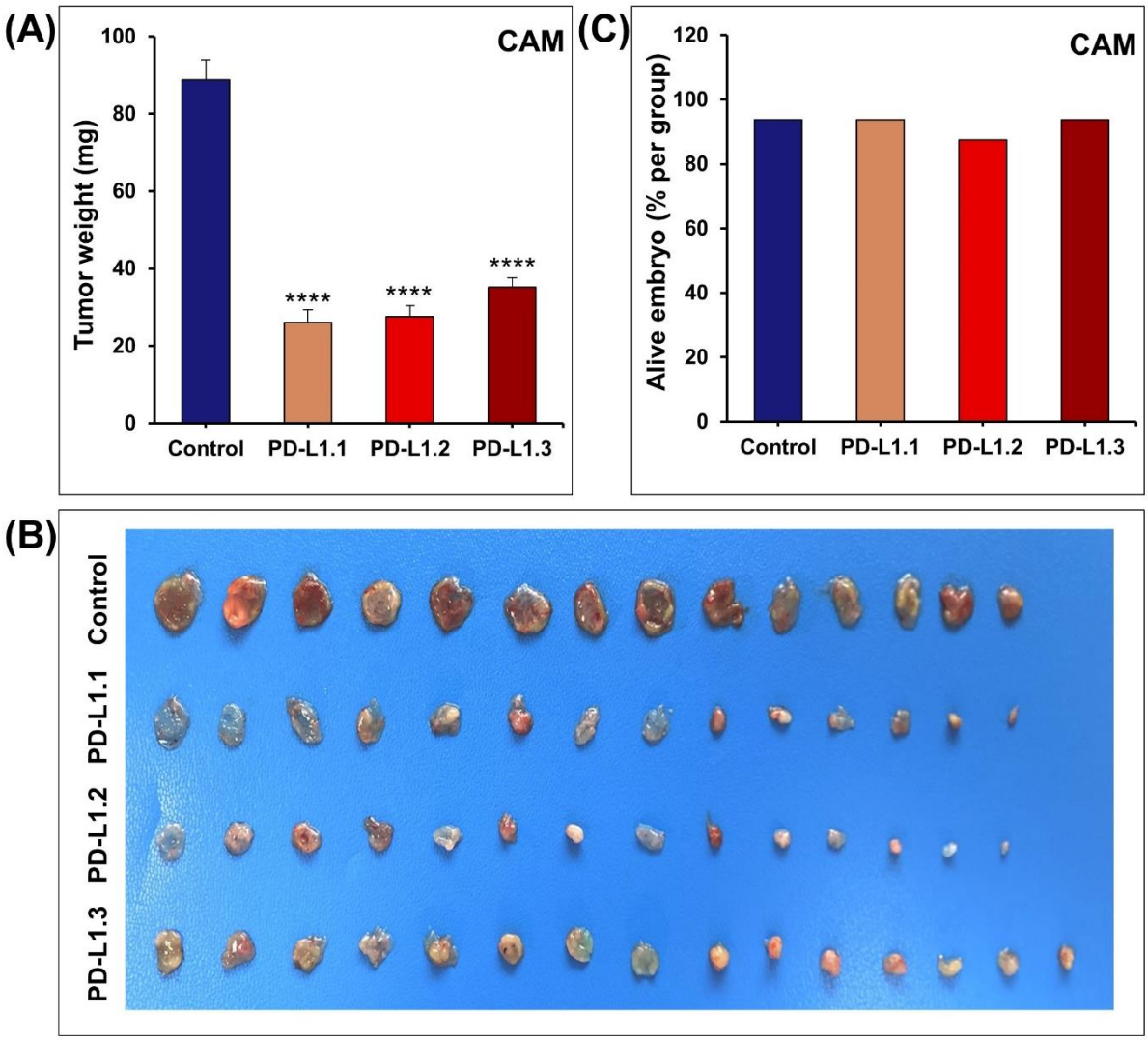

Figure 7: PD-L1 knockout decreased MDA-MB-231 tumors growth in chick embryo CAM model. (A) The cancer cells were inoculated on CAM and allowed to grow for 8 days. The columns represent the average tumor weights of the control and PD-L1 knockout cells. (**B)** Tumors pictures following extraction at Day 17. (**C)** The percentage of living embryos in each group. Columns are means; bars are S.E.M. ****P < 0.0001.

PD-L1 regulation of cancer cells proliferation and growth have been recognized in earlier studies and our results on TNBC comes in agreement. In human gastric cancer, PD-L1 knockdown using shRNAs significantly inhibited SGC-7901 and AGS cancer

cells proliferation (J. Li et al., 2017). In ovarian cancer and melanoma, PD-L1 attenuation significantly slowed proliferation rate of ID8agg and B16 cancer cells (Clark et al., 2016). In addition, PD-L1 knockdown using siRNA in oral squamous cell carcinoma SAS and YD38 cells decreased the cells viability and colony formation ability (Geum et al., 2022). Similar results were obtained in NSCLC where PD-L1 knockdown in H460 and H358 cells was reported to inhibit cell viability and colony formation (Yu et al., 2020). Our results also come into agreement with Chen et al. who reported a decrease in TNBC MDA-MB-231 cell growth and colony formation in soft agar upon PD-L1 knockout using CRISPR/Cas9 or knockdown using siRNA (C. Chen et al., 2021). The inhibitory effect of PD-L1 knockdown by siRNA was also reported on MDA-MB-231 cells viability and clonogenicity (Lotfinejad et al., 2021).

PD-L1 knockdown have been reported to inhibit in vivo tumor growth in melanoma B16 tumors xenografted in NSG mice, gastric SGC-7901 tumors in nude mice, glioma xenografts in nude mice, and NSCLC H460 in BALB/c-nude mice (Clark et al., 2016, J. Li et al., 2017, Qiu et al., 2018, Yu et al., 2020). In addition, a study on the chick embryo CAM model documented the inhibitory effects of PD-L1 inhibitors atezolizumab and avelumab on MDA-MB-231 cells growth without inducing significant toxicity on the chick embryos (Wang et al., 2022).

3.3 PD-L1 Knockout Decreases MDA-MB-231 Cell Migration and Invasion *In Vitro*

Cancer metastasis is a life-threatening event in which cell migration and invasion are considered as critical steps. Here, we investigated the impact of PD-L1 knockout on MDA-MB-231 cell migration using the scratch wound-healing migration assay. As shown in (Figure 8A-C), the migration of PD-L1 knockout MDA-MB-231 cells at 2, 6 and 24 h decreased significantly compared to the control cells. At 24 h, the wound was completely closed in MDA-MB-231 control. However, the three PD-L1 knockout cells failed to heal their scratch wounds.

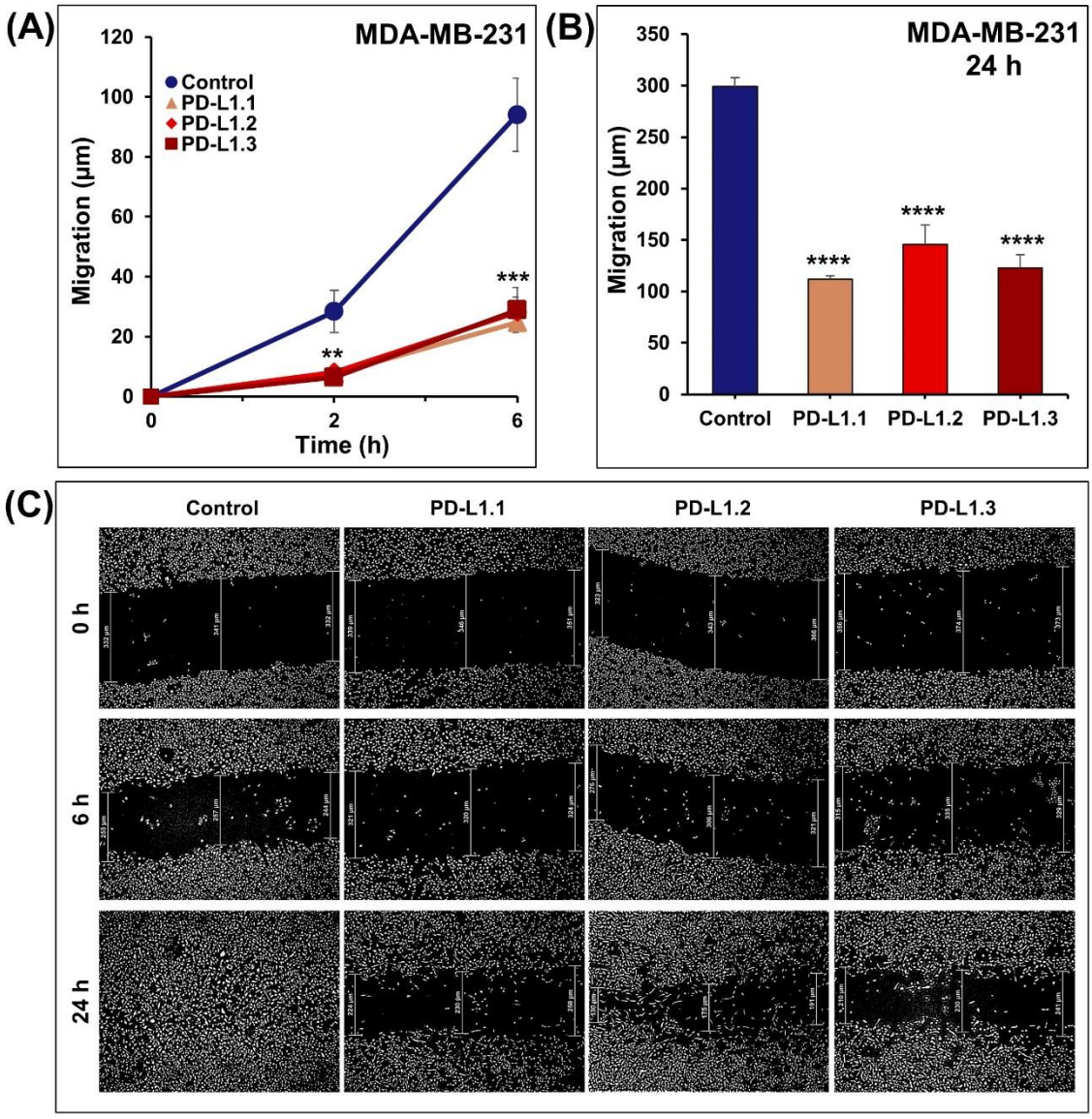

Figure 8: PD-L1 knockout decreased MDA-MB-231 cells migration in vitro. Using wound healing assay, scratches were introduced to the adherent monolayer of control and PD-L1 knockout cells. (A, B) Migration distance was determined after 2, 6 and 24 hours. (C) Representative pictures of the healing progress of the induced wounds at the indicated time points. Experiments were repeated at least three times. Columns or shapes are means; bars are S.E.M. **P < 0.01. ***P < 0.001. ****P <0.0001.

Next, we investigated the impact of PD-L1 knockout on the invasiveness of MDA-MB-231 cells compared to the control cells using the Boyden Chamber Matrigel invasion assay and the Oris™ Matrigel Cell Invasion Assay. We demonstrated in the two invasion assays that the invasiveness of the PD-L1 knockout cells decreased significantly compared the control cells (Figure 9A-C). These data clearly showed that PD-L1 protein is a strong promoter of breast cancer cell migration and invasion.

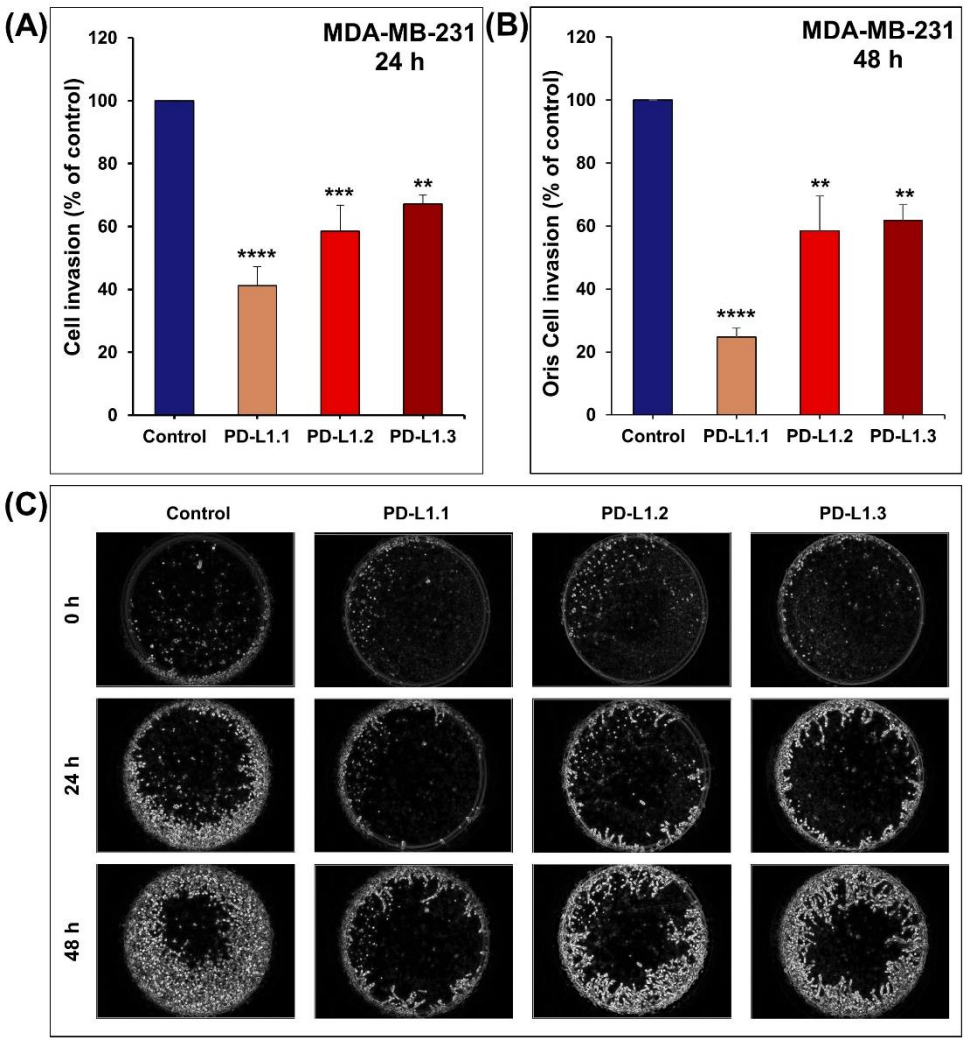

Figure 9: PD-L1 knockout decreased MDA-MB-231 invasion in vitro. (A) using BD-Invasion assay, cells were seeded in the upper chamber for 24 hours incubation. The invading cells into the lower chamber were determined using CellTiter-Glo Luminescent assay. (B,C) Using Oris™ Matrigel invasion assay, the three dimensional invasion of the control and PD-L1 knockout cells into the detection zone was monitored for 24 and 48 hours. Experiments were repeated at least 3 times. Columns are means; bars are S.E.M. **P < 0.01. ***P < 0.001. ****P <0.0001.

Our results come in support to earlier studies demonstrating the impact of PD-L1 knockdown on in vitro migration and invasion of some types of cancer including gastric cancer, oral squamous cell carcinoma, head and neck squamous carcinoma and NSCLC cells (J. Li et al., 2017, Geum et al., 2022, Eichberger et al., 2020,Yu et al., 2020). Additionally, similar findings were reported on migration of human esophageal cancer Eca-109 cells and migration of glioblastoma multiforme (L. Chen et al., 2017, Qiu et al., 2018). Lotfinejad et al, reported that PD-L1 siRNA knockdown in TNBC decreased cells migration rate using a wound healing model after 24 h, and 48 h of culture (Lotfinejad et al., 2021). On the contrary, PD-L1 knockout in osteosarcoma cells didn't cause

significant effects on the cell migration and invasion (Liao et al., 2017). Taken together, these data confirm the contribution of PD-L1 in tumor cell migration and invasion is independent of PD-1 receptor interaction.

3.4 PD-L1 Role on ERK Signalling Pathway

To explore the various molecular pathways through which PD-L1 might contribute to tumor progression of breast cancer cells, ERK and Akt pathways in addition to Rho GTPases have been investigated in our PD-L1 knockout MDA-MB-231 cells.

Mitogen-activated protein kinase (MAPK) signaling pathways are critical for cancer cell survival, proliferation, differentiation, invasion, metastasis, and tumor growth (Braicu et al., 2019). The current study demonstrated that PD-L1 knockout significantly decreases ERK phosphorylation without impacting total ERK expression (Figure 10A, B). It was reported that treatment of MDA-MB-231 cells with Atezolizumab, an anti-PD-L1 mAbs, altered a number of affected genes related to MAPK signaling pathway (Saleh et al., 2019). These findings come in agreement with Geum et al, study reporting that PD-L1 siRNA knockdown decreased p-ERK level in oral squamous cell carcinoma cells (Geum et al., 2022). In addition, increased phosphorylation of ERK was reported in glioblastoma multiforme upon PD-L1 overexpression (Qiu et al., 2018). Atezolizumab showed significant inhibitory effects on p-ERK and slight effect on p-P38 level but no effect on p-JNK level in SK-BR-3 human breast tumor cells (Passariello et al., 2019). Though there are not yet clear evidence on a complete specific pathway downstream PD-L1. Thus, we further investigated PD-L1 knockout effects on proliferation and invasion markers downstream PD-L1/ERK in TNBC, MDA-MB-231 cells.

MAPKs, including ERK, p38 and JNK, activation increases the expression and the activity of AP-1 complex families like Fos family (c-Fos, FosB, Fra-1, and Fra-2) (Gazon et al., 2018; Monje et al., 2005). c-Fos has an important role in tumor formation by regulating of proliferation, cell cycle progression, differentiation, and apoptosis (Angel & Karin, 1991; You et al., 2016). In this context, we showed that the decrease in ERK phosphorylation in PD-L1 knockout cells was associated with a significant reduction in c-Fos expression compared to the control cells (Figure 10C). Additionally, ERK1/2 phosphorylation activates many transcription factors such as CREB, c-Myc, and NFκB, leading to the expression of genes encoding proteins that regulate key functions

in cancer progression (Braicu et al., 2019). Myc, an oncoprotein highly expressed in TNBC and significantly associated with short overall survival, is also regulated by MAPK/ERK signaling pathway (Gupta et al., 2017). c-Myc executes its activities through transcriptional repression of cell cycle inhibitors p15, p21, p27 which contributes to its effect on promoting proliferation and oncogenesis (Gartel & Shchors, 2003). In this study, we demonstrated that PD-L1 knockout in MDA-MB-231 cells was also associated with a significant decrease in c-Myc expression (Figure 10 D). Our result is supportive to Kim et al study that demonstrated a significant correlation between c-Myc and PD-L1 expression in NSCLC tissue (E. Y. Kim et al., 2017).

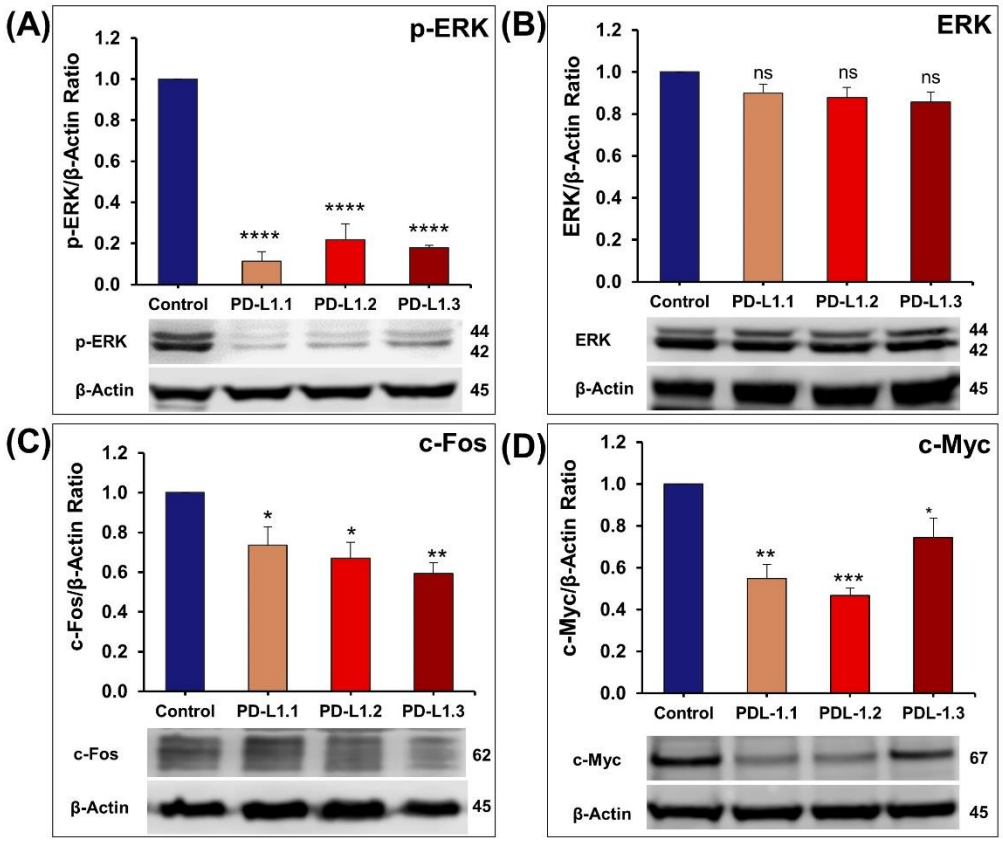

Figure 10: Quantification of the western blot showing the impact of PD-L1 knockout on the expression of; p-ERK and total ERK (A, B), c-Fos (C) and c-Myc (D). Columns represent mean of at least three independent experiments; bars represent S.E.M. *P < 0.05. **P < 0.01. ***P < 0.001. ****P < 0.0001. ns—non-significant.

3.5 PD-L1 Role on Akt Signaling Pathway

The Akt serine/threonine kinase, also known as protein kinase B (PKB), is the central node of the PI3K/Akt pathway that is a key regulator of cellular processes involved

in cell metabolism, proliferation, survival, migration, and invasion (Song et al., 2019). PDK1 induced the phosphorylation of Akt at Thr308. To achieve its full activation, Akt needs to be phosphorylated at Ser473 mainly by mTORC2 (Hinz & Jücker, 2019). Phosphorylation of Akt at Ser473 has been reported to promote breast cancer metastasis (Qiao et al., 2007; Song et al., 2019). The western-blot analysis results show that PD-L1 depletion in TNBC cells inhibits Akt phosphorylation at Ser473 and Thr308 (Figure 11A, B) without affecting the total Akt level (Figure 11C). In this context, it has been reported that PD-L1 overexpression increased the level of p-Akt in H1299 lung cancer cells and in HT-29 and HCT-116 colorectal cancer cells(Wei et al., 2019; Yu et al., 2020). In addition, transient knockdown of PD-L1 in oral squamous cell carcinoma decreased the phosphorylation of AKT (Geum et al., 2022). Similarly, decreased p-Akt was documented in pancreatic cancer and liver metastasis tissues in mice treated with anti-PD-L1 antibody (Zhao et al., 2017). PD-L1 has been reported to promote activation of PI3k/Akt pathway in nasopharyngeal cancer and breast cancer stem cells (Fei et al., 2019; Almozyan, Colak, Mansour, Alaiya, Al-Harazi, et al., 2017). On the contrary, it has been reported that PD-L1 knockout or transient knockdown in MDA-MB-231 slightly increases the AKT phosphorylation (C. Chen et al., 2021).

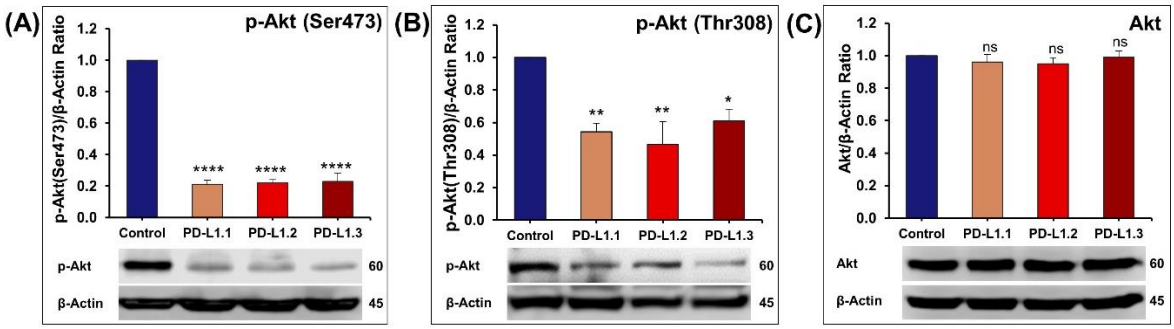

Figure 11: Quantification of the western blot showing the impact of PD-L1 knockout on the expression of; p-Akt (Ser473), p-Akt (Thr308) and total Akt (A-C). Columns represent mean of at least three independent experiments; bars represent S.E.M. *P < 0.05. **P < 0.01. ****P < 0.0001. ns non-significant.

3.6 PD-L1 Knockout Effect on ERK and Akt Downstream Proteins

Multiple hallmarks in cancer cells including sustained proliferation signaling and activated invasion and metastasis are the primary drivers of tumor progression especially in TNBC (He et al., 2019; Lyons, 2019). The contribution of Akt and Erk signaling to

tumor progression by inhibiting apoptosis and promoting cell proliferation, migration, and invasion is widely accepted. The complexity of the intrinsic crosstalk of MAPK with other signaling pathways, including the Akt pathway, is very challenging. It has been reported that the loss of phosphatase PTEN, the main inhibitor of the Akt-phosphorylation, also leads to Ras/MAPK activation (Braicu et al., 2019). Theoretically, the crosstalk between these two signaling pathways may lead to a reduction of the efficacy of single inhibition approaches (Mendoza et al., 2011). Therefore, the recent trend in clinical trials is to combine MAPK and AKT inhibitors since the dual inhibition of Akt and ERK signaling may achieve greater tumor suppression than single inhibition (Braicu et al., 2019). Here, we demonstrated that PD-L1 acts as a key activator of the two major ERK and Akt signaling pathways. Encouraged by these results, we decided to look further into some of the downstream oncoproteins, such as P21, COX-2, and survivin.

$p21^{Cip1/WAF1}$ is one of the primary cell cycle inhibitors. $p21^{Cip1/WAF1}$ mediates cell cycle arrest in G1 phase by inhibiting the activity of cyclin/CDK2 complexes leading to the inhibition of pRb phosphorylation, and consequently E2F transcription factors remain sequestered and unable to promote entry into S phase (al Bitar & Gali-Muhtasib, 2019). It has been reported that Akt directly phosphorylates $p21^{Cip1/WAF1}$ and inhibit its nuclear translocation preventing its access to nuclear cyclin/CDK targets (Suh et al., 2009; Zhou et al., 2001). Additionally, c-Myc suppress $p21^{Cip1/WAF1}$ transcription at the promoter site (Gartel & Shchors, 2003). In agreement, we demonstrate that both inhibition of c-Myc expression and Akt-phosphorylation induced by PD-L1 knockout lead to more than six folds increase in the expression of CDK inhibitor $p21^{Cip1/WAF1}$ (Figure 12 A).

COX-2 is an established pro-inflammatory protein that is highly expressed in TNBC and correlate with poor survival patient's outcome (Tian et al., 2017). It has been reported that COX-2 expression is correlated with increased phosphorylation of ERK and Akt (Chi et al., 2016; Glynn et al., 2010). In addition, the transcription factor C-Fos positively regulate COX-2 expression (Guo et al., 2001). In this context, we demonstrate that COX-2 inhibition in the PD-L1 knockout cells may be due to decrease in p-Akt, p-ERK and its downstream factor c-Fos (Figure 12 B).

Survivin is the smallest member of the (IAP) family and a potent anti-apoptotic protein that can be upregulated by Akt (Zhao et al., 2010). Moreover, survivin overexpression has been reported to be associated with cancer cells migration and invasion (McKenzie et al., 2010). Hence, The decrease in Akt phosphorylation due to PD-L1 knockout lead to a statistically significant reduction in survivin expression (Figure 12 C). These data are in agreement with the results showing evidence of survivin up-regulation by Akt and HIF–1α and data reporting that PI3K regulates survivin expression through Akt activation (Sun et al., 2014; Zhao et al., 2010). Additionally, in breast cancer tissues survivin level positively correlated with COX-2 level (Barnes et al., 2006). Therefore, survivin decrease seen in this study may also be linked to COX-2 as a study reported that COX-2 inhibit survivin ubiquitination and prevent its proteasomal degradation (Krysan et al., 2004). Our results shed the light on the implication of p21, COX-2, survivin, c-Myc and c-Fos through ERK and AKT signaling pathways in the PD-L1-mediated cancer progression.

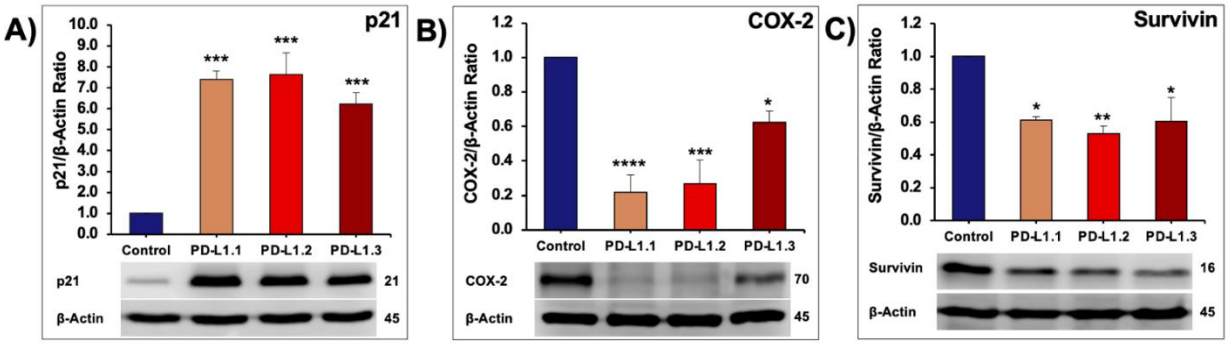

Figure 12: Quantification of the western blot showing the impact of PD-L1 knockout on the expression of; (A) p21, (B) COX-2, and (C) Survivin. Columns represent mean of at least three independent experiments; bars represent S.E.M. *P < 0.05. **P < 0.01. ***P < 0.001. ****P < 0.0001.

3.7 PD-L1 Role on Rho GTPases Expression

Rho GTPases overexpression has been reported in human tumors, including breast cancer (Orgaz et al., 2014; Svensmark & Brakebusch, 2019). Rho GTPases (Rho, Rac and Cdc42) play major role in breast cancer cell proliferation, adhesion, migration, invasion, and metastasis. RhoA stimulates actin-myosin contractility, resulting in the formation of stress fibers and focal adhesions and providing cell–extracellular matrix (ECM) anchoring points needed to pull the cell body in the direction of movement. Rac1 controls the formation of lamellipodia at the leading edge of the migrating cell. Finally,

Cdc42 signaling leads to the formation of filopodia and maintains the cellular polarization required for directional migration (Humphries et al., 2020). RhoA inhibition in MDA-MB-231 reduced cancer cells proliferation and invasion ability (Pillé et al., 2005). Therefore, we have decided to investigate whether PD-L1 regulation of MDA-MB-231 cancer cell proliferation, migration, and invasion involves the Rho GTPase signaling cascade. We demonstrated that PD-L1 knockout was associated with a significant decrease of RhoA protein expression without any impact on Rac1 and cdc42 proteins expression (Figure 13 A-C). Our result partially supports Eichberger et al study that reported a PD-L1-dependent gene regulation of Rho-GTPases Rho and Rac1 in head and neck cancer cells (Eichberger et al., 2020). Liberto et al. (2002) reported that RhoA is required for G1 to S progression in mammary epithelial cells by repression of p21 (Waf1/Cip1) (Liberto et al., 2002). In this context, we suggest that the increased expression of p21 (Waf1/Cip1) observed in our MDA-MB-231 PD-L1 knockout cells is at least in part due to the decrease in the expression of RhoA.

Snail confers a migratory and invasive ability essential for malignant cells MDA-MB-231 to disseminate and form metastasis (Wang et al., 2013). It was reported that Snail contributes to the progression of breast tumors through the regulation of RhoA (Zhang et al., 2013). Here, we showed that the decrease in RhoA expression in PD-L1 knockout MDA-MB-231 cells is also associated with a decrease in the expression of Snail (Figure 13D). This is in agreement with a recent article reporting that PD-L1 knockout or knockdown in MDA-MB-231 caused a reduction in snail level without affecting its mRNA level (C. Chen et al., 2021). The study by Kim et al. (2016) also supports our findings by reporting that in pulmonary adenocarcinoma, the EMT marker snail expression level correlates positively with PD-L1 expression.

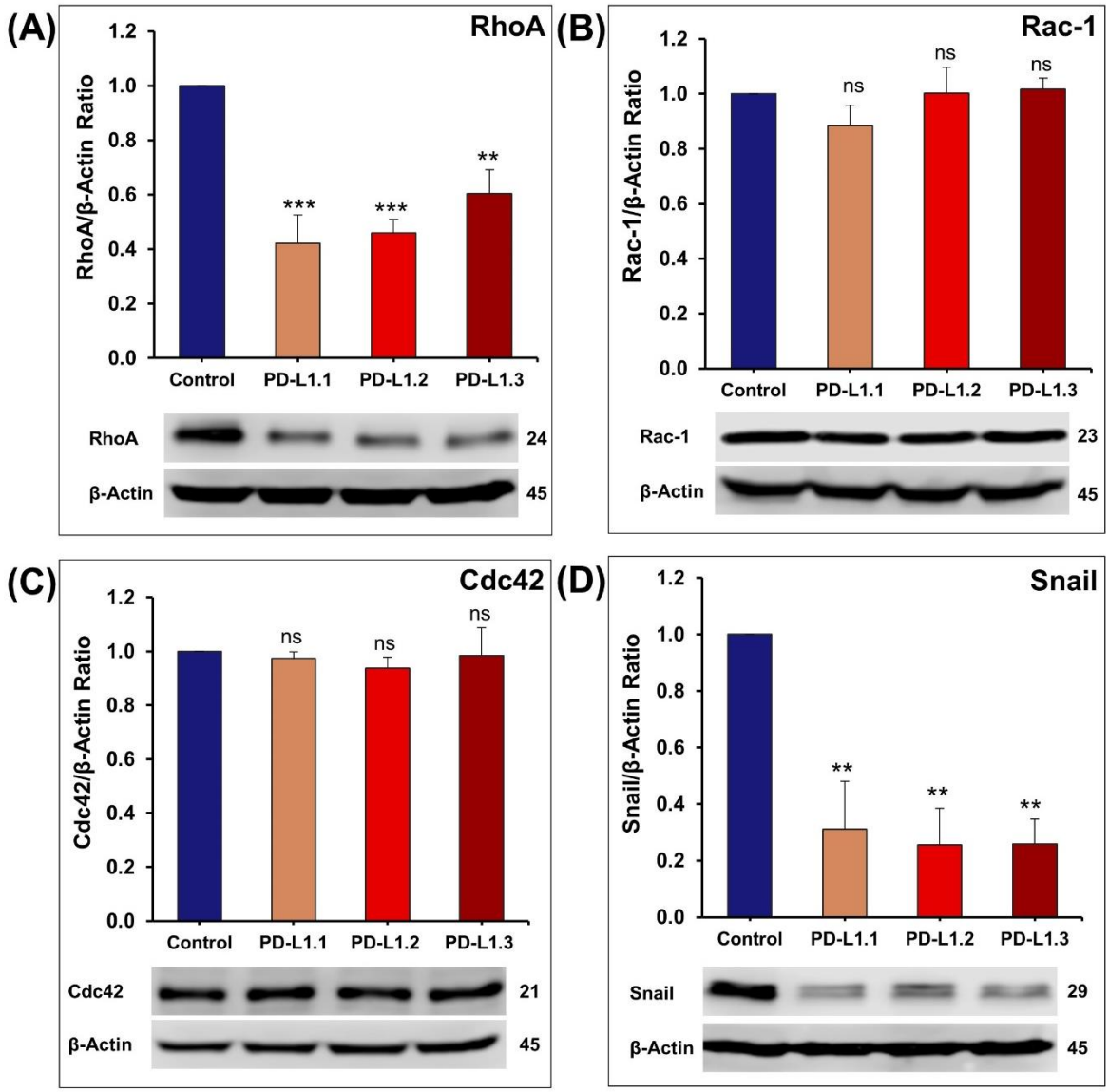

Figure 13: Quantification of western blot showing the effect of PD-L1 knockout on the expression of; RhoA (A), Rac-1 (B), Cdc42 (C) and Snail (D). Experiments were repeated for at least three times. Results are expressed as means ± S.E.M. **P < 0.01. ***P < 0.001. ns—non-significant.

3.8 Overview of the Main Findings

In this study, we were particularly interested in elucidating the Immune-independent role of PD-L1 in regulating TNBC progression. We focused on the deletion of PD-L1 expression in TNBC cells aiming to explore its contribution in promoting tumor progression. We have targeted the PD-L1 gene using CRISPR/Cas9 Knockout system to generate three sub cell lines from MDA-MB-231 each from a different cut design targeting the PD-L1 gene. Our data clearly demonstrate that knockout of PD-L1

expression significantly inhibited MDA-MB-231 cell proliferation, colonies formation, tumor growth, migration and invasion.

The observed changes in MDA-MB-231 pathogenesis are due to PD-L1 interplay with two main oncogenic pathways MAPK/ERK and PI3K/AKT, (Figure 14). PD-L1 deficiency in MDA-MB-231 was able to reduce the activation of ERK and Akt down to 30%. In that regard, our results suggest that PD-L1 is required in maintaining ERK and AKT activation in MDA-MB-231 cells. The PD-L1 knockout mediated inhibition of phosphorylated-ERK1/2 consequently inhibited its downstream targets c-Fos and c-Myc which are known proliferation-related proteins. PD-L1 knockout mediated inhibition of phosphorylated-Akt consequently inhibited its downstream targets survivin and COX-2 which are apoptosis-related proteins while p21 was upregulated. Furthermore, PD-L1 knockout significantly decreased the expression of Snail and RhoA.

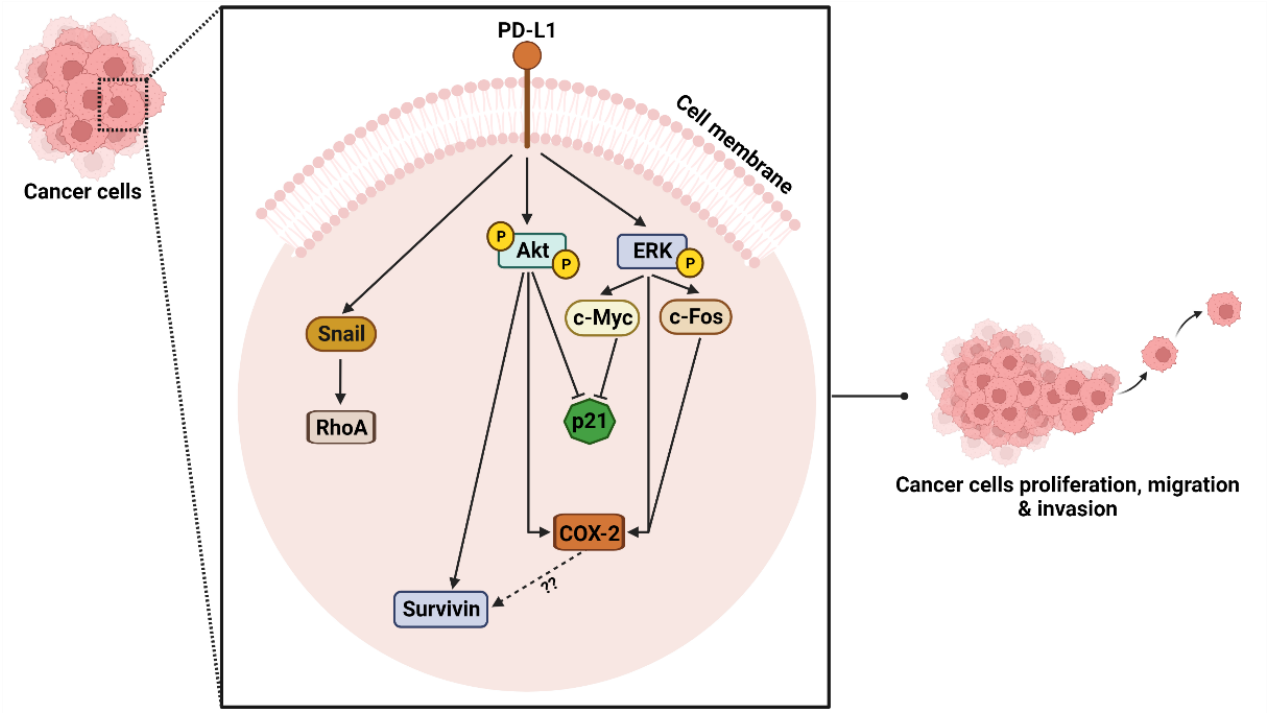

Figure 14: Diagram highlighting the potential downstream signaling of PD-L1 in TNBC.
PD-L1 controls the progression of TNBC by increasing the expression of RhoA through Snail in addition to activating the Akt and ERK pathway. Activation of Akt upregulates the expression of survivin and COX-2 and blocks the expression of p21. Activation of ERK has the ability to increase the expression of c-Fos, COX-2 and c-Myc. Created with BioRender.com.

Chapter 4: Conclusion

PD-L1 is among the most important therapeutic targets in oncology and the clinical use of PD-L1 antibodies is advancing far past basic mechanistic studies. This research aimed to identify how PD-L1 regulates TNBC hallmarks in the absence of immune interaction. This research work identified several intrinsic roles of PD-L1 in promoting cancer hallmarks, including proliferation, growth, migration and invasion. We also found that PD-L1 interplay mainly with two oncogenic pathways ERK1/2 and Akt and consequently their downstream targets. We have reported that PD-L1 regulate the expression of RhoA, c-Fos, c-Myc, Survivin, p21 and COX2. It can be concluded that PD-L1 gene knockout is a promising new strategy to optimize current TNBC therapy. This study provides new insights into understanding the PD-1 independent role of PD-L1 in breast cancer and will open the possibilities for targeted combinatorial interventions. Additionally, agents that regulate intrinsic PD-L1 expression could potentially serve as adjuvant therapies for current immune checkpoint inhibitors.

4.1 Recommendations and Future Study

Although cancers share certain common pathways, researchers should be cautious not to extrapolate results from one cancer type to another, therefore targeting a different type of solid cancer of interest will require to perform similar studies before arriving to a conclusion.

The findings of this study pave the way for further pre-clinical investigation into the impact of PD-L1 knockout on several cancer hallmarks, such as angiogenesis and metastasis.

Angiogenesis promotes progression and metastasis of several tumors. Effect of PD-L1 knockout on angiogenesis can be evaluated by:

- The level of angiogenesis in MDA-MB-231 tumors inoculated on CAM
- The secretion of angiogenic factors from TNBC cells, such as VEGF
- Establishment of stable PD-L1 knockout in immortalized human aortic endothelial cells TeloHAEC to explore: Endothelial cell proliferation, migration, tube formation, and spheroids sprouting.

Metastasis is a critical cancer hallmark that is responsible for 90% of cancer-related deaths. To expand our study further, we will investigate the effect of PD-L1 knockout on the expression of epithelial-mesenchymal transition (EMT) markers. Specifically, we will examine the levels of E-cadherin, N-cadherin, β-catenin, and vimentin to determine how PD-L1 influences the EMT process.

To better understand the impact of PD-L1 on cell behavior, we will investigate the cellular morphology of the PD-L1 knockout cells. Specifically, we will examine the effect of PD-L1 knockout on various cellular structures such as stress fibers, lamellipodia, filopodia, and focal adhesions. We will also study the cell-cell adherence and cell-matrix adherence.

To confirm the impact of PD-L1 on cancer cell invasion seen in vitro, we will conduct an experiment to explore its effect on the metastasis of MDA-MB-231 cells in vivo. Specifically, we will implant our cells orthotopically in the mammary fat pad of nude mice and observe how the absence of PD-L1 affects the development of metastasis.

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List of Publications

1. Alkaabi D, Arafat K, Sulaiman S, Al-Azawi AM, Attoub S. PD-1 Independent Role of PD-L1 in Triple-Negative Breast Cancer Progression. *International Journal of Molecular Sciences*. 2023; 24(7):6420. https://doi.org/10.3390/ijms24076420.

UAE UNIVERSITY MASTER THESIS NO. 2023:9

This research aimed to clarify the PD-1 independent role of PD-L1 in TNBC cells by knocking out the PD-L1 using three designs of CRISPR-Cas9 lentiviral particles. This study revealed that PD-L1 knockout significantly inhibited cell proliferation and colony formation in vitro and tumor growth in the chick embryo chorioallantoic membrane model in vivo. PD-L1 knockout also decreased the migration and invasion of TNBC cells in vitro. In addition, it has been found that PD-L1 knockout cells have low levels of p-Akt and p-ERK impacting some of their downstream proteins, c-Fos, c-Myc, p21, survivin, and COX-2. Furthermore, PD-L1 knockout significantly decreased the expression of Snail and RhoA. It provides insights into new cancer therapeutic strategies.

Duaa AlKaabi received her Master of Medical Sciences from the Department of Pharmacology and Therapeutics, College of Medicine & Health Sciences at UAE University, UAE. She received her bachelor's in pharmacy from the College of Pharmacy, Ajman University, UAE.