AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF DINACICLIB AND PALBOCICLIB ON
NORMAL AND BREAST CANCER CELLS IN 2D AND 3D
CULTURE

by
ZAYNAB ABD EL RAHMAN FATFAT

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Biology
of the Faculty of Arts and Sciences
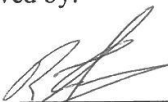at the American University of Beirut

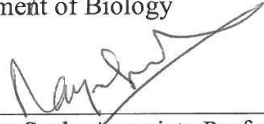Beirut, Lebanon
September 2018

AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF DINACICLIB AND PALBOCICLIB ON
NORMAL AND BREAST CANCER CELLS IN 2D AND 3D
CULTURE

by
ZAYNAB ABD EL RAHMAN FATFAT

Approved by:

_____   _____
Dr. Rabih Talhouk, Professor   Advisor
Department of Biology

_____   _____
Dr. Raya Saab, Associate Professor   Co-Advisor
Department of Anatomy, Cell Biology and Physiological Science

_____   _____
Dr. Marwan El-Sabban, Professor   Member of Committee
Department of Anatomy, Cell Biology and Physiological Science

_____   _____
Dr. Noel Ghanem, Associate Professor   Member of Committee
Department of Biology

Date of thesis defense: September 4, 2018

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name: ____ FATFAT _____ Zaynab _____ Abd El Rahman _____
LAST   FIRST   MIDDLE

⬤ Master's Thesis   ◯ Master's Project   ◯ Doctoral Dissertation

[X]   I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

[ ]   I authorize the American University of Beirut, to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes
after: **One ---- year from the date of submission of my thesis, dissertation, or project.**
             Two ---- years from the date of submission of my thesis, dissertation, or project.
             Three ---- years from the date of submission of my thesis, dissertation, or project.

Zaynab   _September 18, 2018_

Signature   Date

ACKNOWLEDGMENTS

AN ABSTRACT OF THE THESIS OF

Zaynab Abd El Rahman Fatfat   for   Master of Science
                                             Major: Biology

Title: The effect of Dinaciclib and Palbociclib on normal and breast cancer cells
       in 2D and 3D culture

Triple-negative breast cancer (TNBC), which accounts for 15–20% of all breast cancers, are extremely aggressive and have a poor prognosis due to the high tendency for metastatic progression and absence of specific targeted treatment. Since deregulation of cell cycle control is a hallmark of cancer, targeting cell cycle regulators such as cyclin- dependent kinases (CDK) may be an efficacious therapeutic approach for TNBC treatment. Palbociclib is a specific inhibitor of CDK4/6 and has been shown to induce senescence in several types of cancer. Dinaciclib, a CDK2 inhibitor, has shown promising antitumor activity in preclinical studies in broad spectrum of cell lines and in early phase clinical trials of hematological and solid malignancies. Here we examined the effect of Dinaciclib and Palbociclib, added alone, in combination or in tandem in a 2D as well as in 3D model of TNBC which shares similar properties to its in vivo counterpart. Our preliminary data showed that treatment of TNBC MDA- MB 231 cells with Dinaciclib, Palbociclib and combination for 48 hours decreased cell proliferation. Dinaciclib and combination increased the percentage of cells in pre-G1 whereas Palbociclib induced cell cycle arrest in G0/G1. Palbociclib significantly increased the percentage of Senescence associated β- galactosidase positive cells when treated for a longer period compared to those untreated or treated with Dinaciclib, combination or tandem. The drugs effect was also assessed in HMT3552 series including normal S1, intermediate Cx43 KO-S1, and cancer T4-2 cells. Our preliminary data suggested that treatment of S1, Cx43 KO-S1 and T4-2 cells cultured in 2D decreased cell proliferation. This decrease was progressive and became more significant after 10 days of treatment. Moreover, dual drug combination had most potent effect compared with single treatment and tandem. Preliminary data of Western-blot analysis in 2D culture proposed that Palbociclib and combination increased the expression of p21 in S1 and T4-2 cells compared to that of untreated or treated cells with Dinaciclib or tandem. Dinaciclib and Palbociclib also increased p21 expression in Cx43 KO-S1 cells compared to that of untreated or treated cells with combination. Dinaciclib, Palbociclib and combination increased the expression of p15 and Dec1 in Cx43 KO-S1 cells compared to that of untreated or treated cells with tandem. Dinaciclib effect on Dec1 expression was similar to that of combination but higher than that of Palbociclib. S1 and T4-2 cells cultured in 3D were treated with Dinaciclib and Palbociclib during S1 growth phase, and with each drug alone, combination and tandem during S1 quiescence phase. Preliminary data proposed that treatments did not affect neither lumen structures nor β- catenin localization of S1 acini like structures, treated during their growth or quiescence phase, suggesting that treatment did not affect apical polarity of normal S1 acini. Upon treatment of S1 and T4-2 cells in 3D during S1 growth phase with

Dinaciclib and Palbociclib, there was a growth arrest of S1 acini like structures and T4-2 nodules with a marginal preferential effect on T4-2 nodules. Treatment of S1 acini like structures and T4-2 nodules during S1 quiescence phase did not affect S1 acini diameter but prevented T4-2 outgrowth. Dinaciclib and combination effect on T4-2 outgrowth was higher than that of palbociclib and tandem. We suggest that CDK2 and/or CDK4 inhibition may be a promising approach to TNBC. However, future studies are needed to evaluate senescence markers expression in treated S1 and T4-2 cultured in 3D, and to decipher the drugs mechanisms of action.

CONTENTS

CHAPTER

ILLUSTRATIONS

xi

LIST OF TABLES

Table

ABBREVIATIONS

2D	2 Dimensional
3D	3 Dimensional
APC/C	Anaphase Promoting Complex/Cyclosome
ATM	Ataxia-Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-related protein
bHLH	Basic Helix-Loop-Helix
BMI1	B lymphoma Mo-MLV Insertion region 1
CAR	Cocksackie Adenovirus Receptor
Cdc42	Cell division control protein 42 homolog
CDK	Cyclin-Dependent Kinase
CEBPβ	CCAAT/ Enhancer Binding Protein-β
Cip/Kip	CDK-interacting protein/Kinase inhibitor protein
CKI	CDK Inhibitor
COX-2	Cyclooxygenase 2
Cx	Connexon
Cx 43 KO	Connexin 43 Knockout
DAPI	4',6-diamino-2-phenylindole
DCR2	Decoy receptor 2
DDR	DNA Damage Response
DEC1	Differentiated Embryo-Chondrocyte Expressed gene 1
DMEM	Dulbecco's Modified Eagle's Medium

DMSO	Dimethyl Sulfoxide
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
ETS	Epithelium-specific Transcription Factor
FDA	Food and Drug Administration
FOXM1	Forkhead box protein M1
GM-CSF	Granulocyte–Macrophage Colony-Stimulating Factor
GSK3β	Glycogen Synthase Kinase 3β
HER2	Human Epidermal growth factor Receptor 2
HP1γ	Protein 1 Homologue-γ
HUVEC	Human Umbilical Vein Endothelial Cell
INK4	Inhibitor of CDK4
LOC	Lab-on-Chip
MAPK	Mitogen- Activated Protein kinase
MCP	Monocyte Chemoattractant Proteins
MDM2	Mouse Double Minute 2 homolog
MIP	Macrophage Inflammatory Proteins
MPF	M phase-promoting factor
NF-kB	Nuclear Factor kappa B
PI3K	Phosphoinositide 3 Kinase
Pin1	Peptidyl- prolyl cis- trans isomerase NIMA- interactin 1

PR	Progesterone Receptor
PRC2	Polycomb-Repressive Complex 2
ROS	Reactive Oxygen Species
STAT	Signal Transducer and Activator of Transcription
T14	Threonine 14
T161	Threonine 161
TGFβ	Transforming Growth Factor-β
TNBC	Triple Negative Breast Cancer
TNF	Tumor Necrosis Factor
Y15	Tyrosine 15
ZEB-1	Zinc finger E- Box
ZO	Zonula Occludens

CHAPTER I

INTRODUCTION

Breast cancer is the most commonly encountered form of cancer and the second leading cause of cancer related mortality among women in the world (Wahba et al. 2015). Every year, an estimated 1 to 1.3 million breast cancer cases are diagnosed worldwide. Of these, approximately 15 -20% belong to the triple-negative subtype (Anders et al. 2008). Histologically, approximately 80-90% of TNBC tumors are invasive ductal carcinomas (Yuan at al. 2014). In spite of the general susceptibility to standard chemotherapy, TNBCs exhibit an overall poorer survival compared to non-TNBCs (Chacón et al. 2010). Unfortunately, many years of study have failed to identify a single pathway that is targetable in TNBC. Therefore, research efforts to develop targeted therapies, and assessment of their efficacy in relevant culture models is an urgent necessity.

The eukaryotic cell division cycle is a tightly regulated series of events coordinated by the periodic activation of multiple cyclin dependent kinases (CDK) upon interaction with their partner cyclins and ATP molecules (reviewed in Lim et al. 2013). Since cancer is characterized by uncontrolled cellular proliferation due to the loss of cell cycle control, driving tumorigenic cells into a senescent state may prevent cancer cells proliferation and abrogate tumor progression. Recently, senescence induction has emerged as an attractive therapeutic approach to treat cancer. Importantly, the therapeutic potential of senescence induction strongly relies on induction of irreversible cancer cells proliferation arrest. In addition, it relies on the engagement of immune

system to clear off malignant cells, since senescent cells were found to recruit cells of the immune system (Yun et al. 2015). During the onset of senescence, CDK2 and CDK4/6 inhibitors are upregulated and contribute together to cell cycle exit (Besson et al 2008). It was found that CDK2 repression was temporally followed by CDK4 repression during senescence mechanism (Zalzali et al. 2012). CDK2 and CDK4/6 are major regulators of the cell cycle; they phosphorylate and inactivate the retinoblastoma family of tumor suppressors Rb, p107 and p130, negative regulators of the cell cycle (reviewed in Harper et al. 2005). Several studies have shown that inhibition of these CDK was necessary for induction of senescence as a tumor suppressor mechanism. Inhibition of CDK2 was found to induce senescence in multiple models of cancer, including RasV12-induced proliferation (Zalzali et al. 2015), Cyclin D1-driven pineoblastoma (Zalzali et al. 2012), and MYC-driven leukemia (Perna et al. 2010). In addition, CDK4 inhibition was shown to promote senescence in melanoma (Anders et al. 2011), non-small cell lung cancer (Puyol et al. 2010) and neuroblastoma (Rader et al. 2013). Collectively, these results indicate that CDK inhibition and senescence induction is a candidate strategy for cancer treatment. In this study, we hypothesized that pharmacologic inhibition of CDK2 and/or CDK4 will potentially decrease TNBC tumor progression without affecting normal breast cells when used in a suitable 3D context. CDK2 and CDK4 inhibitors effect will be tested in a 3D model each one alone, in combination or in a temporally orchestrated approach that parallel events that occur physiologically during cellular senescence. We used small ATP-competitive molecules Dinaciclib and Palbociclib to inhibit CDK2 and CDK4 respectively. Dinaciclib has entered phase III clinical development for the treatment of refractory chronic

lymphocytic leukemia (Blachly et al. 2016). Palbociclib in combination with letorzole (aromatase inhibitor) has been approved by the Food and Drug Administration (FDA) for the treatment of patients with ER-positive and HER2-negative advanced breast cancer (Finn et al. 2016). Our results suggested that CDK inhibition induced senescence in normal, intermediate and cancer cells cultured in 2D. However, CDK inhibition when studied in a proper 3D context did not affect size and polarity of normal S1 acini like structures, but prevented the outgrowth from cancer T4-2 nodules.

CHAPTER II

LITERATURE REVIEW

A. Breast cancer

1. Incidence and mortality rates of breast cancer in the world and Lebanon

Cancer is the major burden disease, that affects human being worldwide. GLOBOCAN study demonstrated that the number of new cancer cases is of about 14.1 million cases and 8.2 million deaths occurred in 2012 worldwide. Among the different types of cancer, breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women with an estimate of 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers) (Torre et al. 2012). American Cancer Society estimated that 252,710 new cases of invasive breast cancer and 63,410 cases of in situ breast carcinoma were expected to be diagnosed among women in the United States in 2017 (American Cancer Society 2017). Lebanon had the second highest incidence rate and the third highest death rate of breast cancer among Asian countries (Ghoncheh et al. 2016).

2. Mammary Gland Anatomy and Breast Epithelium Structure

The mammary gland is composed of series of ducts that connect the lobes to the nipple. A lobe contains 20 to 40 lobules composed of alveoli or acini. A lobule consists of branching ducts that divide into subsegmental structures and terminate in the terminal duct lobular unit. The terminal duct lobular unit is formed of the terminal duct

and the acinus which is the structural and functional glandular unit (Gusterson et al. 2012).

The acini and the ducts are constituted of two cell layers. The first is an inner layer of secretory luminal epithelial cells. These cells have apical microvilli, are radially organized, and surround a central lumen. The second is a layer of contractile myoepithelial cells (Vidi et al. 2013). Both layers are surrounded by the basement membrane which is a specialized form of extracellular matrix constituted of a polymeric network of collagen IV and laminins (Tzu et al. 2008). The main function of the luminal epithelial cells is milk production during lactation. Milk transportation through the ducts towards the nipple is powered by the contraction of the myoepithelial cells (Vidi et al. 2013).

The mammary gland undergoes massive remodeling during puberty, pregnancy and lactation, and regression during involution following lactation. During puberty, there are an increase in the amount fatty tissue, elongation and branching of the ducts and formation of the lobular structures as well as terminal duct lobular units (Howlin et al. 2006). During pregnancy, epithelial cells proliferate rapidly within the ductal branches and developing alveoli. This increase in the number of epithelial cells as well as in the area of epithelial surface is essential for sufficient milk production during lactation. When lactation is complete, weaning induces post-lactational involution during which milk-producing epithelial cells are removed by programmed cell death and the mammary epithelium returns toward its pre-pregnant state (Oakes et al. 2006).
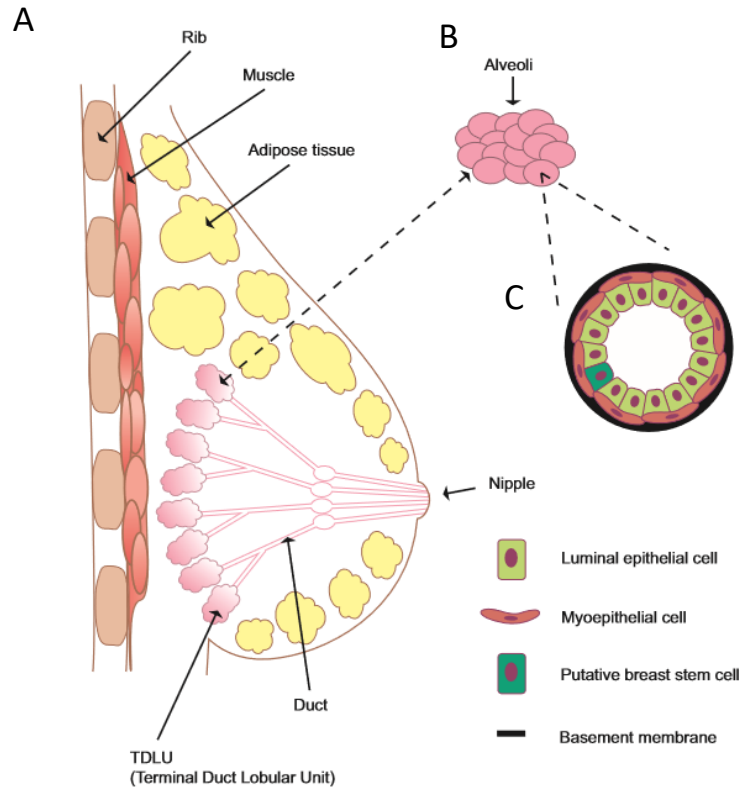
Figure 1. Schematic illustration of the normal breast structure.

The acini or aveoli are the functional units of the breast (B). During lactation, milk is produced and secreted by the luminal epithelial cells of the alveoli and transported to the nipple through the ducts. A cross section of the alveoli shows the luminal epithelial and myoepithelial cells arranged in bilayer and surrounded by a basement membrane (C). As modified from Lehn 2013.

3. Breast cancer initiation and progression

Breast cancer is mainly initiated in the epithelial compartment of the terminal duct lobular units. Aberrant proliferation, hallmark of cancer, leads to atypical ductal hyperplasia which may be followed by ductal carcinoma in situ (DCIS). This latter is defined as a nonlethal type of cancer because cancer cells remain within the lumen of the breast ductal system surrounded by an intact basement membrane. Once cancer cells break and invade the basement membrane, DCIS progresses into invasive ductal

carcinoma (IDC) which is potentially lethal. Corresponding stages for lobular invasive carcinoma would be atypical lobular hyperplasia and lobular carcinoma in situ (LCIS) (Bombonati et al. 2007).

4. Types of Breast Cancer

In general, breast cancer is classified according to receptor status and molecular subtypes (Peppercorn et al. 2008). Hormonal and growth receptors that determine basic breast tumor molecular subtypes are estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Breast cancers may be (ER+ and/or PR+, HER2-), (ER+ and/or PR+, HER2+), triple negative (ER-, PR-, HER2-) or HER2 type (ER-, PR-, HER2+). On the other hand, the molecular subtypes of breast cancer are luminal A, luminal B, basal-like or claudin-low. Luminal tumors have a gene expression pattern similar to that of the breast luminal epithelial cells. They are marked by the expression of luminal cytokeratins 8 and 18 (Perou et al. 2000). Luminal A tumors are characterized by low expression of proliferation-associated genes including Ki-67(Sørlie et al. 2001). Conversely, luminal B tumors are TP53 mutant and tend to be highly proliferative (Cornen et al. 2014). Basal-like tumors are marked by the expression of cytokeratins 5, 6, 14, and 17 that are typically expressed within basal epithelial cells of the skin and airways (Gusterson et al. 2005). Claudin-low tumors are characterized by the low expression of genes involved in tight junctions and cell–cell adhesion including claudin 3, 4, 7, Occludin, and E-cadherin, as well as by the high expression of many mesenchymal genes including Vimentin, Snail1, Snail 2, and Twist1 (Forrest et al. 2009).

5. Cell Junctions and Polarity

Maintenance of cells adhesion, cells communication and polarity is correlated with a normal phenotype of breast epithelial cells. In contrast, disruption of cell-cell and cell- basement membrane contacts as well as cell polarization is implicated in breast tumor initiation and progression.

Basoapical polarity axis is a fundamental feature of normal breast epithelia. The apical side of the cell faces the lumen, whereas its basolateral side interacts with the basement membrane. Basoapical polarity is characterized by the asymmetric distribution of polarity proteins to distinct membrane domains. It controls the localization of key mediators of signaling pathways involved in regulating proliferation, apoptosis, and differentiation. It also provides a proper functional barrier to regulate vectorial secretion and intake of molecules. Polarity formation is dependent on cell junctions: apical tight, adherens and gap junctions that connect laterally epithelial cells (Plachot et al. 2009).

Tight junctions contribute in apical polarity establishment by providing physical segregation between the basolateral and apical domains of the cell membrane. They consist of peripheral and transmembrane proteins that form belt-like structures at the apical surface. These junctions are composed of 3 different groups of proteins. The first includes integral membrane proteins occludin, claudin and Junctional adhesion molecule (JAM)) involved in the formation of the tight junctions strands. The first group also includes MarvelD2 and MarvelD3 proteins that has been proposed to contribute with occludins to membrane apposition and/or microdomain organisation. The second includes peripherally associated cytoplasmic proteins (Zonula Occludens

(ZO-1, ZO-2, and ZO-3) Par 3 and 6, cingulin and JACOP) that organize the integral membrane proteins and connect them to actin filaments. ZO-1 was found to be a critical organizer of tight junctions and consequently of the apical compartment (Mitic et al. 1999). The third contains signaling proteins (protein kinase A and C) involved in junction assembly, barrier regulation, and gene transcription (reviewed in Zihni et al. 2014).

Lateral cell- cell contacts are also mediated by adherens junctions located underneath tight junctions. These junctions consist of interactions among transmembrane cadherin, such as E-cadherin, and peripheral catenin such as p120-catenin, β-catenin, and α-catenin. Apart of initiation and stabilization of cell-cell adhesion, adherens junctions regulate actin cytoskeleton and intracellular signaling (reviewed in Hartsock et al. 2008). E-cadherens interaction with actin cytoskeleton and Cdc42 (a small GTPase of the Rho family) is required for the establishment of apical polarity (Desai et al. 2009). In addition, β-catenin was found to contribute in apical polarity formation through its interaction with Connexin 43 in the mammary epithelium (Submitted Bazzoun at al. 2018).

Another type of cell junctions located underneath the tight junctions along the remainder of the lateral cell membranes is gap junctions. A gap junction is a channel composed of a family of proteins called connexins. This channel allows intercellular communication through the exchange of ions, small metabolites, and electrical signals. Connexins are involved in luminal cell proliferation and differentiation, in proper production and ejection of milk, and in tumor suppression (reviewed in Banerjee et al. 2016). They interact by their intracellular C-terminal domain with proteins from other

junction complexes such as occludins, claudins, ZO-1, ZO-2, cadherins and catenins thus controlling their metabolism and function (Hervé et al. 2004, Dbouk et al. 2009). For example, C-termini of both Cx43 and Cx26 form a complex with ZO-1 and β-catenin at the membrane of mammary epithelial murine cells and contribute to the differentiated state of these cells as shown by the production of β-casein, a milk precursor (Talhouk et al. 2008). Cx43 drives the establishment of apical polarity in the mammary epithelium. Its silencing caused loss of epithelial polarity by decreasing the expression of claudin-1 and mislocalizing both ZO-1 and β-catenin (Submitted Bazzoun at al. 2018).

Concerning cell- basement membrane interaction, it is mediated by the binding of basement membrane components to specific receptors such as integrin and α-dystroglycan. It is the interaction between laminin-332 and α6/β4 integrin that specifically determines the basal polarity in the mammary epithelim (Taddei et al. 2003). Changes in the expression of β1, β4, α2, α3, and α6integrins have been found in mammary tumor cell lines and in tissue sections and have been shown to be associated with tissue disorganization, loss of polarity, increased tumor aggressiveness, and metastasis (Natali et al. 1999, Berdichevsky et al 1994, Rossen et al. 1994, Gui et al. 1995). In addition, the importance of β1 integrins in directing cell–cell and cell-ECM polarity and differentiation has been shown by studies in kidney cells (Ojakian et al. 1994, Schoenenberger et al. 1994) and keratinocytes (Symington et al. 1993, Watt et al. 1993).

6.Cell Junctions and Polarity in Breast Oncogenesis

Disruption of cell junctions due to deregulation of polarity proteins was thought to play a key role during tumorigenesis and invasiveness. Cx43 silenced HMT3522 S1 breast epithelial cells cultured in 3D show an intermediate level of tumor progression. Cx43 loss primed the cells to enter the cell cycle. It also disrupted lumen formation by misorienting the mitotic spindle (Submitted Bazzoun at al. 2018). On the other hand, reduced levels of claudins 1, 4, and 6 were reported in 2D and 3D cultures of human brain metastatic breast cancer cells MDA-MB-361 (Hoevel et al. 2004). Similar to claudins, a decrease in the protein level of ZO-1 and ZO-2, core tight junction proteins, was observed in patients with primary breast cancer (Chlenski et al. 2000, Martin et al. 2004). In addition, relocalization of ZO-1 away from the apical domain of the cell membrane in HMT3522 S1 cells cultured in 3D perturbed the apical polarity and pushed the cells into the cell cycle upon induction of chromatin alterations (Lelièvre et al. 2010). Delocalization of ZO-1 was also related to invasive potentials. ZO-1 was observed at the plasma membrane in noninvasive MCF-7 tumor cells and in the cytoplasm of invasive BT549 tumor cells (Polette et al. 2005). Similar to gap and tight junction proteins, adherens junction proteins were involved in breast tumorigenesis. E-cadherin loss enhanced metastasis of breast cancer cells by inducing the up-regulation of the transcription levels of EMT markers such as Twist and ZEB-1 (Onder et al. 2008). As for proteins involved in cell- basement membrane interactions, their deregulation was also implicated in breast oncogenesis. β1 integrin was reported to be abnormally expressed and localized in the tumorigenic HMT3522 T4-2 cells (Nisticò at al. 2014). Blocking β1-Integrin reverted T4-2 acini to a nonmalignant phenotype.

11

Reverted T4-2 acini re-assembled a basement membrane and re-established E-cadherin–catenin complexes and re-organized their cytoskeletons (Weaver et al. 1997).

B. Breast Cancer Treatment

There are two types of breast cancer treatment local and systemic. Local treatments such as surgery and radiation therapy removes the tumor from a limited local area. In contrast, systemic treatments reach cancer cells wherever they may be in the body, and include chemotherapy, hormone therapy, and targeted therapy (SweBCG, 2011).

1. Local treatments

Surgery has an important role in breast cancer treatment. Two types of surgery are used to remove breast tumors: breast-conserving surgery and mastectomy. In breast-conserving surgery, only the part of the breast that contains the tumor as well as some surrounding normal tissue are removed, whereas, in mastectomy the entire breast is removed. Radiotherapy, the second type of local treatment, is typically used in early breast cancer after breast conservation surgery and in locally advanced breast cancer patients after mastectomy (Matsen et al. 2013). Meta-analysis of individual patient data by Early Breast Cancer Trialists' Collaborative Group (EBCTCG) showed that, radiotherapy, following breast conserving surgery decreased the rate of disease recurrence and that of breast cancer death to the half and sixth, respectively (Darby et al. 2011).

2.Systemic treatments

Chemotherapy helps in the treatment of inoperable primary tumors when applied before the surgery, and in the eradication of dormant micrometastases when done after the surgery. The more common chemotherapy treatments used in breast cancer are the anthracyclin-based polychemotherapies FAC (fluorouracil, doxorubicin, cyclophosphamide) and FEC (fluorouracil, epirubicin, cyclophosphamide) as well as the combination therapy CMF (cyclophosphamide, methotrexate, fluorouracil) (Abe et al. 2005). Women with hormone receptor-positive (ER-positive and/or PR-positive) breast cancers are treated with hormone therapy. This therapy works by decreasing the amount of estrogen in the body or blocking estrogen from binding to its receptor (de Cremoux et al. 2011). Tamoxifen, a nonsteroidal drug classified as a 'selective estrogen receptor modulator' (SERM), competitively inhibits the binding of estrogen to its receptor on breast tissues. It was initially approved by the FDA in 1977 for treatment of metastatic breast cancer (Wolff et al. 2001). Raloxifene, another member of the SERM class, has a high binding affinity for ER. It has been approved as a chemopreventive drug in women having a high risk for developing breast cancer. However, it has failed to replace tamoxifen in breast cancer treatment (Jordan et al. 2008). Hormone therapy also uses FDA approved Aromatase inhibitors such as anastrozole, letrozole and exemestane. They decrease estrogen biosynthesis by targeting the enzyme aromatase, involved in the process of estrogens production (Chumsri et al. 2011). Aromatase inhibitors have proven to be more efficient in ER+ postmenopausal patients compared to treatment with tamoxifen and are now the recommended treatment for this patient group (Howell et al. 2005). Women with HER2- positive breast cancers are treated with

targeted therapy such as trastuzumab (Herceptin). Trastuzumab is a monoclonal

antibody that targets and inhibits HER2. It was approved by the FDA in 1988 (Masoud

et al. 2017).

3.Drug resistance

There are two types of drug resistances de novo drug resistance and acquired

drug resistance. Cancers that do not respond to the therapy at all from the beginning

have de novo drug resistance. However, in acquired drug resistance cancers respond

initially to the therapy but develop later this resistance by activating alternate cell

proliferation pathways, which function to restore cell cycle and lead to cancer

progression (Fan et al. 2015).

The progression of cancer and the development of drug resistant phenotype is

particularly challenging because of the excessive cross-talk between multiple signaling

molecules and pathways. Regulation of cell cycle proteins plays a crucial role in most of

these processes as well. Upregulation of positive regulators of the cell cycle, as well as

downregulation of negative regulators of the cell cycle have been documented to

interrupt the antiproliferative effects of endocrine and targeted therapies, leading to

resistance. For instance, it has been shown that tamoxifen-resistant MCF-7 cells,

developed by long term exposure of MCF-7 xenografts to tamoxifen, express higher

levels of cyclin D and E1 and cyclin-dependent kinase CDK2, compared to parental

cells (Louie et al. 2010). In addition, somatic deletion of p21 gene in human breast

cancer cells mediated Tamoxifen resistant phenotype (Abukhdeir et al. 2008). Nahta et

al. created trastuzumab-resistant pools from the SKBR3 HER-2-overexpressing breast

cancer cell line. They found that trastuzumab-resistant cells have decreased expression of cyclin-dependent kinase inhibitor p27, and increased activity of CDK2 (Nahta et al. 2004). Moreover, Scaltriti et al. established trastuzumab-resistant HER2 amplified breast cancer cells by chronic exposure to trastuzumab treatment, and found that these cells had cyclin E amplification/overexppresion (Scaltriti et al. 2011). Akli et al. found that overexpression of low molecular weight (LMW) cyclin E mediated the resistance to the aromatase inhibitor letrozole by using an aromatase-overexpressing MCF-7/Ac1 cells in the presence or absence of full-length cyclin E and LMW-E (Akli et al. 2010).

C. The need of cell cycle blockers (Senescence as a therapeutic approach)

In addition to their contribution in drug resistance, cell cycle mediators are involved in breast cancer initiation and progression. Previous studies have shown that the gene encoding cyclin D is amplified in 15% and overexpressed in 30-50% of primary human breast cancers (Bartkova et al. 1994, Gillett et al. 1994). In addition, cyclin E1 was found to be overexpressed in around 40% of breast cancers (Lodén et al. 2002). CDK4 gene amplification was also reported in about 15% of breast cancers (An et al. 1999). Since CDKs have a central role in the control of cell division, numerous drugs that target CDK activity have emerged and have been tested in the clinic over the past 30 years (reviewed in Roskoski 2016). The goal of the use of CDK inhibitors in breast cancer is to induce an irreversible cell cycle arrest and thus senescence. By this way, senescence induction may stop breast tumor cells proliferation, decrease their metastatic potential and promote their elimination by the immune system.

1.Cell Cycle

Eukaryotic cells dividing actively pass through 4 phases known collectively as the cell cycle. During two of these phases, cells duplicate its genetic material (S phase) then partition it between two identical daughter cells (Mitosis). During the two other phases called G1 and G2 ("Gap" periods) phases, cells prepare themselves for the S phase and mitosis, respectively. Cells stop dividing in the absence of mitogenic signals and in the presence of antimitogenic signals. They exit the cycle and enter G0 which is a quiescent state. They resume their proliferation once stimulated again with mitogenic signals. In this chapter, we will discuss the main cell cycle regulators, the internal control checkpoints, in addition to an overview of the mammalian cell cycle.

a. Cyclins

Cyclins are a large family of 29 proteins that belongs to 3 main groups. Group I or the cyclin B group include cyclins A, B, D, E, F, G, J, I and O; group II contains cyclin Y; and group III or the cyclin C group contains cyclins C, H, K, L, and T. All these proteins have a 100 amino-acid-residue domain which constitutes the cyclin box. The role of this cyclin box is to associate cyclins with specific kinases called cyclin dependent kinase. By this way, the kinase activity of these enzymes will be regulated. The A, B, D, E, F, J, and O type cyclins are formed of two cyclin boxes while the rest have only one (Ma et al. 2013). The oscillation of cyclins accounts for their names as they cycle up and down during the cell cycle. Cyclin expression is related to the cell cycle-dependent activation of the E2F and FOXM1 transcription factors. However, cyclin degradation occurs via ubiquitin mediated proteasome pathway. Therefore, fluctuation of cyclin levels causes changes in CDK activity which consequently drives

the cell cycle (reviewed in Roskoski, et al. 2016). Cyclin A expression accumulates at the beginning of S phase (Erlandsson et al. 2000) whereas expression of cyclin B accumulates in late G2 (Pines et al. 1989). Cyclin D1 levels increases in G1 and remains elevated until mitosis (Aktas et al. 1997). Cyclins E mediates progression through G1/ S phases (Ekholm et al. 2001). Cyclin F mediates a DNA damage-induced checkpoint response in G2 (Klein et al. 2015). Whereas cyclin H forms a complex with CDK7 and constitutes an enzyme known as CDK-activating kinase (CAK) which activates CDK2 and CDK 4/6 by phosphorylating them (Martin et al. 1997). Cyclin T forms a complex with CDK9 and contributes in basal transcription, signal transduction, and differentiation but not in cell cycle progression (Simone et al. 2001).

b. Cyclin- Dependent Kinases

Cyclin-dependent protein kinases belong to a family of 20 members. They are protein-serine/threonine kinases and become activated once they interact with cyclins. CDKs activity changes throughout the cell cycle and not their level. It increases with cyclin synthesis and decreases with cyclin degradation (reviewed in Rocca et al. 2014). CDKs have a conserved catalytic core with an ATP-binding pocket, a PSTAIRE-like cyclin-binding domain, and an activating T-loop motif which is a conserved threonine residue. CDKs are called proline-directed protein kinases because they transfer the phosphoryl group from an ATP molecule to a protein-serine or threonine residue coming immediately before a proline residue. By their association with cyclins via the PSTAIRE helix there will be a displacement of the T-loop and exposition of the substrate-binding interface. Most CDK family members have inhibitory and activating phosphorylation sites. Phosphorylation at T14 and Y15 within the ATP-binding site by

inhibitory kinases Wee1 and Myt1 prevents the binding of ATP. Whereas T-loop phosphorylation at T161 by CDK activating kinases (CAKs) allows substrate binding (reviewed in Lim et al. 2013).

c. Checkpoints

Cell cycle progression is always under control. It is stopped once any of the phases is unsuccessfully completed or DNA is damaged. It is only resumed when the damage is repaired. Otherwise, cell undergoes cell death. DNA damage may be intrinsic, such as intermediates of metabolism, attrition of telomeres, oncogene overexpression, and DNA replication errors. Also, it may be extrinsic ranging from sunlight, to carcinogens, ionizing radiation or other anticancer therapeutics. 3 checkpoints control the progression of the cell cycle.

i. G1 phase checkpoint

The first is before entry in S phase (G1/S phase checkpoint), cells check their genome integrity and check whether it is ready to undergo replication .In the case of DNA damage, ATM (ataxia-telangiectasia mutated) a serine/threonine protein kinase is recruited and activated by DNA double-strand breaks. ATM inactivates MDM2 (Mouse double minute 2 homolog) by phosphorylating it. The role of MDM2, an E3 ubiquitin-protein ligase, is to bind and block the N-terminal trans-activation domain of the tumor suppressor gene p53. Mdm2 inhibition causes p53 accumulation which promotes the transcription of gene encoding cyclin dependent kinase inhibitors. Moreover, p53 accumulation induce apoptosis by activating 2 signaling apoptotic pathways which activates aspartate-specific cysteine proteases caspases. The role of these caspases is to degrade the cellular components. In addition, ATM phosphorylates and activates protein

18

kinases called Chk1 and Chk2, which then phosphorylate p53 and Cdc25. This phosphorylation of p53 makes it more stable. Cdc25 protein phosphatases are activators of Cyclin A–CDK2 and Cyclin E–CDK2 involved in G1/S transition. However, Cdc25 phosphorylation by Chk2 leads to its degradation. Therefore, cyclin–CDKs involved in G1/S transition will not be activated which will result in cell cycle arrest in G1.

ii. S phase checkpoint

The second checkpoint is progression into M phase and relies on cyclin B dependent kinase activity. Cyclin B–CDK1 forms M phase-promoting factor (MPF). Its role is to promote the early events of mitosis. Before starting mitosis, cells check whether the DNA is replicated without DNA structural abnormalities, such as strand breaks or base modifications. If not, wee1, a protein kinase, phosphorylates tyrosine and threonine residues of the catalytic subunit of MPF. MPF phosphorylation lower CDK1 activity and thus prevents mitosis entry. The G2 to M transition occurs when MPF is dephosphorylated by Cdc25C. In the case of DNA damage, Chk1 and/or Chk2 phosphorylates Cdc25C and sequesters it in the cytosol making it unable to act on the nuclear MPF. Moreover, ATR (ataxia telangiectasia and Rad3-related protein) transduces unreplicated DNA signals by phosphorylating Chk1. This will prevent Cdc25C inhibition by phosphorylating and sequestrating it in the cytosol.

iii. Metaphase (Spindle) checkpoint

MPF mediates Cdc20-dependent activation of the anaphase promoting complex/cyclosome (APC/C). APC/C is a multisubunit E3 ubiquitin ligase that causes ubiquitination of different cell-cycle regulators targeting them for destruction by the 26S proteasome (Fang et al. 1998). Once activated, Cdc20–APC causes the destruction

19

of a protein called securin whose role is to inactivate a protease named separase during most of the cell cycle. The function of separase is to destroy the proteins that links sister chromatids together. By this way, Cdc20–APC initiates anaphase. In addition, Cdc20–APC also causes the degradation the B-type cyclin component of MPF. Anaphase occurs only when all chromosomes are attached to the bipolar mitotic spindle via their kinetochores. Kinetochores that are not bound to the mitotic spindle activate Mad2 protein, which binds and blocks Cdc20 activity. This results in cell cycle arrest in metaphase (reviewed in Barnum et al. 2014).

d. <u>Rb protein</u>

The retinoblastoma gene, a tumor suppressor gene, was first discovered in the cancer of the eye (Dunn et al. 1988). It has an essential role in the G1/S checkpoint, blocks phase S entry, and drives cell cycle arrest (Weinberg 1995). The Rb gene family includes 3 members Rb/p105, p107 and Rb2/p130. They are called 'pocket proteins' because they have a conserved binding pocket region by which they interact with cellular targets. The pocket region has two conserved functional domains A and B separated by a spacer S. E2F, family of transcription factors, binds to the A and B pocket domains of Rb proteins (Gillet et al., 2000). Rb-E2F complex represses the promotor of the genes required for G1/S transition. pRb is expressed throughout the cell cycle. It is hyper-phosphorylated and thus inactivated in late G1, then it is dephosphorylated during mitosis. p130 expression is elevated in G0, then it diminishes as cells progress into S phase. However, p107 expression is low in G0, and then increase as cells progress through G1 into S. Inactivation of Rb protein is required for the progression of the cells through G1 and S phases. The phosphorylation status of

these proteins is regulated by CDK inhibitor (CKI) by their binding to the cyclin/CDK complexes (reviewed in Henley et al. 2012).

e. E2F transcription factors

The E2F family of transcription factors includes at least seven members, E2F1, E2F2, E2F3, E2F4, E2F5, E2F6 and E2F7. A functional E2F transcription factor is a heterodimer formed by an E2F polypeptide and a DP polypeptide whose role is to stabilize the interaction between E2F factors and the Rb proteins. Each of the E2F polypeptides can heterodimerize with either DP1 or DP2 (DP3). E2F1, E2F2 and E2F3 are transcriptional activators, and bind to pRB. E2F4 and E2F5 are transcriptional repressors and interact with Rb2/p130 and p107. E2F6 does not to have a pocket protein interaction domain, and interacts with Polycomb proteins and represses transcription. The recently identified E2F7 and E2F8 repress specific promoters. During the cell cycle, E2F3 expression increases in early-G1 to mid-G1. E2F1 and E2F2 expression increases at the G1/S boundary. E2F4 and E2F5 are expressed throughout the cell cycle, but in G0 and early G1, they are bound by p107 and Rb2/p130, forming transcriptional repressor complexes. p107 and Rb2/p130 recruit E2F4 into the nuclei of the cell, inducing the repression of S phase genes transcription and cell proliferation (reviewed in Giacinti et al. 2005, reviewed in Henley et al. 2012).

f. CDK inhibitors

Cyclin-CDK complexes are down regulated by small molecules called the CDK-inhibitors. CKIs are divided into two groups. The INK4 (inhibitor of CDK4) family binds and inhibits CDK4/6 by this way they weaken their binding to cyclin D.

The members of this family are p16INK4a, p15INK4b, p18INK4c, and p19INK4d. These four proteins share a similar structure dominated by several ankyrin repeats. They only bind to CDK4 and CDK6 but not to other CDKs or to cyclins D and they are involved in G1 phase control. p15INK4b expression induced by TGF-b causes G1 phase arrest. p16INK4a accumulates progressively as cells age, possibly being induced by a senescence timer. Whereas, p18INK4c and p19INK4d are expressed during fetal development and may have roles in terminal differentiation. The second family is Cip/Kip (CDK-interacting protein)/KIP (kinase inhibitor protein) family and it includes p21Cip1, p27Kip1, and p57Kip2. These members interact with, and inhibit the kinase activities of, cyclin E-CDK2, cyclin D-CDK4, cyclin D-CDK6, cyclin A-CDK2, and cyclin B-CDC2 complexes and function throughout the cell cycle. p21 expression induced by the tumor suppressor gene p53 in response to DNA damage causes cell cycle arrest (reviewed in Lim et al. 2013). However, p21 phosphorylation on Thr-57 by glycogen synthase kinase 3β (GSK3β) induces its binding to cyclin B1-CDK1 at the G2/M transition leading to cell cycle progression (Dash et al. 2005). p27 expression increases in mitogen-starved cells and other quiescent states. However, its expression decreases as cells enter the cell cycle. In addition, p27 phosphorylation on Tyr-74, -88 and/or -89 by Src, Lyn, or Abl, prevents this protein to inhibit complexes containing CDK2 (Chu et al. 2007). The role of p57 is to control the cell cycle during embryonic development (Matsuoka et al. 1995).

g. Cell cycle machinery

Cell cycle is influenced by external signals during the G0 and G1 phases, such as the Ras/ Raf/MAPK pathway, NF-kB pathway, Jak/STAT pathway, hedgehog

pathway, Wnt/b-catenin pathway (reviewed in Rocca et al 2014). For example, the mitogen-activated protein kinase (MAPK) cascade whose activation is mediated sequentially by a Ras-dependent manner consists of three protein kinases MAPK kinase kinase(MAPKKK), MAPK kinase (MAPKK), and MAPK. The Ras/MAPK pathway directly regulates cyclin D gene expression. MAPK contributes in the activation of the activation protein-1 (AP-1) and ETS transcription factors which transactivate the cyclin D promoter that contains specific binding sites for both AP-1 and ETS. Ras signaling pathway involving phosphatidylinositol 3-kinase (PI3K) and Akt (protein kinase B) is another mitogenic pathway that plays an important role in the progression of the cell cycle. It triggers a network that positively regulates G1/S cell cycle progression. This pathway inactivates GSK3β, therefore preventing cyclin D degradation (Liang et al. 2003). Cyclin D1 forms complexes with their partners, CDK4 and CDK6 then enter the cell nucleus where they are phosphorylated and fully activated by CDK-activating kinase (CAK).

Once activated, cyclinD1-CDK4/6 phosphorylates Rb in mid-G1 phase. Rb phosphorylation leads to the release of the transcription factor E2F which promotes cyclin E, cyclin A, CDK1, and CDK2 transcription. Other E2F target genes encoding proteins that are essential for DNA synthesis, such as DNA polymerase α, thymidine kinase (Dyson, 1998). Cyclin E is synthesized during G1 phase of the cell cycle, and its level increases sharply in late G1. This is followed by accumulation of Cyclin E protein and then down regulation in S-phase. Cyclin E association with CDK2 mediates Rb phosphorylation on additional sites. As cells approach the G1/S border, control of the cell cycle becomes dominated by cyclin E-CDK2 complexes. In addition, binding of

cyclin A with CDK2 is required during S phase. However, CDK2 activation does not only reinforce CDK4 to complete Rb phosphorylation but also triggers the destruction of the CDK inhibitor p27Kip. Cells become independent of mitogens for completion of the cell cycle and become committed to enter S phase.

Cyclin A- and B-dependent CDKs become activated later during the cell division cycle maintaining Rb in a hyper-phosphorylated form. The messenger RNA (mRNA) of cyclin A2 starts to accumulate during S phase and diminishes at mitosis, whereas cyclins B accumulates in late G2. During G2 phase, the cell prepares itself to undergo mitosis and cytokinesis. Cyclin B-CDK1, MPF, drives with cyclin A-CDK1 G2-M transition.

Phosphorylation of CDK1 on Tyr15 by the kinases Wee1 and on Tyr15 and Thr14 by the kinase Myt1 keeps cyclin B-CDK1 complex inactive. Once dephosphorylated by the protein phosphatase CDC25C, it becomes activated. Cyclin B-CDK1 complex is involved in the initiation of mitotic events in both the cytoplasm and the nucleus. Cyclin B- CDK1 contributes in centrosome separation by phosphorylation of the centrosome-associated motor protein Eg 5 in the fragmentation of the Golgi network, in the breakdown of the nuclear lamina and cell rounding.

Exit from mitosis requires degradation of both cyclin A and cyclin B, and this occurs via a ubiquitin-mediated pathway that itself is regulated by the APC pathway. Degradation of both cyclin A and cyclin B reestablishes the requirement for cyclin D and reinstitutes a period of mitogen dependence in the ensuing G1 phase of the next cell cycle (reviewed in Harper et al. 2005, Vermeulen et al. 2003).
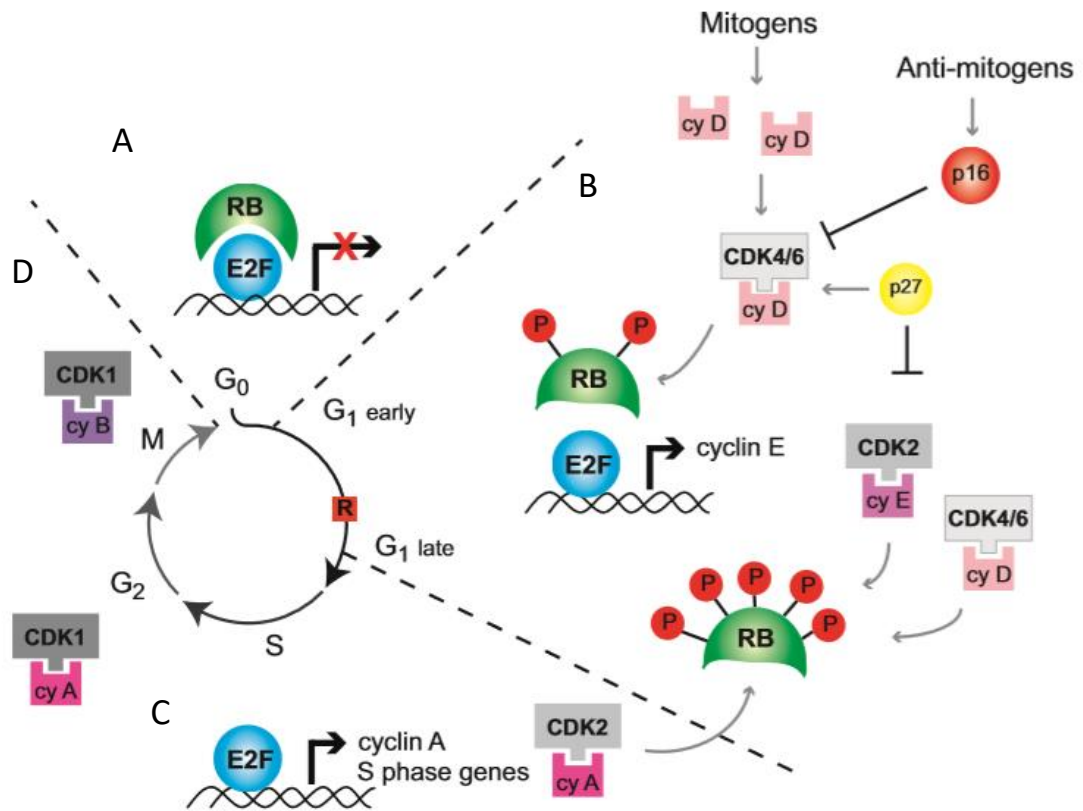
Figure 2. The main phases of the cell cycle.

In a quiescent cell (G0), RB inactivates E2F-mediated transcription (A). Upon receiving a mitogenic signal, the cell synthesizes cyclin D. Cyclin D-CDK4/6 complexes phosphorylate RB which leads to E2F release and transactivation of cyclin E expression. Cyclin E-CDK2 complexes phosphorylate RB on additional sites leading to its inactivation (B) and transcription of S phase genes (C). R symbolizes the restriction point. As modified from Lehn 2013.

2. Senescence

Senescence is defined as an irreversible cell cycle arrest, which can be induced when a certain finite number of divisions has been reached (replicative senescence or when cells are exposed to pro-tumorigenic stresses (He et al. 2017). Although they remain metabolically, and synthetically active, senescent cells are not able to divide even when they are stimulated by mitogens. Senescence supports a variety of functions,

including tumour suppression, wound healing, embryonic development (Storer et al. 2013, Demaria et al. 2014, Chuprin et al. 2013, Li et al. 2012, Muñoz-Espín et al. 2013), and tissue regeneration (Storer et al. 2013). In this chapter, we will discuss the factors that induce senescence, morphology and biomarkers of senescent cells, and signaling pathways initiating and maintaining senescence.

a. <u>Causes</u>

i.<u>Telomere shortening and DNA damage response</u>

Telomeres are tandem TTAGGG repeats found at the ends of chromosomes, associated with several telomere-binding proteins. Telomeres length decreases progressively as cells divide. This loss of telomeres sequences prevents endless proliferation by mediating cellular senescence. Cells recognize telomeres loss as a DNA damage, then mediate DNA damage response DDR by inducing DNA damage kinases ATM. This kinase phosphorylates and activates p53. In turn, phosphorylated p53 protein induces the expression of p21 causing G1 phase arrest (Herbig et al. 2004).

ii. <u>CDKN2A locus derepression</u>

CDKN2A locus encodes two essential tumour suppressors, p16 and ARF which are also key regulators of cellular senescence. It was demonstrated that CDKN2A locus expression was very low in young rodent tissues and increased with ageing (Krishnamurthy et al. 2004). Repression of this locus is mediated by EZH2-containing Polycomb-Repressive Complex 2 (PRC2) complex. The expression of EZH2, a histone methyltransferase which primarily trimethylates histone H3 on lysine 27 (i.e. H3K27me3), was found to be decreased in a stressed and senescing populations of cells.

This was accompanied by a decreased level of associated H3K27me3 and activated transcription of p16 and ARF (Bracken et al. 2007).

iii. <u>Stress-induced senescence</u>

Different types of stresses such as chemotherapeutic drugs, Ultra-Violet radiation (UV), hydrogen peroxide, ionizing radiation, and DNA damage increase the levels of reactive oxygen species (ROS) and induce senescence. For example, treatment of normal human epidermal keratinocytes with hydrogen peroxide resulted in an increase in ROS level which promoted cellular senescence through epigenetic regulation of p16INK4a (Sasaki et al. 2014). In addition, Fitzgerald et al. reported that DNA-damaging ROS induced by ionizing radiation led to the activation of p53, transcription of p21 and caused senescence in head and neck squamous cell carcinoma (Fitzgerald et al. 2015).

iv. <u>Oncogene induced senescence</u>

It is considered as a mechanism of tumor suppression that restricts the progression of benign tumors. Expression of oncogenic *ras* in primary human and rodent cells resulted in a permanent G1 arrest. This was accompanied by accumulation of p53 and p16 (Serrano et al. 1997). In addition, Baek et al. used a transgenic mouse model for tumor initiation in which the oncogenic KRas V12 allele expression was induced in the lung. The authors found that the oncogenic Kras induced senescence in a p53-dependent manner in the premalignant lung tumors (Baek et al. 2013).

b. Characteristics of senescent cells

i. Morphology

In vitro, senescent cells become large, flat, vacuolized and, multinucleated. They also exhibit an increase in the granularity and in the size of their nuclei (Kuilman et al. 2015, Singh et al. 2010).

ii. Cell cycle arrest

Cell cycle of senescent cells must be arrested. Flow cytometry analysis displays decreased number of cells in S phase (cell uptake of BrdU) and increased number of cells in G1 or sometimes also G2/M phase, but no change in the number of cells in sub-G1 phase (dead cells) (Fang et al. 1999).

iii. Senescence Associated β-Galactosidase

Senescent cells are characterized by having a specific β-galactosidase enzymatic activity detected at pH 6.0, different from the normally observed at pH 4.0 within lysosomes. This enzyme has been called senescence-associated β-galactosidase (SA-β-Gal). β-galactosidase is a hydrolase that cleaves β-D-galactose residues in β-D-galactosides. It is thought that this enzymatic activity is a consequence of the increase in the mass and activity of lysosome in senescent cells. However, the relationship between this enzyme activity and the establishment and maintenance of senescence is still unknown (Yang et al. 2005).

iv. Senescence Associated Secretory Phenotype

Coppe et al. showed that human cells induced to senesce by genotoxic stress secrete many factors associated with inflammation and malignancy (Coppe et al. 2008). Senescent cells are able to implement a complex pro-inflammatory response known as

senescence-associated secretory phenotype (SASP). The SASP is induced by the transcription factors NF-κB and CCAAT/ enhancer binding protein-β (CEBPβ). This complex contains pro-inflammatory cytokines (interleukin-6 (IL-6) and IL-8), chemokines (monocyte chemoattractant proteins (MCPs) and macrophage inflammatory proteins (MIPs)), growth factors (transforming growth factor-β (TGFβ) and granulocyte–macrophage colony-stimulating factor (GM-CSF)) and proteases (Kuilman et al. 2008, Hernando et al. 2007, Ender et al. 2012). The production of these molecules by senescent cells causes inflammation and thus mediates the clearance of senescent cells by phagocytosis in vivo (Yun et al. 2015). In addition, SASP components, most notably TGFβ, induce senescence in neighboring cells in a paracrine manner, through a mechanism that produces ROS and DNA damage (Hubackova et al. 2012).

v. Senescence Associated Heterochromatic Foci

Another characteristic of senescent cells is senescence-associated heterochromatic foci (SAHF). Narita et al. described SAHF as bright and punctate DNA-stained dense foci that can be readily distinguished from chromatin in normal cells (Narita et al. 2003). Zhang at al. reported that a single SAHF focus resulted from the condensation of a single chromosome (Zhang et al. 2007). These foci contain trimethylation at Lys9 of histone 3 (H3K9me3), heterochromatin protein 1 homologue-γ (HP1γ) and macroH2A which are hallmarks of heterochromatin (Zhang et al. 2007). Aird et al. also showed that SAHF heterochromatin domains contains certain genes involved in cell proliferation, including E2F target genes such as cyclin A, which is required for the progression through S-phase of the cell cycle (Aird et al. 2013). To

note, not all senescent cells display SAHF, and therefore SAHF absence does not mean that cells are not senescent (Mercurio et al. 2011).

vi. Proteins markers

Senescent cells are characterized by a high expression of p16, ARF, p53, p21, p15, p27, Dec1 (also known as TNFRSF10C), Decoy receptor 2 (DCR2; also known as TNFRSF10D) and by a downregulation of Lamin B1 (Freund et al. 2012, Sagiv et al. 2013, He et al. 2017).

Dec1 belongs to the bHLH (a basic helix-loop-helix) family of transcription factors. Qian et al. found that Dec1 expression was induced by p53 which binds to the p53-responsive element found in Dec1 promoter. The authors also found that overexpression of Dec1 alone initiated G1 arrest and senescence, however, Dec1 knockdown attenuated DNA damage-induced premature senescence (Qian et al. 2008). Dec1 targets are epithelium-specific ETS (Epithelium-specific Transcription Factor Subfamily) gene-2 (*ELF5/ESE2*) and -3 (*EHF/ESE3*). ELF5 and EHF are members of ETS family of transcription factors and play a role in the regulation of cellular proliferation, differentiation, and tumorigenesis (Zhou et al. 1998, Ohtani et al. 2001). However, the mechanism by which Dec1, ELF5 *and* EHF are involved in senescence is still unknown.

DCR2 is a member of TNF receptor superfamily. DCR2 was proposed as a biomarker of oncogene-induced senescence. Sagiv. et al reported that upregulation of DCR2 in senescent cells was responsible for their preferential killing through the granule exocytosis pathway and not through the activation of death receptors. DCR2 receptors overexpressed in senescent cells bind to the death receptor ligands. By this

way, they prevent downstream signaling through competitive inhibition of the death-receptor pathway (Sagiv et al. 2013).

Lamins constitute an integral structural component of the nuclear lamina which contributes to the size, shape, and mechanical stability of the nucleus (Freund et al. 2012). Freund et al. showed that lamin B1 was lost from primary human and murine cell strains when they are induced to senesce by DNA damage, replicative exhaustion, or oncogene expression. The authors also showed that the decline of lamin B1, but not lamin B2, lamin A, or lamin C, was associated with senescence. They also reported that Lamin B1 loss was dependent on the activation of either p53 or pRB tumor suppressor pathway (Hallstrom et al. 2008).

vii. Signaling pathway involved in senescence

Two main signaling pathways initiate and maintain this growth arrest: p53–p21–retinoblastoma protein (RB) and p16INK4A–RB. DNA double-strand breaks, or uncapped telomeres activate DDR. The kinases ATM and ATR phosphorylate and stabilize p53. In addition, p14ARF- or p19ARF inhibit (human or mouse, respectively) the ubiquitin ligase MDM2 (Utz et al. 2003, Wong et al. 2009). In turn, p53 transcription factor activity increases expression of the p21, which arrests the cell cycle. Permanent arrest is mediated by p16INK4A, which is transcriptionally upregulated by BMI1, and ETS1 and ETS2 under the control of p38 MAPK and extracellular signal-regulated kinase (ERK) signaling, respectively (Takahashi et al. 2006). p16INK4A inhibits CDK4 and CDK6 activity, which leads to RB hypophosphorylation, blockade of S phase entry, and cell cycle arrest. Furthermore, p16INK4A cooperates with mitogenic signalling to trigger a ROS–protein kinase C δ-type (PKCδ)-positive

31

feedback loop that stably inhibits cytokinesis (Johmura et al. 2014). Accordingly, senescent cells in vitro can display polyploidy, being left with a 4n genome following failed cytokinesis (Pampalona et al. 2012).

viii. Unified model of senescence

During tissue remodeling process senescent cells recruite immune cells including T helper lymphocytes and macrophages through the SASP. These immune cells eliminate senescent cells, and progenitor cells regenerate the damaged tissue. This model can be applied, with some modifications, in developmental senescence. This latter allows the elimination of transitory embryonic structures (such as the mesonephric tubules and the interdigital webs) and that of one cell population in favor of another (as in the endolymphatic sac of the inner ear) (Muñoz-Espín et al. 2014). In the case of pre-malignant tumors, oncogenically stressed cells become senescent and may also trigger clearance and tumor elimination (Ender et al. 2012).
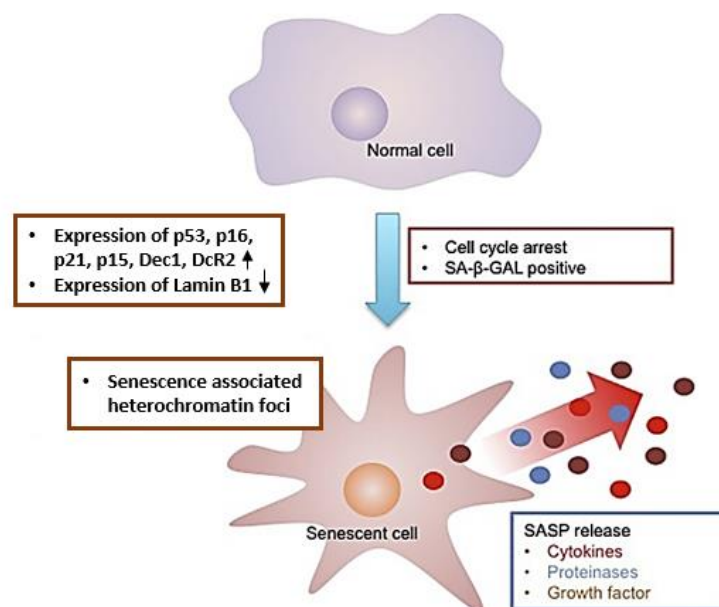
Figure 3. Features of senescent cells.

The characteristics of in vitro senescent cells are large cell size, cell cycle arrest, regulation of specific proteins markers expression, enzymatic activity of senescence-associated β-galactosidase (SA-β-GAL), expression of an SASP factor that is a secretome consisting of a several proinflammatory molecules and growth factors, and formation of senescence associated heterochromatin foci. As modified from Kang et al. 2018.

D. Cell culture models

1. 2D and 3D cell cultures

2D cultures have been very useful models to understand cell biology, mechanisms of diseases, signaling pathways, and genetic interactions of mutated oncogenes and tumor suppressor genes. However, they do not form a relevant physiological model because cells cultured on rigid substrates have flattened morphologies, are different from those observed in vivo, and do not behave as they would in the body. The development and maintenance of any normal tissue in the body depends on cellular interactions in a microenvironment composed of various growth factors, hormones, and adhesion molecules as well as a complex extracellular molecular matrix. Therefore, it is necessary to culture cells under conditions that consider this microenvironment. As extracellular matrix components have become recently available for this purpose, it is now possible to study cells growing in a more relevant structural architecture (3D structure) and recapitulating cells behavior better than cellular monolayers.

Many human breast cancer cell lines can be cultured in 3D; however, the recapitulation of phenotypically normal acinar phenotypes is more challenging. A few non-neoplastic breast epithelial cell lines have been used in 3D cultures including HMT3522 S1 cells.

HMT3522 S1 cells were derived from a human breast epithelial sample from a 48-year old woman (Briand et al. 1987). S1 cells cultured in 3D differentiate into basoapically polarized acinus-like structures that resemble the acini in the resting mammary gland (Plachot et al. 2009). It was demonstrated that 3D culture allows S1 cells to self-organize transcriptionally (Schmidhauser et al. 1992, Xu et al. 2007), architecturally (Petersen et al. 1992) and at the level of nuclear organization (Lelievre et al. 1998) to express differentiated functions lost in cells grown on plastic. Culture of S1 cells of passage 118 in medium without EGF for 2–3 months led to the establishment of S2 subline. S2 cells kept for another 120 passages in culture, were inoculated subcutaneously in nude mice that consequently developed a tumor. Cells taken from this tumor, cultured in vitro, then inoculated again to nude mice induced tumor development. A T4-2 subline established from this tumor was highly tumorigenic (Rizki et al. 2008). T4-2 cells were found to have a triple-negative phenotype (i.e., no expression of ER, PR, and HER2). They develop structures reminiscent of invasive breast tumors when cultured in 3D (Weaver et al. 1997, Rizki et al. 2009).

It was demonstrated that 3D culture plays a role in the control of gene expression in normal and tumor breast cells. Genome-wide expression analyses using cDNA microarrays, of S1 and T4-2 cells, grown in 2D and 3D showed that 166 transcripts of 8000 genes exhibited statistically significant differences between S1 and T4–2 when cells were cultured in 2D, while 445 transcripts showed altered expression when the cells were cultured in 3D. In addition, HMT3522 cells cultured in 3D showed that Cocksackie adenovirus receptor (CAR) mRNA and protein expression was higher in tumorigenic T4-2 cells than in the normal S1 cells, although CAR expression levels

were similar when the two cell types were grown in 2D (Bissell et al. 2002). On the

other hand, only in 3D cultures could the deregulated pathways in T4-2 cells be clearly

elucidated and could the malignant phenotype be reverted. In T4-2 cells, where the

levels of β1-integrin and epidermal growth factor receptor (EGFR) are significantly

elevated with respect to S1 cells, treatment of tumor cells with inhibitory β1-integrin

antibody attenuated the levels of β1-integrin and EGFR and led to a morphological and

functional reversion of T4-2 to a normal phenotype. In addition, Inhibition of EGFR led

to the phenotypic reversion as well as downregulation of β1-integrin. This cross-

modulation of β1-integrin and EGFR could only be observed when cells were cultured

in 3D and received signals from the extracellular matrix (Wang et al. 1998).
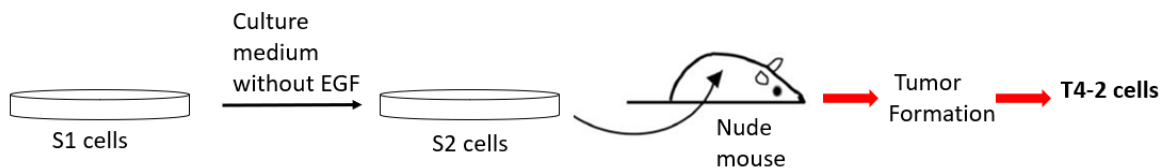
Figure 4. HMT3552 progression series.

Malignant transformation of the S1 cells (normal) and establishment of S2 subline
(malignant) were achieved in vitro by growing S1 cells independently of EGF. S2 cells
were found to produce tumors in nude mice. T4-2 subline (malignant invasive) was
obtained from a tumor after two rounds of in vitro–in vivo mouse passage.

2. LOC (Lab-on-Chip)

3D culture model is an indispensable method to study normal human mammary

functions as well as breast cancer. However, it still has some limitations. For example,

in 3D cultures acini-like structures are isolated, whereas in the mammary glands acini

35

are connected to ducts. Breast cancer is initiated in these ducts and especially in the

terminal mammary ducts. Therefore, this 3D model does not perfectly mimic the

microenvironment where breast tumors develop. Vidia et al. created a Lab-on -Chip

model, a micro-manipulated complex 3D in vitro model, in which breast cells grew on

semicircular acrylic support mimicking portions of mammary ducts. They showed that

T4-2 tumor nodules, cultured with S1 cells, developed well in these hemichannels

mimicking the development of the breast tumor within the epithelium that delineates the

ducts. They also showed cancer cells contacting the ECM-coated acrylic substratum

instead of the non-neoplastic epithelium, which mimic cancer cells infiltration in the

epithelium usually observed in sections of biopsies of cancer tissue (Vidi et al. 2013).

On the other hand, 3D culture model could not be used to study cell migration

mechanisms as well as the invasive potential of cancer cells. Thus, researchers designed

different LOC in order to mimic the functional parameters of migration across to

different tissues. Jeon et al. created an organ-specific human 3D microfluidic model

which can be used to investigate the extravasation mechanism of metastatic tumor cells.

They used their model to study human breast cancer cell extravasation into different

microenvironments. They used the katushka-expressing bone seeking clone (BOKL) of

the MDA-MB 231 metastatic breast cancer cells and showed these cells were able to

adhere to endothelial cells aligned with microvessel axes. These cells were also able to

metastasize through human microvascular networks to matrices engineered to mimic

three different microenvironments: bone, muscle, and acellular collagen matrix. They

found that the extravasation rates of the cancer cells in the bone-mimicking

microenvironment were significantly higher compared with the two other

microenvironments (Jeon et al. 2015). In addition, Chen et al. created an in vitro model of the human microcirculation that allows to visualize and quantitate tumor cell extravasation dynamics. Using this model, they were able to show the transmigration of MDA-MB 231 through the microvascular network. They scored extravasated cells, evaluated the extravasation efficiencies of a panel of tumor cell lines as well as the effects of various treatments on extravasation efficiencies of MDA-MB 231 cells. In addition, they visualized the temporal and spatial organization of intra or extracellular proteins of endothelial and tumor cells on a single cell level, in addition to the position of transmigrating tumor cells relative to the vascular basement membrane (Chen et al. 2017). Apart of studying the biological mechanisms, LOC might also help to design and test anticancer therapies with increased accuracy.

E. Pharmacological CDK inhibitors

Pharmacological CDK inhibitors are small molecules that have shown a potent anticancer activity in preclinical studies. Besides blocking cell cycle progression, they can also promote apoptosis. Moreover, they were found to exert antiangiogenetic effects and to potentiate chemotherapy efficacy.

The first generation CDK inhibitors developed were relatively nonspecific, an example of which is flavopiridol. Flavopiridol, a flavonoid alkaloid, was the first CDK antagonist to enter clinical trials. It was shown to inhibit CDK 1, 2, 4, 6 (Sedlacek et al. 1996). However, clinical trials showed that flavopiridol had high toxicity associated to tumour lysis syndrome, a potentially fatal complication that results in the breakdown of large numbers of cells (Blum et al. 2011). Efforts have been focused toward minimizing

this syndrome by finding safe dosage ranges of flavopiridol in addition to identifying other CDK inhibitors with higher efficacity, and fewer off-target effects.

Dinaciclib, a new second generation CDK inhibitor and an ATP competitive molecule, was found to have superior therapeutic index and better side effects profile than those of flavopiridol. Dinaciclib, compared to flavopiridol, appeared to be an equally potent inhibitor of CDK1 and CDK9, but a much stronger inhibitor of CDK2 and CDK5, and more selective in an in vitro kinase screen.

Dinaciclib antiproliferative activity was tested on different tumor cell lines, including breast cancer, and was found to arrest cell cycle in all of them. Moreover, apoptosis following a single exposure to the drug was detected in around 85% of the cell lines tested and in 6 out of 7 breast cancer cell lines (Parry et al. 2010). Dinaciclib antiproliferative activity was also assessed in vivo. Dinaciclib was found to inhibit tumor growth in human ovarian carcinoma, pancreatic cancer, and anaplastic thyroid cancer mice xenograft (Chen et al. 2015, Feldmann et al. 2011, Lin et al. 2017). Dinaciclib effect was evaluated in clinical trials. Phase I clinical trials in patients with advanced solid tumors confirmed safety and tolerability of Dinaciclib (Nemunaitis et al. 2013, Mita et al. 2011). The most common adverse effects were nausea (33% of the patients), anemia (21%), neutropenia (17%) vomiting (17%) and fatigue (15%) (Mita et al. 2011). A Phase I trial evaluated side effects and recommended dose of dinaciclib when administered with epirubicin (anthracycline drug) in patients with triple-negative breast cancer. The combination of dinaciclib and epirubicin was found to cause substantial toxicities and not to be effective for TNBC treatment (Mitri et al. 2015). Additional Phase I clinical trial assessing the potential use of dinaciclib in breast cancer

treatment are ongoing. A Phase I/Ib dose-escalation trial investigates dinaciclib in combination with weekly paclitaxel (antimicrotubule agent.) in patients with advanced solid cancer, including breast cancer, after assessment of MYC oncogene overexpression (NCT01676753). Moreover, another phase I clinical trial explores the side effects and the best dose of veliparib (PARP inhibitor) and dinaciclib given with or without carboplatin (chemotherapy drug) in patients with advanced solid tumors, including breast cancer (NCT01434316).

The second generation of CDK inhibitors include AT7519, a pyrazole 3-carboxyamide compound that acts as an inhibitor of CDK1, CDK2, CDK4, CDK6 and CDK9. AT7519 was found to induce apoptosis in colon cancer, multiple myeloma and neuroblastoma in vitro and in vivo (Squires et al. 2009, Santo et al. 2010, Dolman et al. 2015). It has been evaluated in combination with bortezomib in a phase II clinical trial enrolling patient with previously treated multiple myeloma. The combination was well tolerated, and more than one-third of patients achieved partial response (Raje et al. 2013). R547 is an inhibitor of CDK1, CDK2 and CDK4 with less potency for CDK7. R547 was found to have anti- tumor activity in colon, lung, breast, prostate cancer and melanoma in vitro and in vivo (DePinto et al. 2006). It was tested in a Phase I study in 2007 as an intravenous weekly infusion. Although reported to have manageable side effects, there have not been further clinical trials with this compound (Camidge et al. 2006). SNS-032, an inhibitor of CDK1, 2, 4, 7, and 9, was found to be effective as a single agent and in combination with Cytarabine in primary acute myeloid leukemia samples (Walsby et al. 2011). SNS-032 was shown to have anti-neuroblastoma activity in vitro through CDK7 and CDK9 inhibition-mediated suppression of RNA synthesis

and subsequent depletion of antiapoptotic proteins (Löschmann et al. 2013). SNS-032 completely prevented U87MG cell–mediated capillary formation of HUVECs (Human umbilical vein endothelial *cells*), by preventing the production of VEGF in both cell lines (Ali et al. 2007). Two Phase I clinical studies with SNS-032, one in 2010 in advanced lymphoid malignancies (Tong et al. 2010) and one in 2008 in selected advanced tumours (Heath et al. 2008), were reported, but no further developments are apparent. AZD5438, potent inhibitor of CDK1, CDK2 and CDK9 with less selectivity for CDK5 and CDK6, showed significant antiproliferative activity in human tumor cell lines in vitro and in vivo (Byth et al. 2009). However, the development of AZD5438 was discontinued after it was reported to be intolerable when administered continuously in patients with advanced solid tumors (Boss et al. 2009).

Selective Inhibitors of CDK4/6 have been also developed; they include abemaciclib, ribociclib, and palbociclib. Palbociclib was granted FDA approval as an initial endocrine therapy for metastatic breast cancer for use in combination with letrozole in early 2015 (Beaver et al. 2015). It was also approved in 2016, for use in combination with fulvestrant in patients who had failed prior endocrine therapy (Walker et al. 2016). Soon after, ribociclib was also approved by the FDA for the first-line treatment of postmenopausal women with metastatic HR+/HER2- breast cancer in combination with an aromatase inhibitor. Finally, abemaciclib garnered an FDA approval as monotherapy in women with HR+/HER2- advanced or metastatic breast cancer with disease progression following endocrine therapy and prior chemotherapy in the metastatic setting. Of these selective inhibitors, palbociclib is the most extensively investigated and has been evaluated in vitro and in vivo till recent times.

Palbociclib, an ATP competitive molecule, was found to be a potent inhibitor of cell proliferation, preventing progression of the cell cycle from G1 into the S phase in Rb-positive cells of different tumor types, but showing no activity against Rb-negative tumor cells (Toogood et al. 2005). Palbociclib anti- tumor activity was also evaluated in vivo. It was efficacious against mice bearing the SF-295 glioblastoma xenografts, the Colo-205 human colon carcinoma xenografts, or medulloblastoma patient-derived xenografts (Fry et al. 2004, Sangar et al. 2017). It was tested in a Phase I dose escalation trial in solid tumors, including five patients with breast cancer, one of whom achieved stable disease (Flaherty et al. 2012). Palbociclib plus endocrine therapy showed encouraging results when given to postmenopausal women with hormone receptor positive/HER2-negative breast cancer within Phase II clinical trials (Finn et al. 2012). This combination is currently under evaluation in a number of Phase III clinical trials in the same setting (NCT01740427). A Phase I clinical trial evaluating the potential use of palbociclib in combination with Gedatolisib (PI3K/mTOR Inhibitor) in cancer treatment of patients with advanced squamous cell lung, pancreatic, head & neck and other solid tumors is currently ongoing (NCT03065062). Another phase I / II trial assessing the side effects as well as best doses of palbociclib used in combination with cetuximab (epidermal growth factor receptor inhibitor) in treating patients with squamous cell carcinoma of the head and neck is currently ongoing (NCI-2014-01079).
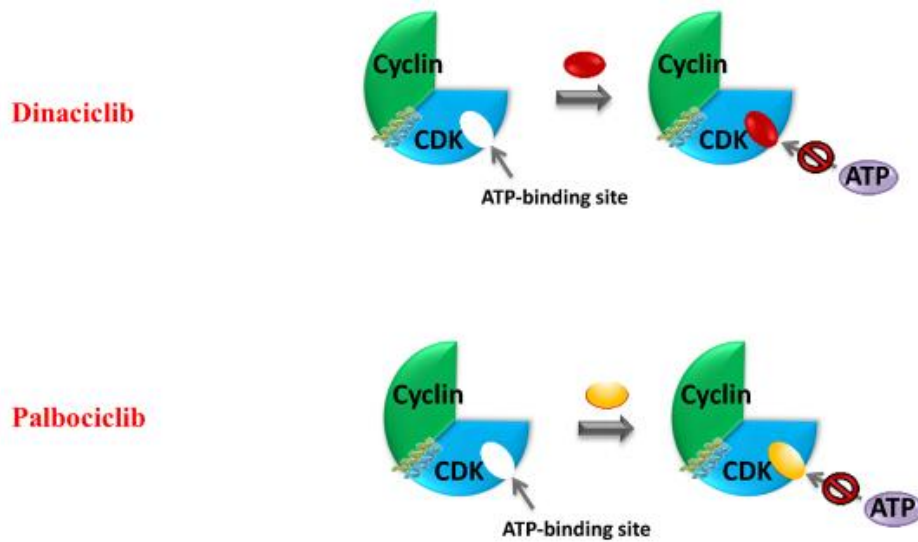
Figure 5. Dinaciclib and Palbociclib mechanism of action.

Dinaciclib and Palbociclib are ATP-competitive inhibitors that bind the ATP pocket and compete with ATP binding. As modified from Peyressatre et al. 2015.

TNBC is an aggressive and invasive subtype of breast cancer. It tends to have the worst prognosis among the major subtypes, due to both limitations in understanding of the underlying biology and the lack of targeted therapeutic agents. Since perturbations in the cell cycle control are described commonly in carcinogenesis, inducing cell cycle arrest and senescence by targeting key regulators of the cell cycle, CDK, may be an attractive therapeutic strategy in TNBC treatment. CDK2 and CDK4 are the major regulators of the cell cycle since they drive the transition from G1 to S phase. In this study, we hypothesize that treatment of TNBC cells with two selective CDK2 and CDK4 inhibitors, Dinaciclib and Palbociclib respectively, will potentially decrease TNBC tumor progression when used in a suitable 3D context.

Aim 1:

Determine the effect of Dinaciclib and Palbociclib drugs added alone, or in combination and tandem on the viability and proliferation of MDA- MB 231 cells in 2D culture.

Aim 2:

Determine the effect of Dinaciclib and Palbociclib drugs (added alone, in combination or in tandem) on the viability and proliferation of premalignant (S1 cells), intermediate (Cx43 KO- S1) and malignant breast cancer cells (T4-2 cells) in 2D culture.

Aim 3:

Determine the effect of Dinaciclib and Palbociclib on the polarity and lumen formation of S1 cells and on the proliferation of S1 and T4-2 cells cultured in 3D and treated during growth or quiescence phase of S1 acini.

CHAPTER III

MATERIALS AND METHODS

A. Cell culture

MDA-MB 231 human mammary adenocarcinoma cell line derived from pleural effusions were grown in humidified incubator (95% air, 5% CO2) at 37°C, in Rose Park Memorial Insitute (RPMI) 1640 media (Sigma) supplemented with 1% penicillin streptomycin and 10% Fetal Bovine Serum (FBS). Cells were seeded at a density of 1.5×10^4 cells/ml and treated with 10 nM Dinaciclib and 1 μM Palbociclib on day 1. Treatment was replenished with every change of media (48 hours).

Non-neoplastic HMT3522 S1 human mammary epithelial cells, between passages 52 and 60, were routinely maintained as a monolayer (2D culture) in a chemically defined serum-free H14 medium consisting of DMEM:F12 medium containing 250 ng/ml insulin, 10 μg/ml transferrin, 2.6 ng/ml sodium selenite, 10^{-10} M estradiol, 1.4 μM hydrocortisone, 5 μg/ml prolactin, and 10 ng/ml EGF (Plachot et al. 2004). Cx 43-KO S1 cells were cultured in selection media consisting of serum-free H14 supplemented with hygromycin- B 150 μg/ml. T4-2 cells were grown on collagen type I (Corning) – coated flasks in serum-free H14 medium without EGF. Cell culture medium was changed every 48 hours during the culture period.

For three- dimensional (3D) cell culture, S1 and T4-2 cells were plated on MatrigelTM (41 μl/cm2, Corning) at a density of 5×10^4 cells/ml and 2.5×10^3 cells/ml respectively, in the presence of culture medium containing 5% MatrigelTM (Plachot et al. 2004). EGF was omitted from the culture medium of S1 cells after day 7 to allow S1 cells to

recapitulate the formation of polarized acini like structures (Plachot et al. 2004; Lelièvre et al. 2005).

Culture media of S1, Cx43 KO-S1 and T4-2 cells in 2D and 3D was supplemented on day 4 with 10 nM Dinaciclib and 1μM Palbociclib. Drugs were replenished with every change of media (every 48 hours).

Some experiments were performed with cells cultured without prolactin due to discontinued production by international suppliers. This shortage of supply lasted for more than one year. This is indicated in figures legends when appropriate.

B. Drug Preparation

Dinaciclib SCH727965 (A8412, Apexbio) is prepared in stock solution of 10mM in DMSO. The stock is diluted 1:1000 in media to obtain an intermediate of 10 μM. 2 μl of this intermediate are added to 2 ml of media in order to obtain a final concentration of 10nM.

As for Palbociclib PD 0332991, HCl (A8316, Apexbio), is prepared in stock solution of 5 mM in water. This stock is then diluted 1:10 in DMSO to obtain an intermediate of 500 uM. 4 μl of this intermediate is added to 2 ml media in order to obtain a final concentration of 1 μM. Equal amount of the DMSO is added to the control.

C. Cell Counting Using Trypan Blue Extraction Assay

MDA MB 231, S1, Cx43 KO-S1, and T4-2 cells were counted from duplicates at different time points. First, media was collected, and cells subsequently trypsinized and collected. After centrifugation, the supernatant was discarded, and the pellet was resuspended in 1 ml media. Cells were than diluted in Trypan Blue (1:1) ratio (V: V) and counted using a hemocytometer.

D. Cell Cycle Analysis

Cells were trypsinized, collected by centrifugation, washed twice with 1x PBS and then fixed in ice-cold 70% ethanol overnight. Cells were then centrifuged, and the pellet was washed with 1x PBS before final re-suspension in 300 µl of 1x PBS containing 1.5 µl RNAase A (Thermo Scientific) and 5.5 µl of 1 mg/ml propidium iodide (life technologies). Cells were kept at 37 °C in the dark for 30 min then analyzed using the flow cytometer machine FACSAria SORP.

E. Senescence Associated β- Galactosidase Assay

Cells were quickly washed with PBS 1x then fixed in 4% paraformaldehyde. They were then incubated in SABG staining solution (1 mg/ml X-gal, 40mM Citric acid/ NaPhos buffer pH 6.0, 5 mM K3CN, 5mM K4CN, 150 mM NaCl, 2mM MgCl2) at 37°C in the dark for 12-24 hours. They were washed with PBS 1x, then counterstained with eosin. 10 random fields were selected for quantification of proportion of positive cells for SABG.

F. Preparation of whole cell protein extracts and Western blot analysis

Cells were lysed with RIPA buffer (25 mM Tris HCL, 150 mM NaCl, 1% NP-40, 1% Sodium Deoxycholate) mixed with 40 µl/ml protease inhibitors. The mixture was sheared by passage through 27- gauge needle, and then centrifuged at 15000 g for 30 min at 4°C. The supernatant was stored at -20 °C. DC Protein Assay (Bio-Rad) was used to quantify proteins using bovine serum albumin (BSA, amresco) as standards. Protein extracts were then mixed with 2x or 4x sample buffer (Bio Rad) with 10 or 20% β- mercapto-ethanol (Sigma) at the time of gel electrophoresis. Equal amounts of proteins were separated and immunoblotted with mouse antibody against p21 (Sc16246,

1:50), rabbit p15 Sc13, 1:500) and mouse Dec1 (Sc101023, 1:500). Equal protein

loading was verified by immunoblotting for rabbit GAPDH (Sc25778,1:1000 and

MAB5476, 1:1000). Protein levels were quantified using ImageJ and normalized to

GAPDH.

G. Immunofluorescence labeling

Cells cultured in 3D were permeabilized with 0.5% peroxide and carbonyl-free Triton

X-100 (Sigma) in cytoskeleton buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES,

pH 6.8, 5 mM MgCl2, 1 mM pefabloc, 10 µg/ml aprotinin, 250 µM NaF) prior to

fixation in 4% formaldehyde. Antibody used was rabbit polyclonal against β- catenin

(Sc7199, 1:100). Secondary antibodies goat ant- rabbit conjugated with fluorescein

(green) (F2765,1:1000). Nuclei were counterstained with 0.5 µg/ml 4',6 diamidino-2-

phenylindole (DAPI, Invitrogen Molecular Probes) and slides were mounted in

ProLong® Gold antifade reagent (Invitrogen Molecular Probes).

- Image processing

Images of immunofluorescence labeling were recorded using laser scanning fluorescent

confocal microscope LSCM. Images were processed using ZEN lite software. A

minimum of one hundred acini were analyzed for each immunostaining.

H. Measurement of acinar diameter and cell devoid spaces between nodules

Photomicrographs of the cells were recorded using OLYMPUS IX71 microscope.

Images were processed using Adobe Photoshop CS2. Acinar diameter and cell devoid

spaces between nodules were measured using imageJ, length and area selection tools.

For quantification of acini diameter and cell devoid spaces, fifty random acini and thirty

random fields were selected respectively.

I. Statistical analysis

Data were presented as means ± SD, except for cell devoid spaces they were presented

as means ± SEM. Statistical comparisons were done using Microsoft Excel.

Comparisons between experimental groups were performed using the Student t test, and

a p value below 0.05 or 0.01 was considered as statistically significant.

* Some experiments were performed with cells cultured without prolactin as indicated

in figures legends.

CHAPTER IV

RESULTS

A. Dinaciclib and Palbociclib decrease MDA-MB 231 cell proliferation in 2D culture.

We examined the effect of Dinaciclib, Palbociclib and combination on the proliferation of MDA-MB 231 cells cultured in 2D, on plastic. The cell count showed that Dinaciclib, Palbociclib and combination decreased the total number of cells by 0.73×10^5, 0.67×10^5, 0.8×10^5 cells respectively compared to the control ($p < 0.01$). The number of dead cells using the trypan blue dye exclusion assay did not exceed 4 % of total cell number in all treatment conditions (Fig 6A). Flow cytometry analysis showed that Dinaciclib and combination increased the percentage of MDA-MB 231 cells in pre-G1, whereas Palbociclib induced cell cycle arrest in G0/G1 phase. Dinaciclib significantly increased the percentage of cells in pre-G1 by 26% and decreased that of cells in S phase by 15% compared to the control ($p < 0.05$). Combination had an effect similar to that of Dinaciclib. It significantly increased the percentage of cells in pre-G1 by 28% and significantly reduced that of cells in S phase by 17% compared to the control ($p < 0.05$). However, Palbociclib significantly increased the percentage of cells in G0/G1 phase by 27% compared to the control. It significantly decreased the percentage of cells in S phase by 20% and that of cells in G2/M by 11% in respect to the control ($p < 0.05$) (Fig 6B). We could not detect positive staining for senescence associated β-galactosidase, indicative of senescence, under all treatment conditions (Data not shown).
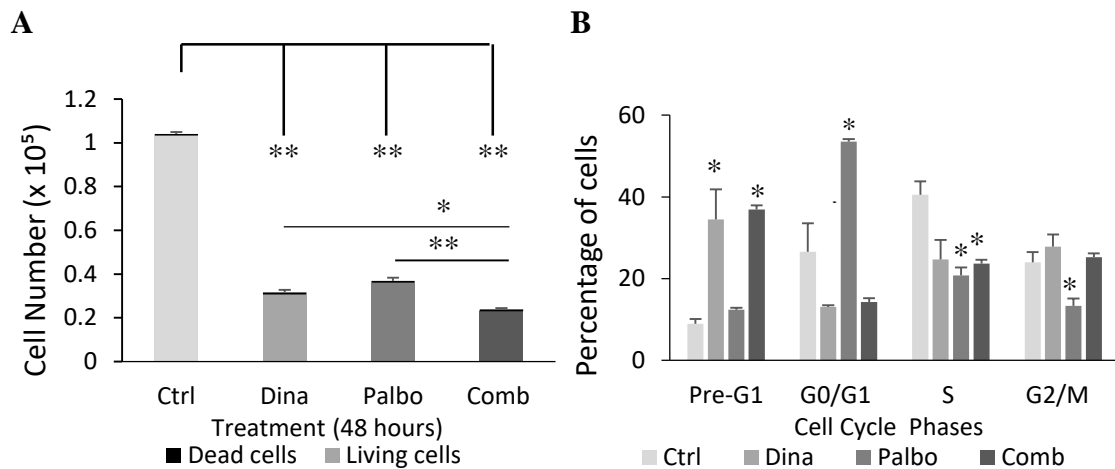
Figure 6. Effect of Dinaciclib and Palbociclib on MDA-MB 231 proliferation in 2D Culture.

MDA-MB 231 cells were untreated (Crtl) or treated with 10 nM of Dinaciclib (Dina), 1 µM of Palbociclib (Palbo) or combination (Comb; treatment of cells with both drugs) on day 1. (A) Cell count was measured using Trypan Blue Exclusion Assay at 48 hours post treatment. Each bar represents duplicate analyses of mean ± SD. Significantly different **($p<0.01$) from control for each treatment using nonpaired t-test. (B) Cell cycle analysis of MDA-MB 231 cells was performed after 48 hours of treatment. Cells were labeled with PI and analyzed by DNA flow cytometry. Each bar represents duplicate analyses of mean ± SD. Significantly different * ($p<0.05$) from control for each treatment using nonpaired t-test. n=2/ treatment (duplicates).

B. Palbociclib markedly elevates SABG activity in MDA-MB 231 cultured in 2D and treated for a longer period.

We prolonged the exposure of MDA- MB 231 cells to Dinaciclib and Palbociclib.

MDA-MB 231 cells were treated with each drug alone, combination or tandem for 7

days instead of 48 hours. The percentage of positive SABG cells (stained blue and

indicated by the arrows) detected in MDA- MB 231 cells treated with Dinaciclib,

Palbociclib, combination or tandem was respectively 5, 43, 21 and 7 % (Fig 7A and B).

Palbociclib significantly increased senescence associated β-galactosidase activity in

MDA-MB 231 treated for 7 days.

A
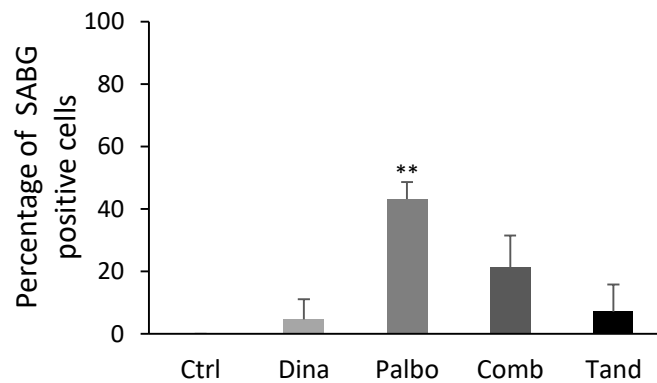
B
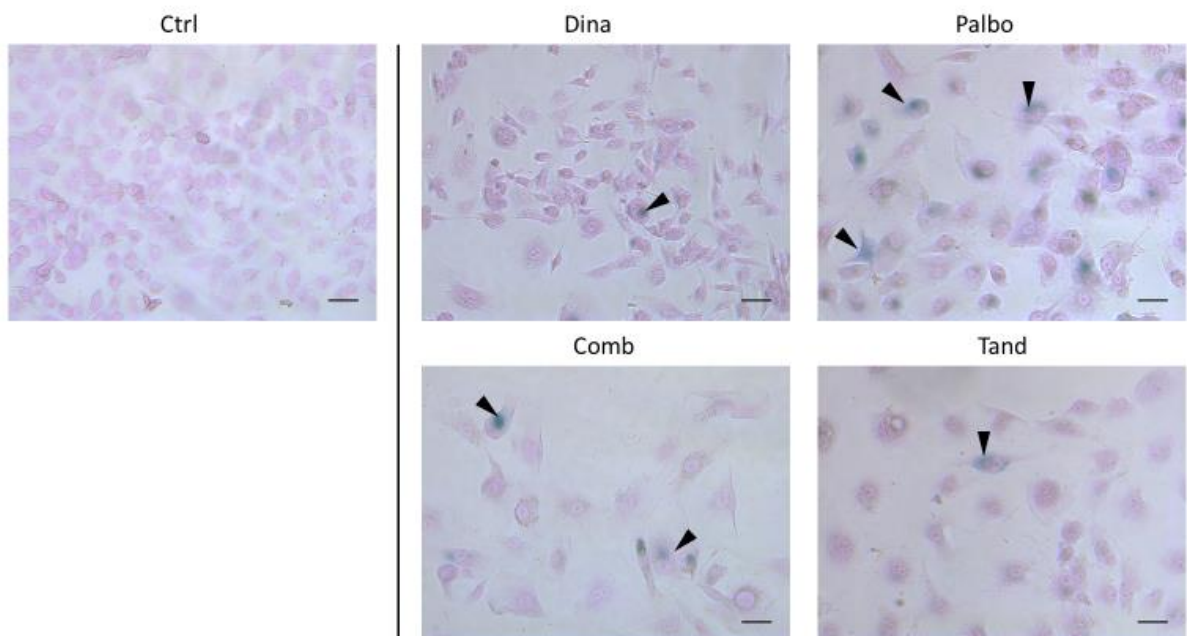
Figure 7. Detection of β -Galactosidase enzymatic activity in MDA- MB 231 cells cultured in 2D.

MDA- MB 231 cells untreated (Ctrl), treated with Dinaciclib (Dina), Palbociclib (Palbo), combination (Comb) or tandem (Tand; treatment of cells with Dinaciclib during the first half of the culture duration followed by treatment with Palbociclib during the second half of the culture duration) on day 1 for 7 days were stained with X-gal (blue) and counterstained with Eosin (pink). (A)Percentage of MDA- MB 231 SABG positive cells. Each bar represents duplicate analyses of mean ± SD. Significantly different ** (p<0.01) from control for each treatment using non paired t-

test. (B) Photomicrographs of MDA- MB 231 cells taken at 20x magnification. Scale bar: 50μm. The arrows point the blue stain. n=2/ treatment (duplicates).

C. Dinaciclib and Palbociclib Reduce S1, Cx 43 KO- S1, and T4-2 cell proliferation in 2D Culture.

Investigating mechanisms of anti- cancer drugs involves studying the effect of the drugs on cancer cells and on normal counterparts in microenvironments that resemble the in vivo conditions (Horning et al. 2008). MDA- MB 231 cell line, a typical aggressive breast cancer cell line, does not have neither a normal counterpart with same genetic lineage nor does it exhibit phenotypic advantages in 3D culture. On the other hand, the HMT-3522 series including normal HMT-3522 S1 and tumorigenic HMT-3522 T4-2 cells permit to model both the normal breast and breast cancer. In 3D culture, S1 cells differentiate into basoapically polarized acinus-like structures whereas T4-2 cells develop structures reminiscent of breast tumors (Plachot et al. 2009, Weaver et al. 1997).

To assess whether Dinaciclib and Palbociclib affect the proliferation of normal S1, tumor initiated Cx 43- KO S1 and tumorigenic T4-2 cells in 2D, cell counts were monitored, using trypan blue exclusion assay, after 3, 7 and 10 days of treatment starting day 4 in 2D cell culture. Cell count showed a decrease in the number of S1, Cx 43- KO S1 (Submitted Bazzoun at al. 2018) and T4-2 cells treated with each drug alone, combination or tandem. This decrease was progressive, noted on day 7, and became more pronounced on day 14. Cell count showed that the drug combination had the most potent effect compared with single treatment in the three cell lines. It is worth noting that Palbociclib had higher effect than Dinaciclib and tandem treatment. The effect of Dinaciclib was similar to that of tandem. The total number of S1 cells treated with

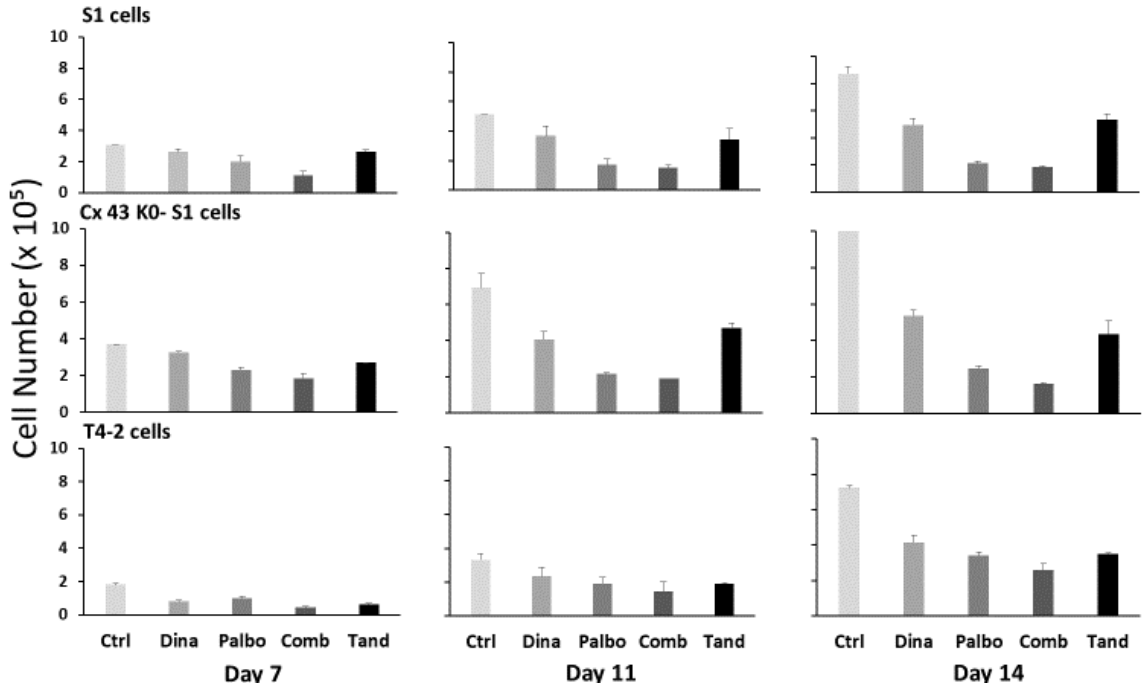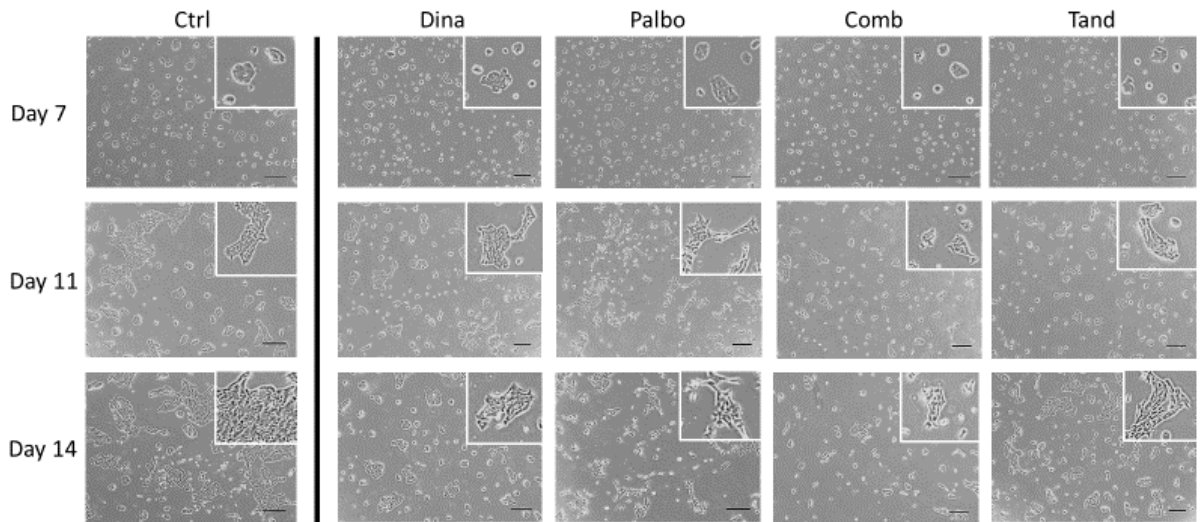Dinaciclib, Palbociclib, combination or tandem was reduced on day 7 by 0.45×10^5, 1.07×10^5, 1.96×10^5 and 0.42×10^5 cells respectively, and by 3.76×10^5, 6.58×10^5, 6.87×10^5, 3.36×10^5 cells on day 14 compared to the control. Cx43- KO S1 cells treated with Dinaciclib, Palbociclib, combination or tandem exhibited on day 7 decreased total cell number by 0.44×10^5, 1.41×10^5, 1.86×10^5 and 1.00×10^5 cells respectively, and by 5.2×10^5, 8.13×10^5, 8.97×10^5, 6.2×10^5 cells on day 14 with respect to the control. T4-2 cells showed a pattern similar to that of S1 and Cx 43- KO S1 cells. The total number of T4-2 cells treated with Dinaciclib, Palbociclib, combination or tandem was attenuated on day 7 by 0.98×10^5, 0.83×10^5, 1.37×10^5, 1.17×10^5 cells respectively, and by 3.1×10^5, 3.85×10^5, 4.65×10^5, 3.75×10^5 on day 14 compared to the control (Fig 8A). Note that the number of S1, Cx 43- KO S1 and T4-2 dead cells did not exceed 4 % of total cell number in all the treatment conditions at the 3 time-points (Data not shown). Photomicrographs of S1 and Cx 43-KO S1 cells reflected results obtained from cell count. They showed that cells treated with combination have lower confluency than that of cells treated with single treatment of either drug or tandem. Cells treated with Palbociclib are less confluent than those treated with Dinaciclib or tandem (Fig 8B and C).

A

B

S1 cells

C

Cx 43 KO- S1 cells
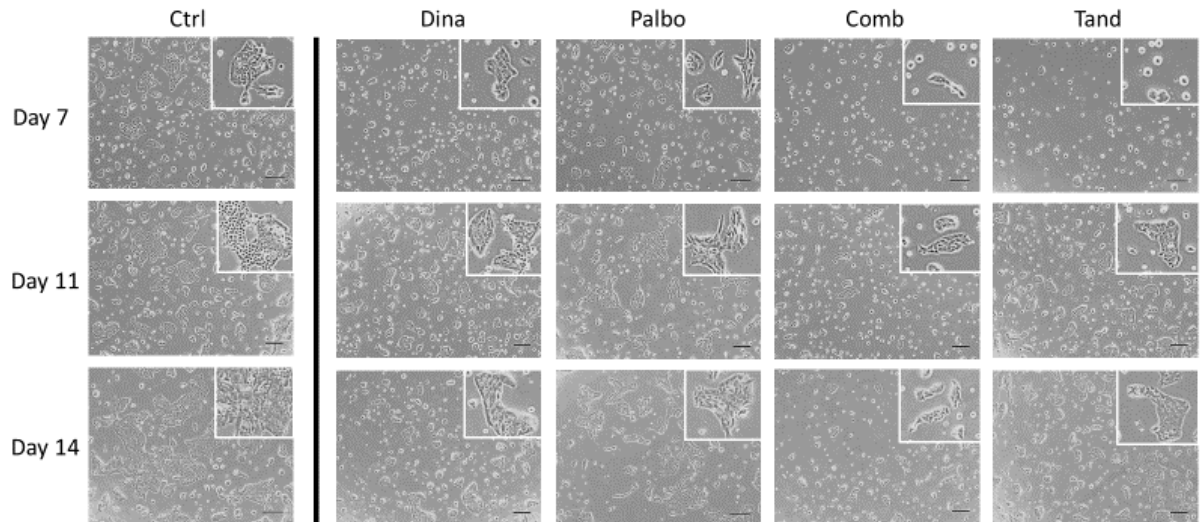
Figure 8. Effect of Dinaciclib and Palbociclib on S1, Cx 43 KO- S1, and T4-2 cells proliferation in 2D Culture.
S1, Cx 43 KO- S1, and T4-2 cells were untreated (Ctrl) or treated with Dinaciclib (Dina), Palbociclib (Palbo), combination (Comb) or tandem (Tand) on day 4. (A) Cell count was measured using Trypan Blue Exclusion Assay at 3 different post-treatment time points. Photomicrographs of S1 (B) and Cx 43 KO- S1 cells (C) were taken at 4x magnification. Scale bar: 300 μm. n=1.

D. Senescence associated β galactosidase assay is not suitable to detect senescence in HMT3522 cells series.

We attempted to determine whether the decrease in cell proliferation was associated

with senescence. S1 and Cx 43 KO- S1 cells treated for 3, 7 and 11 days with a

combination of Dinaciclib and Palbociclib expressed β-Galactosidase (cells stained blue

as indicated by the arrows) as did the control untreated cells (Fig 9A and B). This

suggested that these cells express endogenous β-Galactosidase. Thus, Senescence

Associated β-Galactosidase assay could not be used to detect senescence in our system.

A

S1 cells
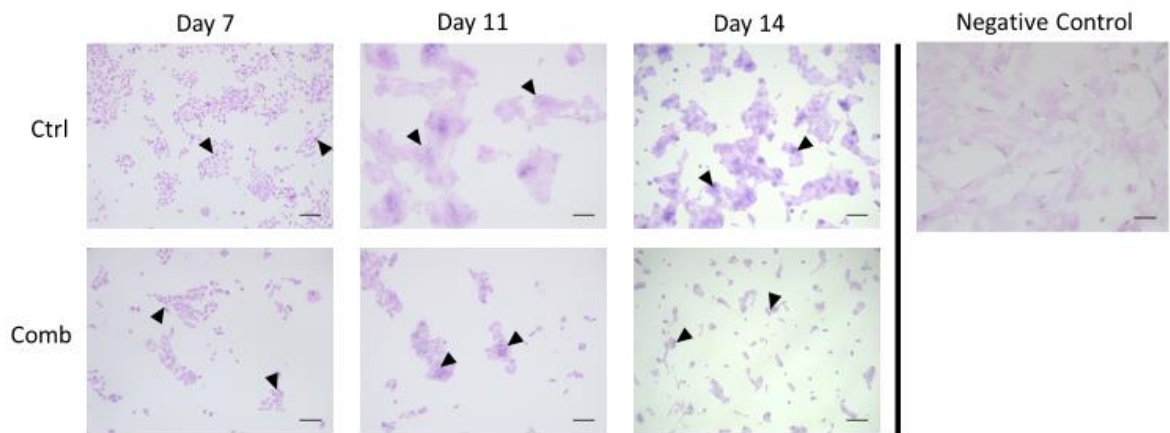
B

Cx 43 KO- S1 cells
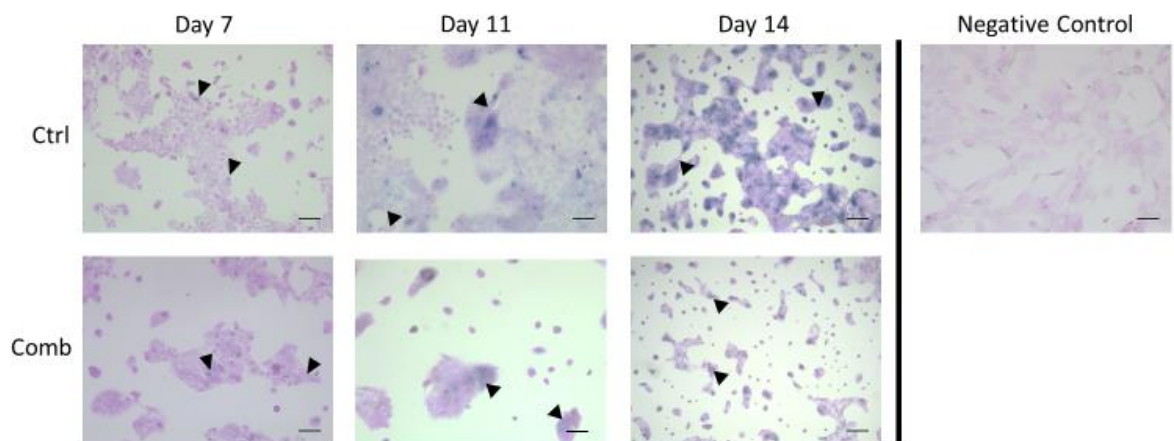
Figure 9. Detection of β -Galactosidase enzymatic activity in S1 and Cx 43 KO-S1 cells cultured in 2D.
S1(A) and Cx 43 KO-S1 (B) cells untreated (Ctrl) or treated with a combination of Dinaciclib and Palbociclib (Comb) on day 4 for the duration of the culture, as well as Rhabdomyosarcoma cells Rh 30 serving as a negative control were stained with X-gal (blue) and counterstained with Eosin (pink). Photomicrographs taken at 10x. Scale bar: 100 μm. The arrows point the blue stain. n=1.

E. Dinaciclib and Palbociclib increase the expression of senescence markers in S1, Cx 43 KO- S1 and T4-2 cells cultured in 2D.

Western blot analysis for the expression of senescence markers was performed on whole protein extracts from S1, Cx 43 KO- S1 and T4-2 cells cultured in 2D and treated on day 4 for 10 days. Treatment regulated the expression of senescence markers in the 3 cell lines.

Preliminary data suggested that S1 cells treated with Palbociclib exhibited an increased expression of p21 compared to those untreated or treated with Dinaciclib, combination or tandem (Fig 10A). On the other hand, the protein level of p21 was upregulated in treated Cx 43 KO- S1 cells with Palbociclib compared to those untreated or treated with Dinaciclib or combination. In addition, the protein level of p15 was upregulated in treated Cx 43 KO- S1 cells with Dinaciclib, Palbociclib, combination and tandem compared to the untreated control. Western blot analysis revealed an up regulation of Dec1 in Cx 43 KO- S1 cells treated with Dinaciclib, Palbociclib and combination with respect to those untreated or treated with tandem (Fig 10B). As for T4-2 cells, the protein level of p21 was increased in cells treated with Palbociclib, combination and tandem compared to those untreated or treated with Dinaciclib. (Fig 10C). GAPDH was used as loading control.

A

S1 cells

B

Cx 43 KO-S1 cells
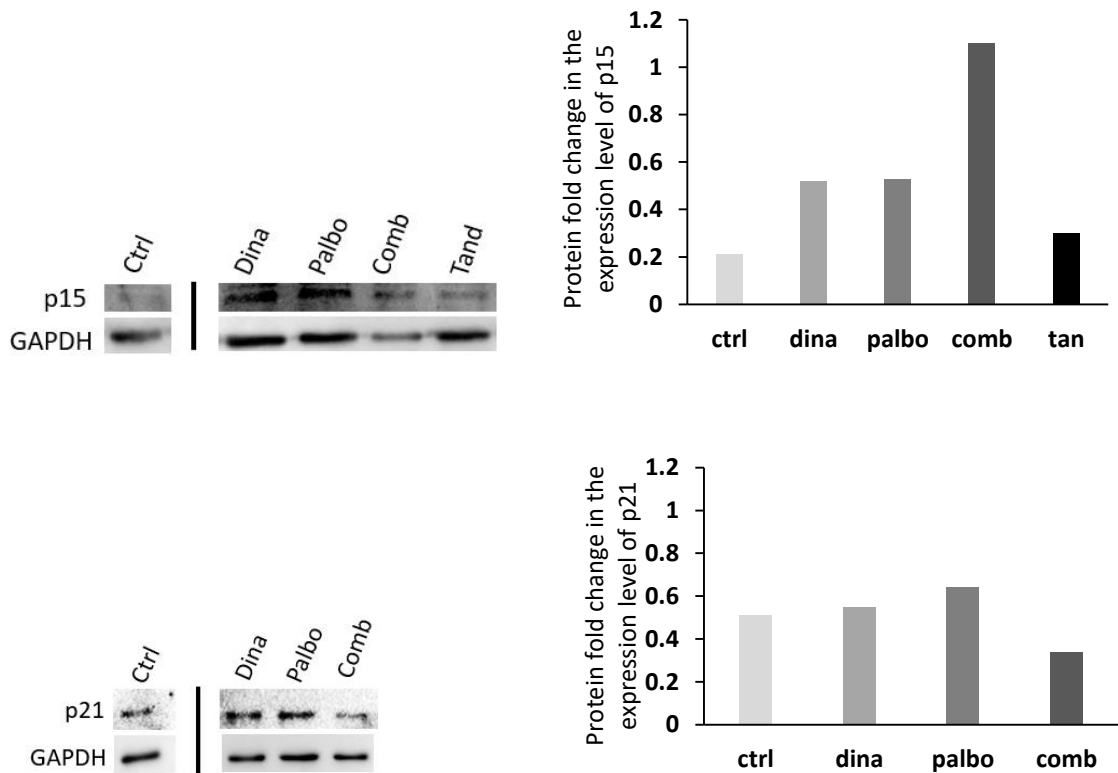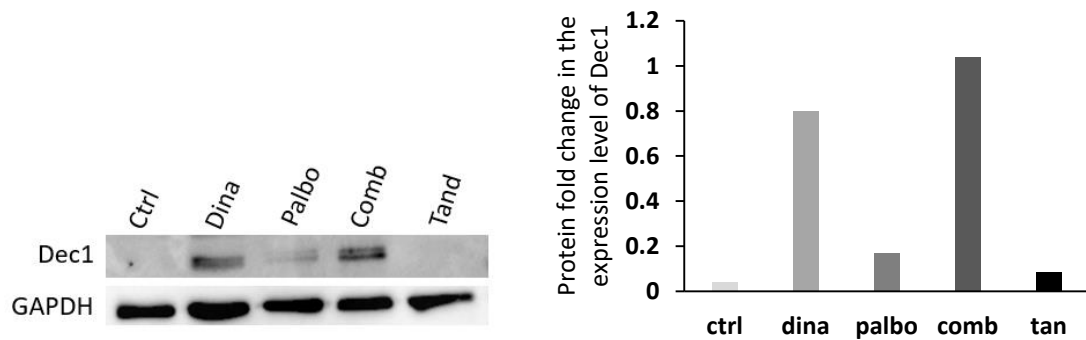
C

T4-2 cells
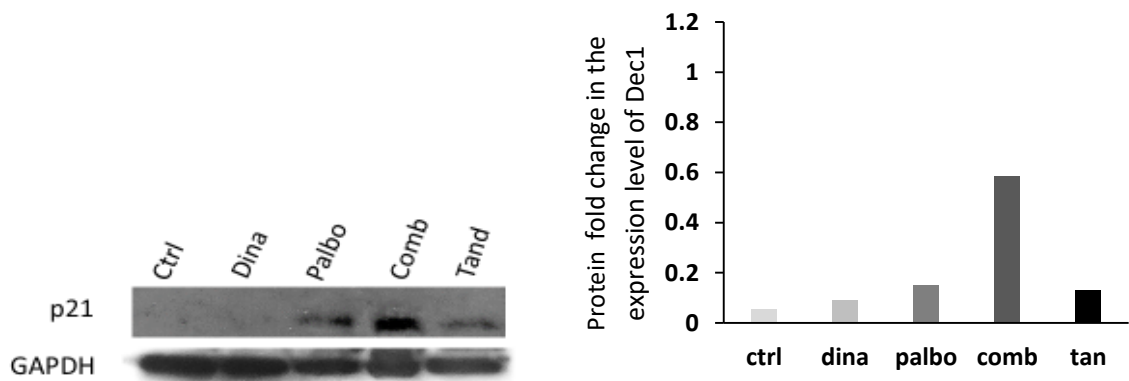
Figure 10. Effect of Dinaciclib and Palbociclib on the expression of senescence markers in S1, Cx 43 KO- S1, and T4-2 cells cultured in 2D.

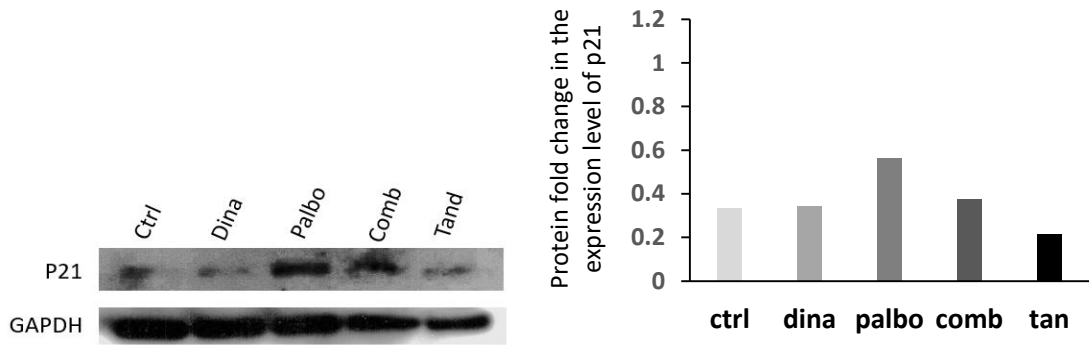S1, Cx 43 KO- S1, and T4-2 cells were untreated (Ctrl) or treated with Dinaciclib (Dina), Palbociclib (Palbo), combination (Comb) or tandem (tand) on day 4 for 10 days. Western blot analysis for the expression of p21 was performed on whole protein extracts from S1 (A) and T4-2 cells (C), and for the expression of p21, p15, Dec1 on whole protein extracts from Cx 43 KO-S1 cells. GAPDH was used as a loading control. Histograms represent the normalized expression levels of the different proteins against GAPDH. n=1. In this experiment, S1 and T4-2 cells were cultured without prolactin due to an international shortage in production from international suppliers that lasted for more than one year. See Material and Methods page 45.

		Dina	Palbo	Comb	Tand
S1 cells	p21		+		
Cx 43 KO- S1 cells	p21		+		
	p15	+	+	++	+
	Dec1	++	+	++	
T4-2 cells	p21		+	++	+

Table 1. Evaluation of Dinaciclib and Palbociclib effect on the expression of senescence markers. ++ and + increase in proteins expression level compared to the control; empty cases no effect.

F. Dinaciclib and Palbociclib do not affect S1 acini like structures and decrease T4-2 outgrowth when cells were treated during S1 quiescence phase.

Preliminary data obtained from 2D culture suggested that Dinaciclib and Palbociclib decreased S1, Cx 43 KO- S1 and T4-2 cell proliferation. However, 2D culture is a poor predictor of in vivo drug response because it does not fully reflect the pathophysiology of real tumors (Horning et al. 2008). Recently, 3D cell culture model has gained increasing interest in drug discovery because cellular responses to drug treatments in 3D cultures have been shown to be similar to those obtained in vivo. 3D culture mimics the tumor progression and development and gives a better reflection of the real tumor setting (Park et al. 2006).

We assessed whether Dinaciclib and Palbociclib affect the growth of S1 acini like structures and T4-2 nodules in 3D, treated during two different phases in culture: growth phase of S1 and T4-2 or quiescence phase of S1 cells. Acini diameter was measured, as an indicator for cell proliferation, at different time points post-treatment. Preliminary data proposed that treatments arrested the growth of S1 acini like structures and T4-2 nodules when treated during growth phase, with a marginal preferential effect on cancer cells. However, treatment of S1 acini like structures and T4-2 nodules during

60

S1 quiescence phase, did not affect the diameter of S1 acini but prevented T4-2 outgrowth from nodules.

Diameters of S1 acini like structures and T4-2 nodules treated as of day 4 in culture during cell's growth phase, were measured after 3, 7 and 11 days of treatment. Diameter of S1 acini treated with Dinaciclib (30 μm) and Palbociclib (29 μm) was smaller than that of the control (32.5 μm) by 7.3 and 9.8% on day 7, by 26% and 32% on day 11 (32 μm,30 μm, compared to the control 43 μm), and by 32 and 36 % on day 14 (30 μm, 29 μm, compared to the control 44 μm) respectively. However, diameter of T4-2 nodules treated with Dinaciclib (115 μm) and Palbociclib (116 μm) was smaller than that of the control (127 μm) by 9 and 8.8% on day 7, by 30% and 39% (134 μm, 117 μm, compared to the control 192 μm) on day 11, and by 48 and 44 % (126 μm, 138 μm, compared to the control 245 μm) on day 14, respectively (Fig 11A and B). Photomicrographs of S1 and T4-2 acini like structures showed that diameter of treated S1 acini like structures and T4-2 nodules was smaller than that of the untreated one (Fig 11C and D).

As for acini treatment during S1 quiescence phase, S1 and T4-2 cells were treated on day 11, after S1 lumen assembly and maturation with Dinaciclib, Palbociclib, combination or tandem till day 30. Acini and nodules diameter was measured after 3, 6, 9, 12, 16 and 19 days of treatment. Acinar diameter measurement showed no change in S1 acini diameter for all treatment conditions. (Fig 12A). On the other hand, although treatment did not affect T4-2 nodules growth, as suggested by T4-2 nodules diameters that keep increasing under all treatment conditions, preliminary data implied that it inhibited T4-2 cellular outgrowth. T4-2 nodules diameter was around 210 μm on day

11, and reached around 270 μm on day 30 under all treatment conditions (Fig 12B).

Decreased outgrowth of treated T4-2 nodules was reflected by the sustained cell- devoid

spaces between nodules. Area of empty spaces between untreated T4-2 nodules was

reduced due to the outgrowth of T4-2 cells from acini. The area of empty spaces

measured on day 30 between untreated or treated nodules with Dinaciclib, Palbociclib,

combination or tandem was respectively 63×10^3, 155×10^3, 94×10^3, 126×10^3, 104×10^3

μm^2. Preliminary data suggested that Dinaciclib and combination had the higher effect

followed by tandem and palbociclib (Fig 12C and D).

A
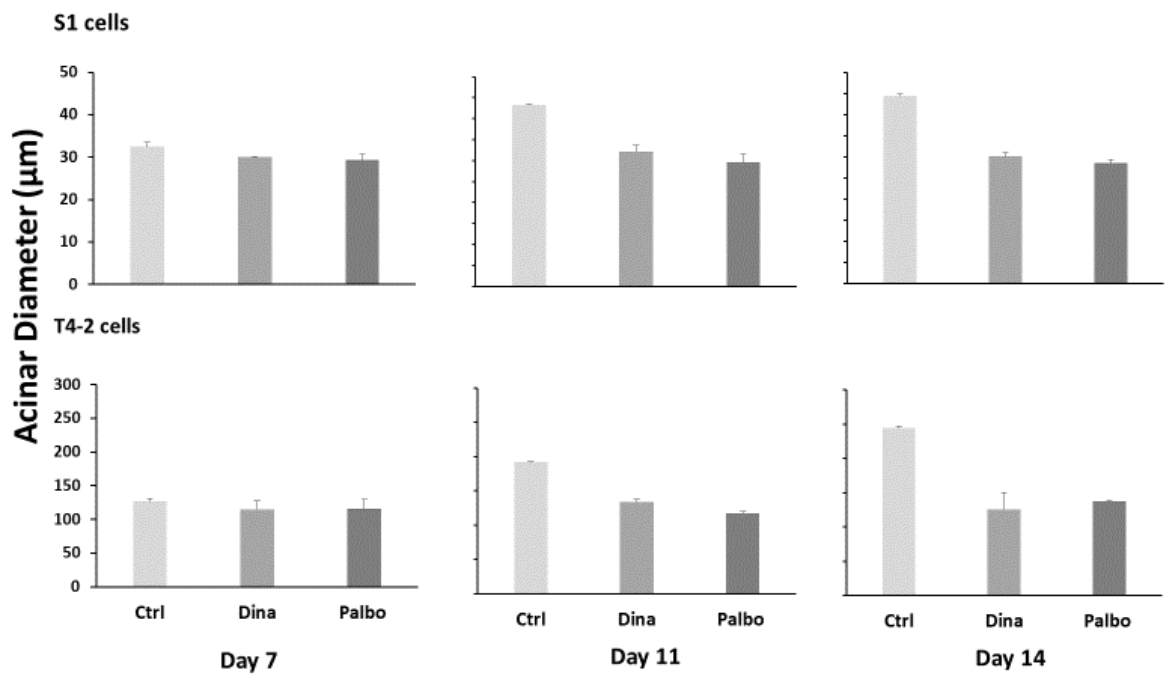
B
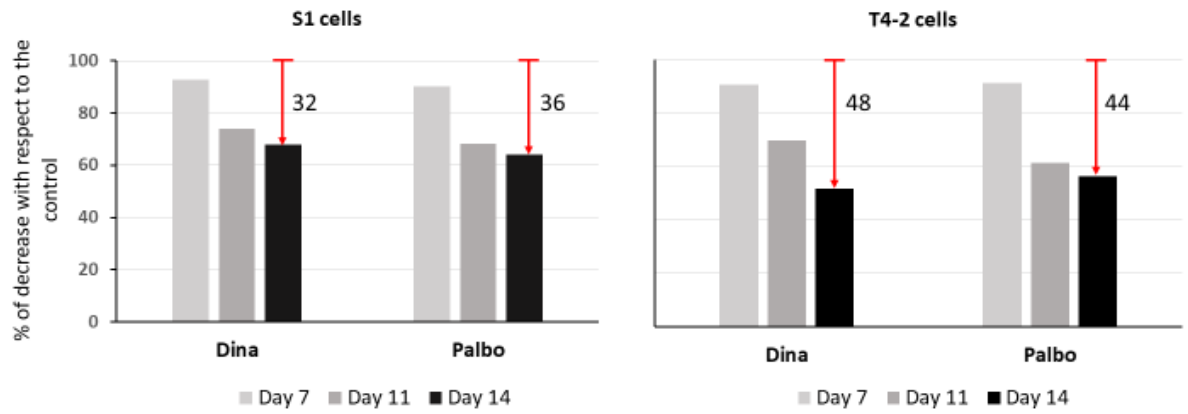
C

S1 cells
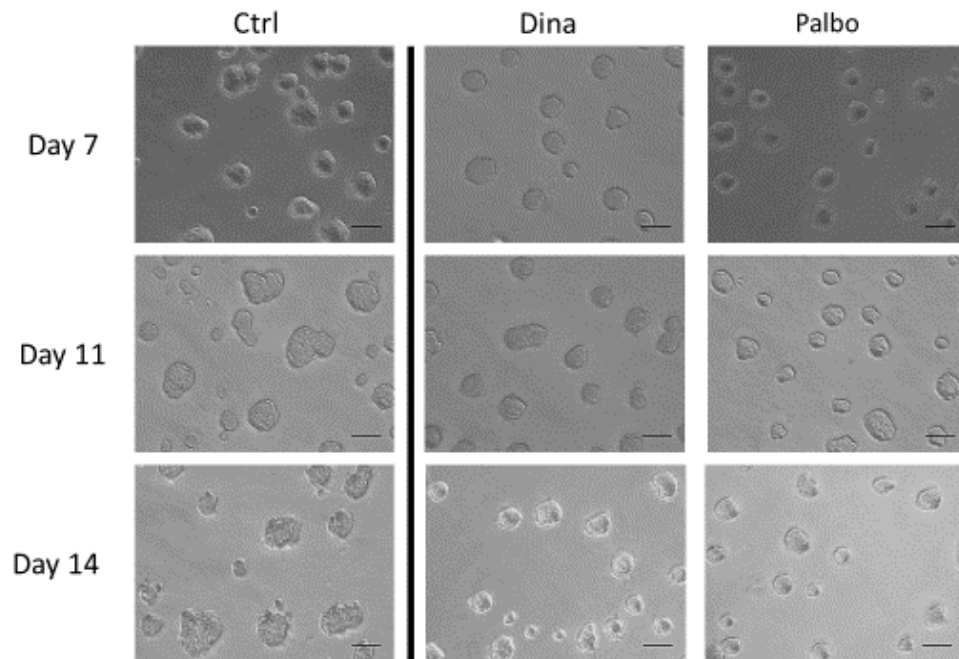
D

T4-2 cells
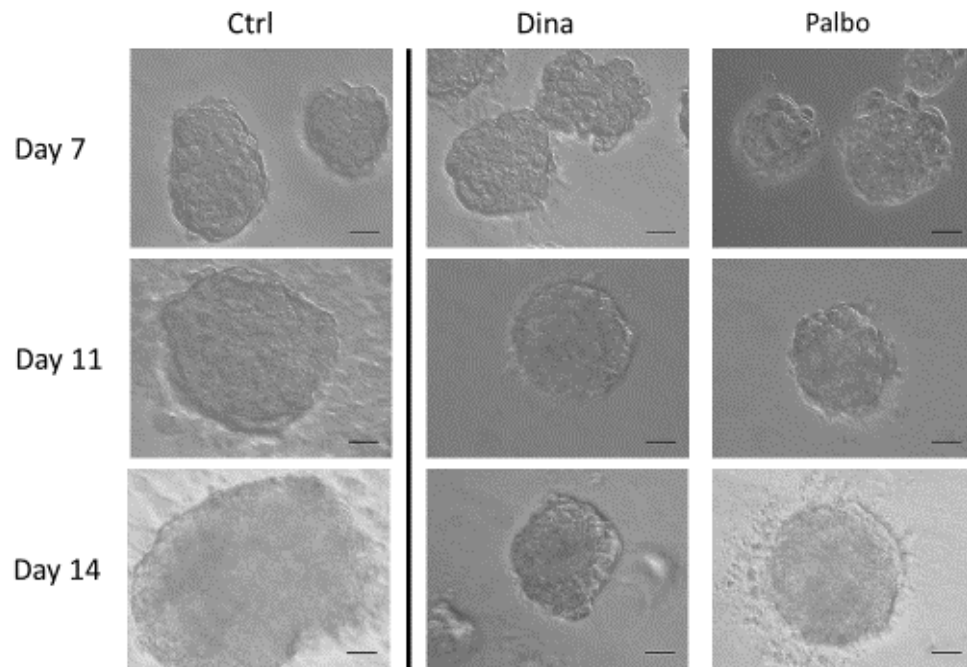
Figure 11. Effect of Dinaciclib and Palbociclib on the growth of S1 and T4-2 acini cultured in 3D and treated during S1 and T4-2 growth phase.
S1 and T4-2 acini were untreated (Ctrl) or treated with Dinaciclib (Dina) or Palbociclib (Palbo) (A) Acini diameter measured at the three-different post treatment time points. (B) Percentage of diameter decrease of treated S1 acini like structures and T4-2 nodules with respect to the control. Photomicrographs of S1 acini like structures (C) and T4-2 nodules (D) taken at 40x magnification. Scale bar: 40 μm. n=1.

A

S1 cells
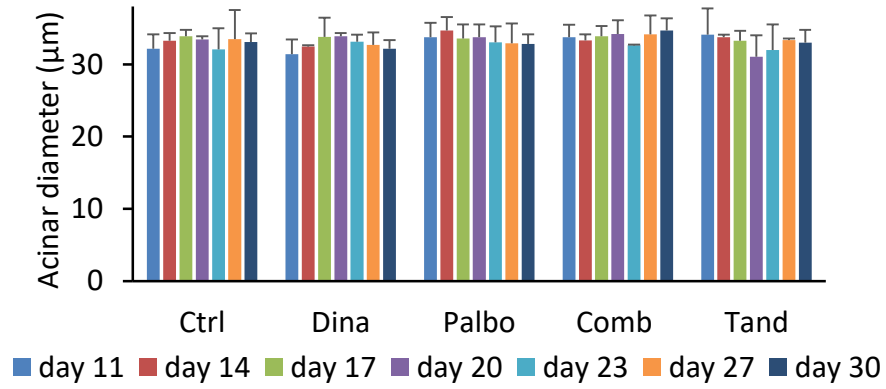
B

T4-2 cells
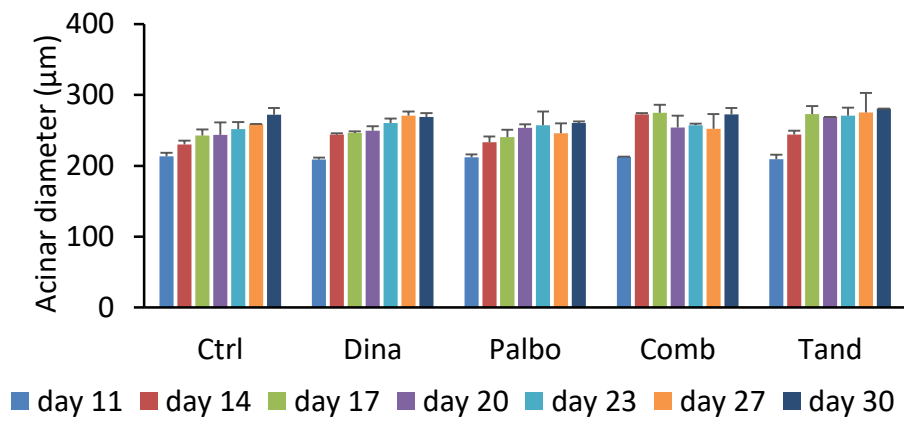
C
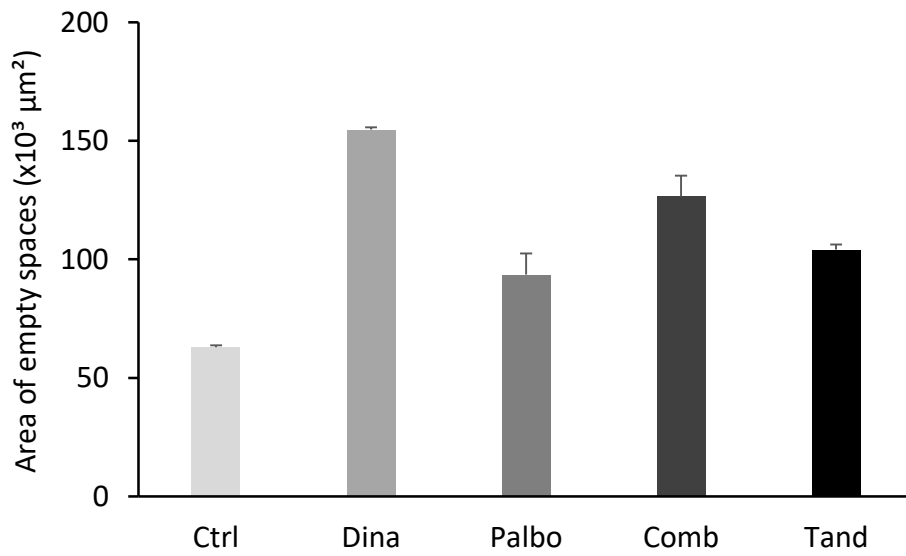
D
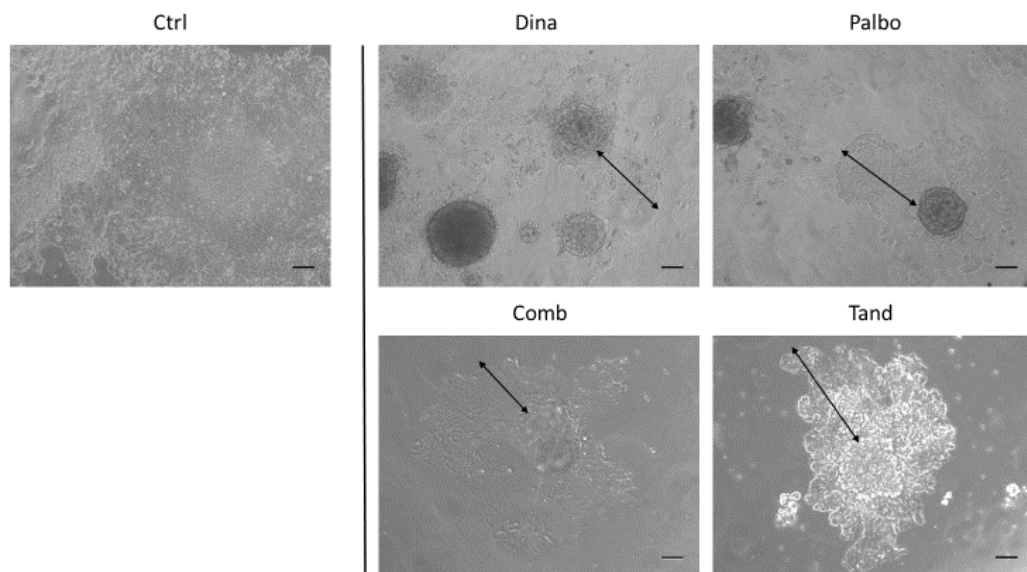
Figure 12. Effect of Dinaciclib and Palbociclib on the growth of S1 and T4-2 acini cultured in 3D and treated during S1 quiescence phase.
S1 and T4-2 cells were untreated (Ctrl) or treated with Dinaciclib (Dina), Palbociclib (Palbo), combination (Comb) or tandem (Tand). S1 acini like structures (A) and T4-2 (B) nodules diameter were measured at six different post treatment time points. (C) Area of cell- devoid spaces between T4-2 nodules was measured on day 30 in all treatment conditions. (D) Photomicrographs of T4-2 nodules were taken at 10x

magnification. Scale bar: 100µm. n=1. In these experiments, S1 and T4-2 cells were cultured without prolactin due to an international shortage in production from international suppliers that lasted for more than one year. See Material and Methods page 45.

G. Dinaciclib and Palbociclib do not disrupt lumen formation of S1 cells cultured in 3D and treated during S1 growth phase.

In 3D culture, S1 epithelial cells are assembled as one layer surrounding a lumen by day 7 in culture (Petersen et al. 1992). To determine whether Dinaciclib and Palbociclib could disrupt lumen of S1 acini during their assembly phase, lumen structures was scored on day 14, 10 days post-treatment. Around 59% of S1 acini treated with Dinaciclib and 61% of S1 acini treated with Palbociclib showed undisrupted lumens compared to 63% in the control untreated S1 cells (Fig 13). Preliminary data implied that treatments did not affect lumen structures of S1 acini.
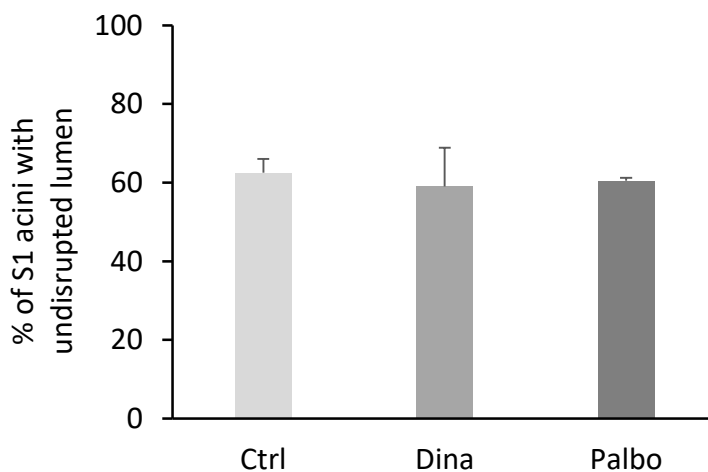
Figure 13. Effect of Dinaciclib and Palbociclib on lumen structures of S1acini treated during their growth phase.
S1 cells cultured in 3D were untreated (Ctrl) or treated with Dinaciclib (Dina), Palbociclib (Palbo)on day 4 for 10 days. Percentage of undisrupted lumenof control untreated and treated S1 acini with Dinaciclib and Palbociclib. 120 acini were scored from each condition. n=1.

H. Dinaciclib and Palbociclib do not disrupt β- Catenin localization in S1 acini treated during S1 growth phase.

Establishment of acini polarity, key for lumen formation, is dependent on cell junctions: tight, adherent and gap junctions. S1 cells were found to exhibit apical polarity as assessed by immunostaining for junctional proteins such as ZO-1, β- catenin, and Cx 43 which were compartmentalized to the cells apicolateral side (Submitted Bazzoun at al. 2018).

We determined whether Dinaciclib and Palbociclib would affect normal acini polarization through assessment of β-catenin localization. Immunostaining for β catenin was performed in S1 acini treated with Dinaciclib and Palbociclib on day 14, 10 days post-treatment. Around 39 % of S1 acini treated with Dinaciclib and 38 % of S1 acini treated with Palbociclib revealed an apicolateral localization of β-catenin with an undisrupted lumen, compared to 42 % in the control untreated S1 cells (Fig 14A). Photomicrographs of treated and untreated S1 acini showed that the treatment did not affect the apicolateral pattern of β- catenin (Fig 14B, C and D).

A
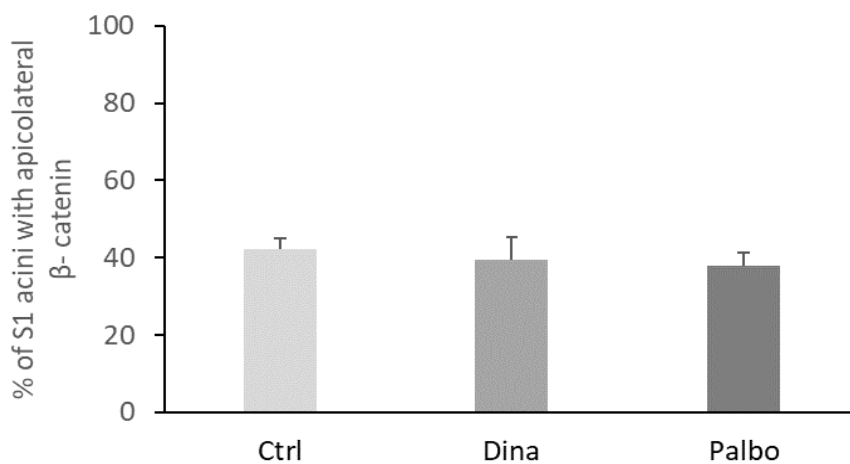
B
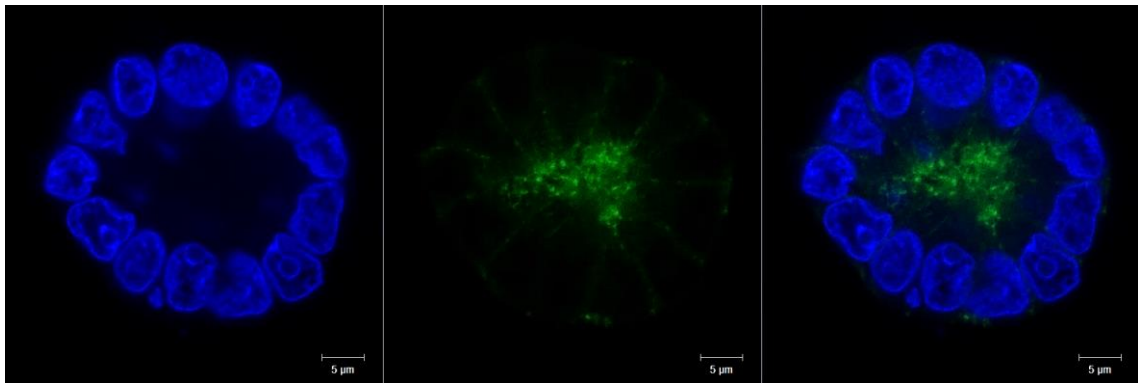
C

D
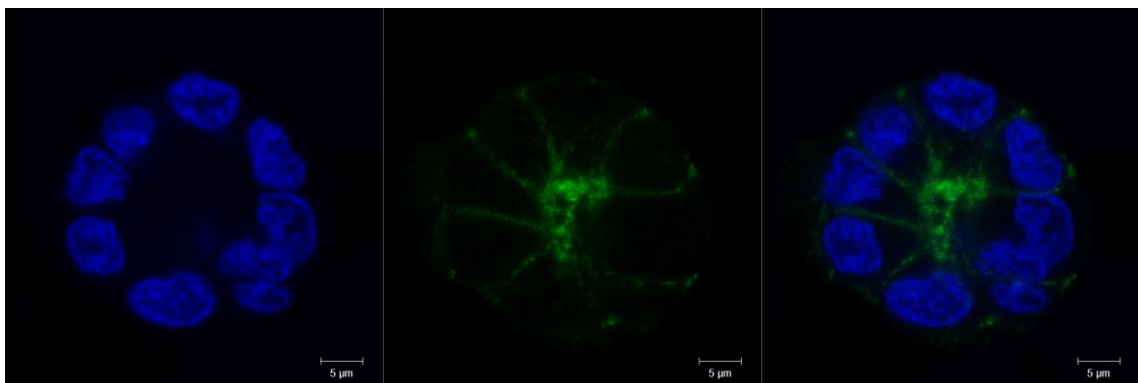
Figure 14. Effect of Dinaciclib and Palbociclib on β- catenin localization in S1 acini treated during S1 growth phase.
S1 cells cultured in 3D were untreated (Crtl) or treated with Dinaciclib (Dina) or Palbociclib (Palbo) on day 4 for 10 days. Histogram shows the quantification data of β-catenin localization. 120 acini were scored from each condition (A). Representative acinus from 3D cultures of (B) control untreated and treated S1 acini with (C). n=1.

Dinaciclib or (D) Palbociclib stained for β-catenin (green) and counterstained with DAPI (blue) on day 14. Images were taken at 63x magnification. Scale Bar: 5µm. Due to time constraints, better images will be taken instead of these.

I. Dinaciclib and Palbociclib do not affect lumen structures of S1 acini treated during their quiescence phase.

To assess whether Dinaciclib and Palbociclib disrupted lumens of S1 acini treated post

acini assembly phase on day 11 in culture, lumen structures were scored on day 30.

Around 60, 62, 60, 62 % of S1 acini treated respectively with Dinaciclib, Palbociclib,

combination or tandem showed undisrupted lumens compared to 61% in the control

untreated S1 acini. Preliminary data suggested that treatments did not affect lumen

structures of S1 acini (Fig 15).
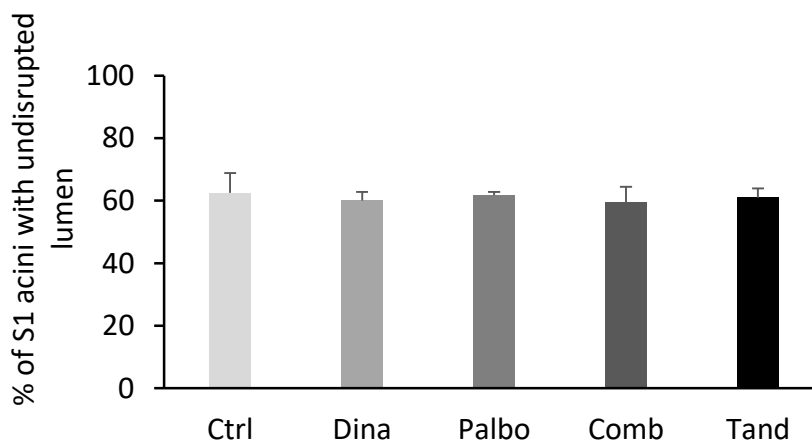
Figure 15. Effect of Dinaciclib and Palbociclib on lumen structure of S1 acini treated during their quiescence phase.
S1 cells cultured in 3D were untreated (Crtl) or treated with Dinaciclib (Dina) or Palbociclib (Palbo), combination (Comb) or tandem (Tand) on day 11 for 19 days. Percentage of undisrupted lumen of control untreated and treated S1 acini. 120 acini were scored from each condition. n=1. In this experiment, S1 and T4-2 cells were cultured without prolactin due to an international shortage in production from international suppliers that lasted for more than one year. See Material and Methods page 45.

J. Dinaciclib and Palbociclib do not disrupt β- Catenin localization in S1 acini treated during S1 quiescence phase.

We attempted to test whether Dinaciclib and Palbociclib would disturb β-catenin localization. Immunostaining for β catenin was performed on day 30 in S1 acini treated with Dinaciclib, Palbociclib, combination or tandem starting day 11. Around 40, 41, 42, 42 % of S1 acini treated respectively with Dinaciclib, Palbociclib, combination or tandem revealed an apicolateral localization of β-catenin with an undisrupted lumen, compared to 39% in the control untreated S1 acini (Fig 16 A). Although β-catenin was still localized on the apical and lateral sides of S1 acini, β-catenin pattern was not the same as we obtained in the control. Fluorescence detected in the center of the treated acini with Dinaciclib was not intense as that detected in the center of untreated acini (Fig 16 B and C). Photomicrographs treated S1 acini with Palbociclib, combination or tandem showed that they have same β- catenin pattern as the untreated ones (Fig 16 B, D, E and F).

A
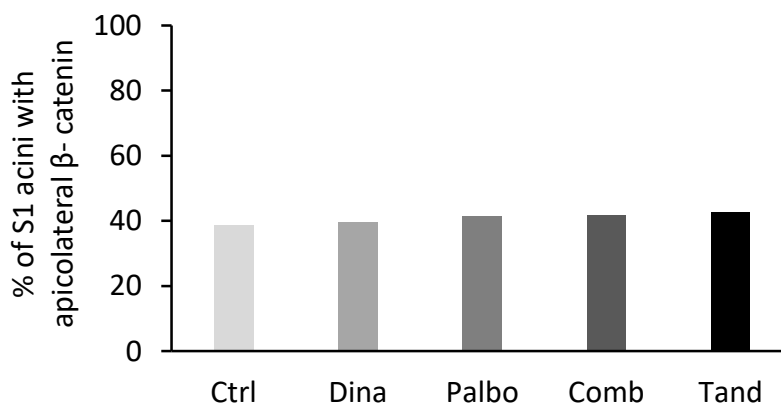
B
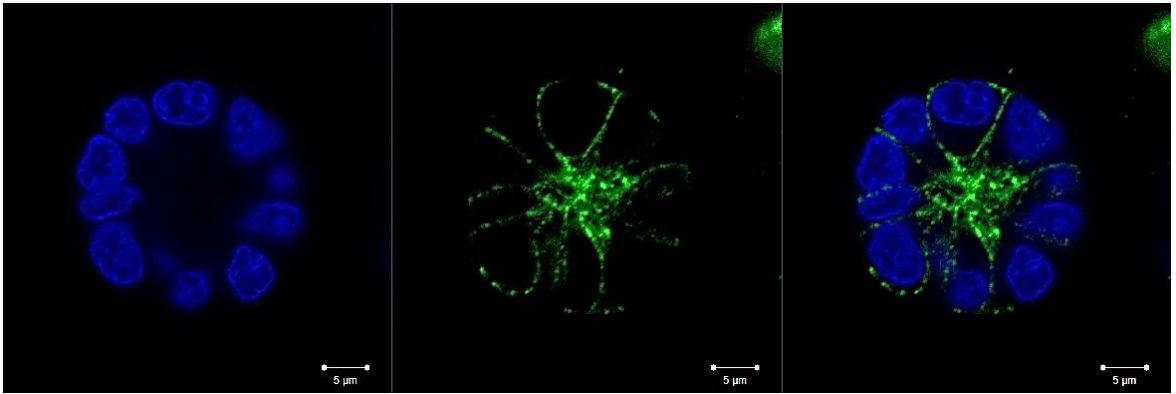
C
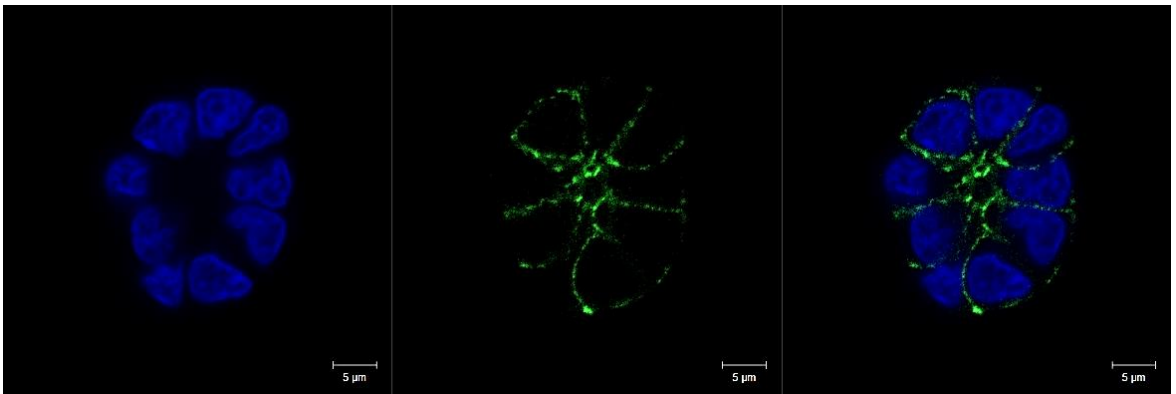
D
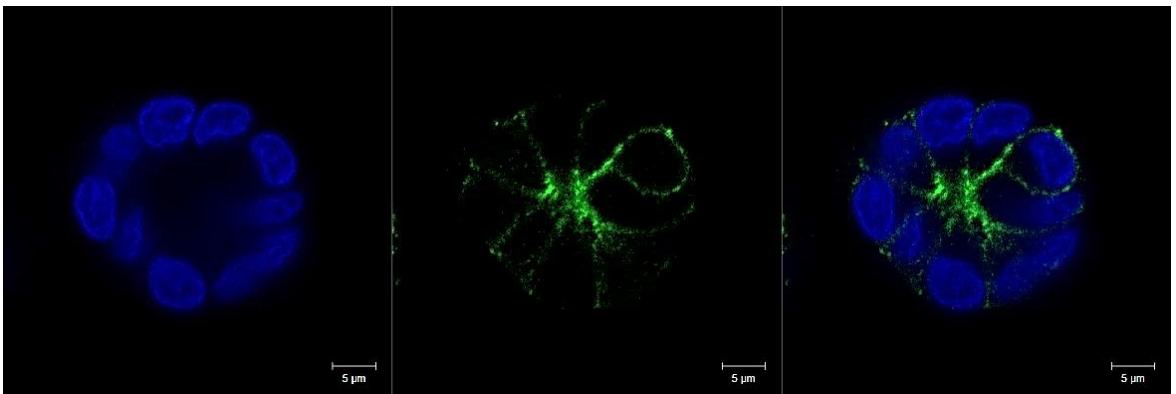
E
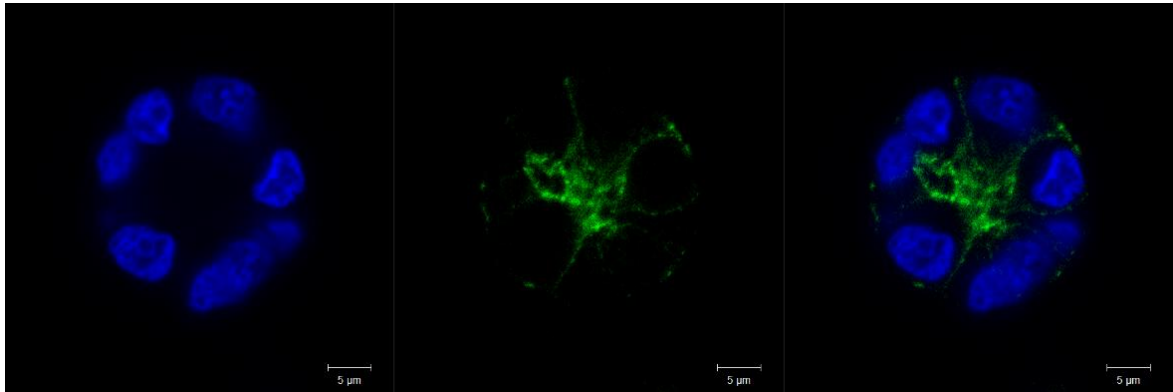
F

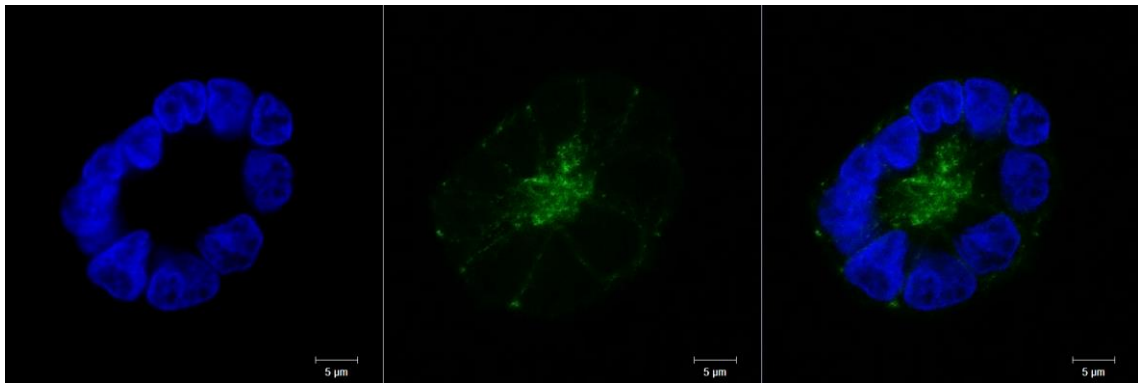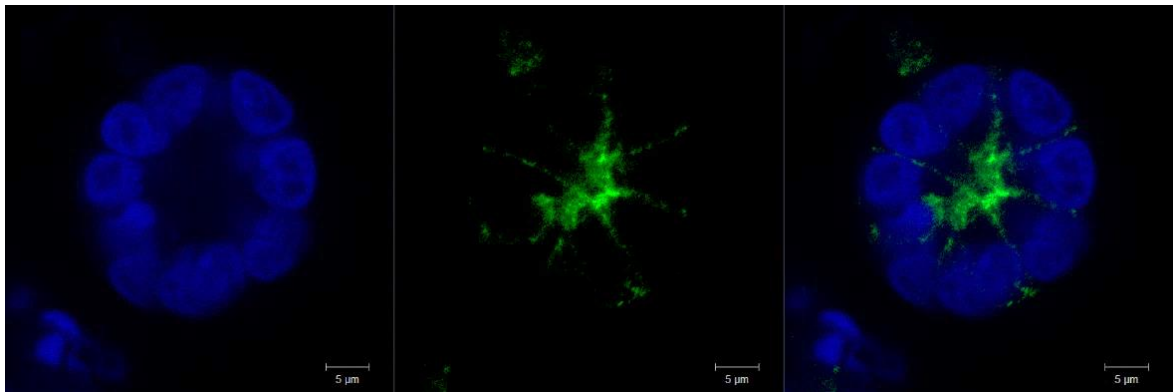**Figure 16. Effect of Dinaciclib and Palbociclib on β-catenin localization in S1 acini treated during their quiescence phase.**
S1 cells cultured in 3D were untreated (Crtl) or treated with Dinaciclib (Dina) or Palbociclib (Palbo), combination (Comb) or tandem (Tand) on day 11 for 19 days. Histogram shows the quantification data of β-catenin localization. 120 acini were scored from each condition (A). Representative acinus from 3D cultures of (B) control untreated and treated S1 acini with (C) Dinaciclib or (D) Palbociclib, (E) combination or (F) tandem stained for β-catenin (green) and counterstained with DAPI (blue) on day 30. Images were taken at 63x magnification. Scale Bar: 5µm. n=1. In this experiment, S1 and T4-2 cells were cultured without prolactin due to an international shortage in production from international suppliers that lasted for more than one year. See Material and Methods page 45.

CHAPTER V

DISCUSSION

TNBC represents approximately 15% of newly diagnosed invasive breast cancers and is associated with a higher incidence of relapse and death than other breast cancer subtypes (Chacón et al. 2010). It does not express hormone receptor, or HER2, thus it is insensitive to many of the treatments used for other breast cancer subtypes, such as molecules that inhibit hormone biosynthesis or antibodies that target cell receptors. Due to the lack of well-defined targets, developing targeted therapies against TNBC has been challenging. There are no approved targeted therapies for this subtype, and treatment remains limited, involving surgery, a small number of first-line systemic chemotherapies, and radiothera (Wahba et al. 2015). Therefore, effort to develop targeted therapies is needed. Since loss of normal cell cycle control is a hallmark of cancer, targeting cell cycle regulators such as CDK with CDK inhibitors may be an effective therapeutic strategy for triple-negative breast cancer. Several in vitro and in vivo studies illustrated the potential of targeting CDKs in several types of cancers. For example, Flavopiridol, an inhibitor of CDK 1, 2 and 4, induced apoptosis in human monoblastic leukemia cells (Decker et al. 2001). PHA-848125, another CDK1,2 and 4 inhibitors, induced a significant tumor growth inhibition in a transgenic mouse model which developed pulmonary cancerous lesions reminiscent of human lung adenocarcinomas (Degrassi et al. 2010). Dinaciclib, an inhibitor of CDK 1, 2, 5, and 9, induced apoptosis and total inhibition of bromodeoxyuridine incorporation in more than 100 tumor cell lines of diverse origin and background (Prostate, Breast, Colon, Ovarian,

Pancreatic, Liver cancers, Melanoma, Leukemia, Mantle cell lymphoma) (Parry et al. 2010). It has already been evaluated in clinical trials, and results showed that it has encouraging activity in relapsed/refractory chronic lymphocytic leukemia (Fabre et al. 2014). CDK inhibitors have been also investigated for their anticancer potential in TNBC. Treatment of TNBC cells with a combination of CDK2 inhibitor and paclitaxel (an antimicrotubule agent) resulted in apoptosis in vitro and decreased tumor size in vivo (Tarasewicz et al. 2014). Moreover, treatment of TNBC cell lines, with CYC065, a CDK2/9 inhibitor, decreased cell migration/invasion through changes in EMT-associated protein levels (Thomas et al. 2017). Another study demonstrated that the combination of CYC065 with eribulin (a non-taxane microtubule dynamics inhibitor) reduced TNBC cell viability, tumor colony size, and cell migration, and induced apoptosis in vitro (Rao et al. 2017). In addition, inhibition of CDK4 with palbociclib decreased the percentage of chemotherapy-resistant cells and breast cancer stem cells in TNBC (Dai et al. 2016). Dinaciclib was also found to have anti- tumor activity in TNBC. It inhibited tumor growth in TNBC patient derived xenograft and cell line models in vitro and in vivo, by inducing significant decrease in pRb (S807/811), arresting cells in the G2-M phase and inducing apoptosis (Rajput et al. 2016). Collectively, these studies indicate that CDK inhibition therapy may be a candidate strategy for patients with TNBC.

CDK2 and CDK 4/6 are major regulators of the cell cycle. They play a key role in regulating the transition of cells from G1 to S phase. A previous study conducted by Zalzali et al. using a 2D culture of irbp-cyclin D1 pineal cells showed that, during tumor suppression due to oncogene-induced cellular senescence (Cyclin D1-induced

75

senescence), CDK2 inhibition precedes CDK4 inhibition, and stable cell cycle arrest is then achieved. This study showed a novel unrecognized temporal sequence of molecular and morphological events that accompany the evolution of an oncogene-driven senescent state. The loss of CDK2 activity temporally correlates with p53-dependent cell cycle exit, whereas the loss of CDK4/6 activity correlates with late RB-associated phenotypes of senescence, such as formation of heterochromatin foci (Zalzali et al. 2012).

In this study, we tested the effect of Dinaciclib and Palbociclib, CDK2 and CDK4 inhibitors respectively, added alone, in combination or tandem (treatment with Dinaciclib followed by treatment with Palbociclib) on normal as well as on breast cancer cells. We used a 3D triple negative breast cancer culture model that shares similar properties of its in vivo counterpart. Dinaciclib has entered phase III trial for refractory chronic lymphocytic leukemia, based on promising responses in earlier phase clinical trials and a favorable toxicity profile (Ghia et al. 2015). Palbociclib treatment in women with ER-positive breast cancer, in combination with the aromatase inhibitor letrozole, resulted in a 3-fold improved outcome over hormonal therapy alone, leading to Palbociclib being labeled in 2013 as a breakthrough drug by the FDA (Morikawa et al. 2015).

As an initial step, we tested the effect of 10 nM Dinaciclib and 1 μM Palbociclib on a highly aggressive triple negative breast cancer cell line MDA-MB 231 cultured in 2D. Treatment of MDA- MB 231 with 10 nM of Dinaciclib for 48 hours decreased cell proliferation and increased the percentage of MDA-MB 231 cells in pre-G1. However, trypan blue cell count did not reflect this cell death. Pre-G1 cells might

still have intact membranes during these 48 hours and excluded trypan blue dye. A previous study has demonstrated that treatment of MDA- MB 231 with 10 nM of dinaciclib for 72 hours, induced substantial cell death and cell cycle arrest in both G1-S and G2-M (Horiuchi et al. 2012). On the other hand, treatment of MDA- MB 231 with 1μM of Palbociclib for 48 hours induced cell cycle arrest in G1 phase. This result is in line with a previous study conducted by Knudsen et al. in 2017. The authors have also shown that cell cycle arrest was dependent on the presence of the Rb tumor suppressor. Deletion of Rb mediated by CRISP/Cas9 was associated with marked reduction in sensitivity to palbociclib. In addition, they have shown that treatment with palbociclib led to repression of genes regulated by the E2F-transcription factor and associated with cell cycle progression (Knudsen et al. 2017). On the other hand, a previous study has demonstrated that treatment of MDA- MB 231 with 7.5 or 15μM of Palbociclib for 48 hours inhibited Rb phosphorylation (Ser 780) in a concentration dependent manner (Qin et al. 2015). Our preliminary data showed that combination of Dinaciclib and Palbociclib had the same effect as Dinaciclib on MDA- MB 231 cells. However, to the best of our knowledge no studies to date have evaluated the combination of Dinaciclib and Palbociclib in cancer.

Investigating mechanisms of anti- cancer drugs necessitates studying the effect of the drugs on cancer cells as well as on normal counterparts in microenvironments that recapitulate in vivo conditions (Langhans, 2018). Dinaciclib effect was evaluated in melanoma cells (Desai et al. 2013), leukemia cells (Gojo et al. 2013), chronic lymphocytic leukemia cells (Chen et al. 2016), ovarian cancer cells (Chen et al. 2015), and thyroid cancer cells (Lin et al. 2017). In addition, Palbociclib effect was

investigated in gastric cancer (Wang et al. 2018) and renal carcinoma cells (Logan et al. 2013). However, all these studies were done in 2D microenvironments on plastic substrata and used only cancer cells. None of these studies tested whether these drugs could affect normal counterparts. On the other hand, accumulated evidence showed that therapeutic strategies performed on 2D cultures gave misleading results compared to 3D cultures, which more mimic cellular responses in vivo and provide more accurate predictions of drug efficacies (Horning et al. 2008). For example, the response of the HER2-amplified breast cancer cell lines AU565, SKBR3 and HCC1569 cells to anti-HER2 agents Trastuzumab, Pertuzumab, and Lapatinib was highly dependent on whether the cells were cultured in 2D monolayer or 3D laminin-rich ECM gels. AU565 cells were significantly sensitive to Trastuzumab, but resistant to Pertuzumab when cultured in 3D compared to 2D culture conditions. SKBR3 cells were resistant to Trastuzumab treatment, but sensitive to Lapatinib when grown in 3D compared to 2D culture conditions. Lapatinib treatment resulted in a clear inhibition of AU565 cells proliferation, in both cell lines culture conditions. As for HCC1569 cells, they were insensitive to Trastuzumab treatment in both 2D and 3D cultures. However, they were significantly more sensitive to treatment with Pertuzumab or Lapatinib when propagated in 3D compared to 2D culture. This study has demonstrated that breast cancer cell lines responded differently to each treatment in 2D and 3D because these two culture conditions induced distinct HER2 downstream signaling in these cell lines. These observations help in unraveling the mechanisms underlying de novo and acquired resistance seen in the majority of patients treated with anti-HER2 tailored therapy. Use of 3D culture model increases the likelihood of translating results of culture models into

patient care (Weigelt et al. 2010). MDA- MB 231 cell line used in assessing Dinaciclib

and Palbociclib in breast cancer does not have neither a normal counterpart with same

genetic lineage nor does it have phenotypic advantages in 3D culture. One such model

of breast cancer that encompasses spectrum of tumor development from normal

mammary epithelium to invasive tumor and recapitulates the in vivo characteristics in

3D culture is the HMT3522 cell line. S1 cells form polarized mammary acini like

structures with a central lumen when grown in 3D extracellular matrix, resembling

normal mammary epithelial differentiation (Plachot et al. 2009). In contrast, T4-2 cells

form disorganized spheroids, have a disrupted cell cycle control and have a triple-

negative phenotype (no expression of ER, PR, and HER2) (Plachot et al. 2009, Rizki et

al. 2008). T4-2 cells also have activated pathways, such as increased signaling through

EGFR, b1-integrin, PI3K, and MAPK (Parikh et al. 2014). Several studies have used

this system to establish that inhibition of such signaling pathways using EGFR inhibitor

tyrphostin AG1478, human EGFR function-blocking antibody mAb225, mitogen-

activated protein (MAP)/extracellular signal-regulated kinase kinase (MEK) inhibitor

PD98059, or PI3K inhibitor LY294002 may be effective in reverting the phenotype

from T4-like to S1-like 3D growth (Wang et al. 1998, Wang et al. 2002). This system

was also used to test whether differentiated mammary epithelial cells are resistant to

apoptosis. S1 and T4–2 cells cultured in 2D were sensitive to a variety of apoptotic

stimuli. In 3D culture, S1 cells became resistant to the apoptotic stimuli, whereas T4-2

cells remained sensitive (Weaver et al. 2002). However, no studies evaluated the effect

of CDK inhibition on cell cycle arrest and senescence induction in cancer and normal

cells. In this study, we determined the effect of Dinaciclib and Palbociclib on normal

S1, and tumor initiated Cx43 KO-S1 (Submitted Bazzoun at al. 2018) and T4-2 cells in 2D culture. Preliminary data suggested that Dinaciclib and Palbociclib decreased the cells proliferation. Combination had the most potent effect and not tandem. Preliminary data obtained from Western- blot analysis suggested that this decrease in cell proliferation was associated to senescence since treated cells exhibited an increase in the expression of senescence markers (p21, p15 and Dec1) compared to the untreated ones. Contrary to our expectations, a positive SABG activity (a marker of senescence) was detected in treated and untreated S1 and Cx43 KO-S1 cells. Similarly, SABG activity was elevated under normal conditions in matured osteoclasts and macrophages (Brusuker et al. 1982, Kopp et al. 2007) suggesting that this assay could not be used to detect senescence in our system. A previous study conducted by Lallena et al. in 2015 has shown that CDK4/6 inhibition induced senescence in ER+ breast cancer. The authors showed that treatment of ER+ breast cancer cells with Abemaciclib, a CDK4/6 inhibitor, induced a decrease in Rb phosphorylation and cell proliferation markers (Ki67 and BrdU). In addition, Abemaciclib induced an accumulation of endogenous beta-galactosidase and an increase in p21 expression.

Given that 3D cell culture enables drug efficacy assessment in a more in vivo–like context than traditional 2D cell cultures, we treated S1 and T4-2 cells in 3D culture during their growth phase, starting day 4. Preliminary data proposed that treatment arrested the growth of S1 acini and T4-2 nodules with a marginal preferential effect on T4-2 nodules. We propose that the size of S1 acini was affected because the S1 cells were treated during their acinar forming growth phase. Since polarized S1 acini have been previously reported to have an apicolateral localization of β-catenin (Submitted

Bazzoun at al. 2018), we evaluated apical polarity of treated S1 acini by immunofluorescence staining for β-catenin. The assembly of S1 cells around a lumen as well as the apicolateral localization of β-catenin observed in treated S1 acini suggested that neither treatment affected normal S1 cells to assemble into polarized acini. The fact that treated S1 cells maintained their polarity in smaller diameter acini suggested that neither treatment induced apoptosis in these cells (Weaver et al. 2002). On the other hand, S1 cultured in 3D proliferate before being induced to differentiate and form acini, after which cells display growth arrest. Previous studies demonstrated that S1 cells become growth arrested and form acini- like structures by days 7-10 in culture (Petersen et al. 1992, Lelievre et al. 1998, Plachot et al. 2004). Howlet et al. reported that treatment of S1 cells with inhibitory anti-β1 integrin antibodies till day 6 in culture (during their growth phase) induced apoptosis (Howlett et al. 1995). Another study conducted by Park et al. tested the effect of the same antibody on S1 and breast cancer cells. The authors treated S1 acini when S1 cells were in their quiescent phase. They found that treatment induced apoptosis in tumor cells, but not in quiescent S1 cells which remained resistant (Park et al. 2006). In the light of this study, we opted to treat S1 acini and T4-2 nodules on day 11 in culture, when S1 cells are quiescent while T4-2 sustained growth phase and formed a nodule of 200 μm of diameter. Our preliminary data showed that treatments did not affect S1 diameter, β- catenin distribution nor their lumen structures. Although treatments did not affect T4-2 nodules diameter, it decreased T4-2 nodules cellular outgrowth. Two different possibilities may explain these observations. This effect may be due to the nodules architectural structure. The nodules 3D structure reduced the number of intra nodule cancer cells exposed to the

drugs. However, these drugs had more accessibility to cells that have migrated away from T4-2 nodules in a monolayer (Lovitt et al. 2014). Alternatively, these observations suggested that these CDK inhibitors affected alternative pathways involved in migration and invasiveness. A previous study demonstrated that Palbociclib inhibited migration and invasion of MDA-MB-231 and T47D triple negative breast cancer cells cultured in 2D by suppressing vimentin and Snail and inhibiting the c-Jun/COX-2 pathway. Palbociclib down-regulated c-Jun, a crucial transcriptional regulator of COX-2 that was found to be linked to EMT and critical for breast cancer motility, invasion and metastasis (Qin et al. 2015). In addition, treatment of triple negative breast cancer cells MDA-MB-231, MDAMB-436, and Hs578T cultured in 2D with CYC065, a CDK2 inhibitor, decreased migration of the triple negative breast cancer cells by reducing Smad3 phosphorylation, a transcription factor in the TGFβ pathway, and Pin1-Smad3 interaction which plays a role in promoting the tumorigenic and metastatic action of non-canonical Smad3 signaling. Inhibition of CDK2 also reduced the expression of the transcription factors Snail and Slug, which are associated with TGFβ-induced EMT (Thomas et al. 2017). Therefore, this decrease in T4-2 outgrowth may reflect a decrease in T4-2 invasion. On the other hand, we tested higher concentrations of Dinaciclib and Palbociclib, 20 nM and 2 μM respectively on S1 acini and T4-2 nodules treated during S1 growth phase. Preliminary data suggested that Dinaciclib affected S1 acini lumen structures; however, Palbociclb did not. As for T4-2 nodules, these concentrations of Dinaciclib and Palbociclib had the same effect of those previously tested (10 nM and 1 μM respectively) (Data not shown). We have found that 10 nM of Dinaciclib and 1 μM of Palbociclib did not affect T4-2 nodule size. Since 20 nM of Dinaciclib affected S1

acini structures, we will investigate the effect of 10 nM Dinaciclib and 2 μM Palbocilcib added alone, in combination or in tandem on S1 acini and T4-2 nodules (treatment starting day 11) and test whether these drugs affect T4-2 nodule size.

In conclusion, we propose that CDK inhibition may be a promising approach to treat triple negative breast cancer. Our study is unique in testing the effect of CDK2 and CDK4/6 inhibitors on cancer as well as on normal breast cells in a 3D model. Our preliminary data suggest that Dinaciclib and Palbociclib have no effect on normal acini-like structures. They also suggest that Dinaciclib and Palbociclib arrested the growth of T4-2 nodule treated during their growth and decreased T4-2 outgrowth and probably invasion when treated after nodule formation on day 11. Further studies are needed to evaluate senescence markers such as p21, p15, Dec1 and DcR2 as well as BrdU incorporation and cell cycle progression in treated S1 and T4-2 nodules cultured in 3D. In addition, the drugs effect will be assessed on proliferation and invasion of T4-2 cells co-cultured with S1 cells in 3D or in Lab-on-Chip device that mimics the 3D ductal system of the human breast. Moreover, the drugs effect will be evaluated in female nude mice injected subcutaneously with T4-2 cells (Park et al. 2006).

- Limitations

Experiments were not replicated due to time constraints. We had to maintain normal and intermediate cells in 2D culture for eleven days then split and seed them. We also had to treat cells till day fourteen or thirty in culture. These cells are very sensitive since we culture them without antibiotics, as a result we had lost twice 3D experiments that should be kept till day thirty in culture because of cell contamination.

In addition, cells were occasionally cultured without prolactin (one of the media additives) because of an international shortage supply (Up to June 2018). Culturing cells without prolactin in 2D resulted in slower growth rate of cells in culyure. This was not quantified but a merely supervised observation over several culture experiments. In addition, acini diameter measurement showed a decrease in the diameter of acini cultured without prolactin compared to those cultured with prolactin (Figure 12 A, page 65). However, culturing cells without prolactin did not affect acini polarity (Figure 16, pages 70-73).

- Future work

1. Reproduce current preliminary (n=1) data.

2. Confirm CDK2 and CDK4 inhibition by assessing RB phosphorylation levels at CDK2-dependent sites (Ser612) and CDK4-dependent sites (Ser780) via western blot.

3. Assess drugs effect on CDK2 and CDK4 activity using in vitro kinase assay as described by James et al. 2005 and Patel et al. 2015.

4. Assess apoptosis in MDA-MB 231 cells treated with Dinaciclib and Palbociclib for 48 hours, by doing TUNEL staining, Annexin V, or evaluating the protein levels of cleaved PARP, cleaved caspase3, Bax, Bcl2 and/or XIAP.

5. Evaluate senescence associated β galactosidase activity in MDA -MB 231 cells and use of a systematic counting approach for the whole plate.

6. Evaluate proliferation markers by performing immunofluorescence for Ki67 and BrdU assay in order to demonstrate that the decrease in S1, Cx 43 KO- S1 and T4-2 cells proliferation is associated with senescence.

7. Test combination and tandem treatment effect on S1 and T4-2 cells cultured in 3D and treated during S1 growth phase.

8. Evaluate β- catenin localization in T4-2 nodules cultured in 3D and treated during growth and quiescence phases of S1 acini.

9. Assess migration of T4-2 cells cultured on matrix gel and treated with Dinaciclib and Palbociclib using Transwell invasion assay and in 3D culture.

BIBLIOGRAPHY

A study of palbociclib (PD0332991) + letrozole vs. letrozole for 1st Line. treatment of postmenopausal women with ER+/HER2- advanced breast cancer (PALOMA-2). 2014. Available from: http://clinicaltrials.gov/ show/NCT01740427.

A study of Palbociclib and Cetuximab in Treating Patients with Squamous Cell Carcinoma of the Head and Neck. Available from: https://www.cancer.gov/clinicaltrials/NCI-2014-01079.

A study of the CDK4/6 Inhibitor Palbociclib (PD-0332991) in combination with the PI3K/mTOR Inhibitor Gedatolisib (PF-05212384) for patients with advanced squamous cell lung, pancreatic, head & neck and other solid tumors. Available from: http://clinicaltrials.gov/ show/NCT03065062.

Abe, O., Abe, R., Enomoto, K., Kikuchi, K., Koyama, H., . . . Department of Clinical Sciences, Malmö. (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. *The Lancet, 365*(9472), 1687-1717.

Abukhdeir, A. M., Vitolo, M. I., Argani, P., Angelo M. De Marzo, Karakas, B., Konishi, H., . . . Park, B. H. (2008). Tamoxifen-stimulated growth of breast cancer due to p21 loss. *Proceedings of the National Academy of Sciences of the United States of America, 105*(1), 288-293.

Aird, K. M., & Zhang, R. (2013). Detection of senescence-associated heterochromatin foci (SAHF). *Methods in Molecular Biology (Clifton, N.J.), 965*, 185-196.

Akli, S., Bui, T., Wingate, H., Biernacka, A., Moulder, S., Tucker, S. L., . . . Keyomarsi, K. (2010). Low-molecular-weight cyclin E can bypass letrozole-induced G1 arrest in human breast cancer cells and tumors. *Clinical Cancer Research, 16*(4), 1179-1190.

Aktas, H., Cai, H., & Cooper, G. M. (1997). Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the cdk inhibitor p27KIP1. *Molecular and Cellular Biology, 17*(7), 3850-3857.

Ali, M. A., Choy, H., Habib, A. A., & Saha, D. (2007). SNS-032 prevents tumor cell-induced angiogenesis by inhibiting vascular endothelial growth factor. *Neoplasia, 9*(5), 370-381.

American Cancer Society. Breast Cancer Facts & Figures 2017-2018. Atlanta: American Cancer Society, Inc. 2017.

An, H., Beckmann, M. W., Reifenberger, G., Bender, H. G., & Niederacher, D. (1999). Gene amplification and overexpression of CDK4 in sporadic breast carcinomas

associated with high tumor cell proliferation. *American Journal of Pathology, 154*(1), 113-118.

Anders, C., & Carey, L. A. (2008). Understanding and treating triple-negative breast cancer. *Oncology (Williston Park, N.Y.), 22*(11), 1233.

Anders, L., Ke, N., Hydbring, P., Choi, Y., Widlund, H., Chick, J., . . . Sicinski, P. (2011). A systematic screen for CDK4/6 substrates links FOXM1 phosphorylation to senescence suppression in cancer cells. *Cancer Cell, 20*(5), 620-634.

Baek, K., Bhang, D., Zaslavsky, A., Wang, L., Vachani, A., Kim, C. F., . . . Ryeom, S. (2013). Thrombospondin-1 mediates oncogenic ras-induced senescence in premalignant lung tumors. *The Journal of Clinical Investigation, 123*(10), 4375-4389.

Banerjee, D. (2016). Connexin's connection in breast cancer growth and progression. *International Journal of Cell Biology, 2016*, 9025905.

Barnum, K. J., & O'Connell, M. J. (2014). Cell cycle regulation by checkpoints. *Methods in Molecular Biology (Clifton, N.J.), 1170*, 29.

Bartkova, J., Lukas, J., Müller, H., Lützhøft, D., Strauss, M., & Bartek, J. (1994). Cyclin D1 protein expression and function in human breast cancer. *International Journal of Cancer, 57*(3), 353-361.

Beaver, J. A., Amiri-Kordestani, L., Charlab, R., Chen, W., Palmby, T., Tilley, A., . . . Cortazar, P. (2015). FDA approval: Palbociclib for the treatment of postmenopausal patients with estrogen receptor-positive, HER2-negative metastatic breast cancer. *Clinical Cancer Research, 21*(21), 4760-4766.

Berdichevsky, F., Wetzels, R., Shearer, M., Maringnone, M., Ramaekers, F., Taylor-Papdimitriou, J., (1994). Integrin expression in relation to cell phenotype and malignant change in the human breast. *Molecular Cell Differentiation*, 2, 255–274.

Besson, A., Dowdy, S., & Roberts, J. (2008). CDK inhibitors: Cell cycle regulators and beyond. *Developmental Cell, 14*(2), 159-169.

Bissell, M. J., Radisky, D. C., Rizki, A., Weaver, V. M., & Petersen, O. W. (2002). The organizing principle: Microenvironmental influences in the normal and malignant breast. *Differentiation, 70*(9), 537-546.

Blachly, J. S., MD, Byrd, J. C., MD, & Grever, M., MD. (2016). Cyclin dependent kinase inhibitors for the treatment of chronic lymphocytic leukemia. *Seminars in Oncology, 43*(2), 265-273.

Blum, K. A., Ruppert, A. S., Woyach, J. A., Jones, J. A., Andritsos, L., Flynn, J. M., . . . Byrd, J. C. (2011). Risk factors for tumor lysis syndrome in patients with chronic lymphocytic leukemia treated with the cyclin-dependent kinase inhibitor, flavopiridol. *Leukemia, 25*(9), 1444-1451.

Bombonati, A., & Sgroi, D. C. (2011). The molecular pathology of breast cancer progression. *The Journal of Pathology, 223*(2), 308-318.

Boss, D. S., Schwartz, G. K., Middleton, M. R., Amakye, D. D., Swaisland, H., Midgley, R. S., . . . Shapiro, G. I. (2009;2010;). Safety, tolerability, pharmacokinetics and pharmacodynamics of the oral cyclin-dependent kinase inhibitor AZD5438 when administered at intermittent and continuous dosing schedules in patients with advanced solid tumours. *Annals of Oncology, 21*(4), 884-894.

Bracken, A. P., Kleine-Kohlbrecher, D., Dietrich, N., Pasini, D., Gargiulo, G., Beekman, C., . . . Helin, K. (2007). The polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes and Development, 21*(5), 525-530.

Briand, P., Petersen, O. W., & Deurs, B. V. (1987). A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. *In Vitro Cellular & Developmental Biology, 23*(3), 181-188.

Brusuker, I., Bursuker, I., Rhodes, J. M., Rhodes, J. M., Goldman, R., & Goldman, R. (1982). β-galactosidase - an indicator of the maturational stage of mouse and human mononuclear phagocytes. *Journal of Cellular Physiology, 112*(3), 385-390.

Byth, K. F., Thomas, A., Hughes, G., Forder, C., McGregor, A., Geh, C., . . . Wilkinson, R. W. (2009). AZD5438, a potent oral inhibitor of cyclin-dependent kinases 1, 2, and 9, leads to pharmacodynamic changes and potent antitumor effects in human tumor xenografts. *Molecular Cancer Therapeutics, 8*(7), 1856-1866.

Camidge, D. R., Eckhardt, S. G., Tan, A., Frenette, G., Diab, S., Depinto, W., . . . Papadimitrakopoulou, V. (2006). 281 POSTER A phase I study of R547, a novel cyclin dependent kinase inhibitor, in patients (pts) with advanced cancer: Preliminary results. *EJC Supplements, 4*(12), 89-89.

Chacón, R. D., & Costanzo, M. V. (2010). Triple-negative breast cancer. *Breast Cancer Research : BCR, 12 Suppl 2*(S2), S3-S3.

Chen, M., Whisler, J., Froese, J., Yu, C., Shin, Y., & Kamm, R. (2017). On-chip human microvasculature assay for visualization and quantification of tumor cell extravasation dynamics. *Nature Protocols, 12*(5), 865-880.

Chen, X., Xie, F., Zhu, X., Lin, F., Pan, S., Gong, L., . . . Yan, X. (2015). Cyclin-dependent kinase inhibitor dinaciclib potently synergizes with cisplatin in preclinical models of ovarian cancer. *Oncotarget, 6*(17), 14926-14939.

Chen, X., Xie, F., Zhu, X., Lin, F., Pan, S., Gong, L., . . . Yan, X. (2015). Cyclin-dependent kinase inhibitor dinaciclib potently synergizes with cisplatin in preclinical models of ovarian cancer. *Oncotarget, 6*(17), 14926-14939.

Chen, Y., Germano, S., Clements, C., Samuel, J., Shelmani, G., Jayne, S., . . . Macip, S. (2016). Pro-survival signal inhibition by CDK inhibitor dinaciclib in chronic lymphocytic leukaemia. *British Journal of Haematology, 175*(4), 641-651.

Chlenski, A., Ketels, K. V., Korovaitseva, G. I., Talamonti, M. S., Oyasu, R., & Scarpelli, D. G. (2000). Organization and expression of the human zo-2 gene (tjp-2) in normal and neoplastic tissues. *Biochimica Et Biophysica Acta, 1493*(3), 319.

Chu, I., Sun, J., Sun, P., Arnaout, A., Kahn, H., Hanna, W., . . . Slingerland, J. (2007). p27 phosphorylation by src regulates inhibition of cyclin E-Cdk2. *Cell, 128*(2), 281-294.

Chumsri, S., Howes, T., Bao, T., Sabnis, G., & Brodie, A. (2011). Aromatase, aromatase inhibitors, and breast cancer. *Journal of Steroid Biochemistry and Molecular Biology, 125*(1), 13-22.

Chuprin, A., Gal, H., Biron-Shental, T., Biran, A., Amiel, A., Rozenblatt, S., & Krizhanovsky, V. (2013). Cell fusion induced by ERVWE1 or measles virus causes cellular senescence. *Genes and Development, 27*(21), 2356-2366.

Coppe, J., Patil, C. K., Rodier, F., Sun, Y., Muñoz, D. P., Goldstein, J., . . . Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biology, 6*(12), 2853-2868.

Cornen, S., Guille, A., Adélaïde, J., Addou-Klouche, L., Finetti, P., Saade, M., . . . Chaffanet, M. (2014). Candidate luminal B breast cancer genes identified by genome, gene expression and DNA methylation profiling. *PloS One, 9*(1), e81843.

Dai, M., Zhang, C., Ali, A., Hong, X., Tian, J., Lo, C., . . . Lebrun, J. (2016). CDK4 regulates cancer stemness and is a novel therapeutic target for triple-negative breast cancer. *Scientific Reports, 6*(1), 35383.

Dash, B. C., & El-Deiry, W. S. (2005). Phosphorylation of p21 in G2/M promotes cyclin B-Cdc2 kinase activity. *Molecular and Cellular Biology, 25*(8), 3364-3387.

Darby, S., McGale, P., Correa, C., Taylor, C., Arriagada, R., . . . Early Breast Cancer Trialists' Collaborative Group (EBCTCG). (2011). Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: Meta-analysis of individual patient data for 10 801 women in 17 randomised trials. *Lancet, the, 378*(9804), 1707-1716.

Dbouk, H. A., Mroue, R. M., El-Sabban, M. E., & Talhouk, R. S. (2009). Connexins: A myriad of functions extending beyond assembly of gap junction channels. *Cell Communication and Signaling, 7*(1), 4-4.

De Cremoux, P. (2011). Hormone therapy and breast cancer. *Bulletin Du Cancer, 98*(11), 1311-1319.

Decker, R. H., Dai, Y., & Grant, S. (2001). The cyclin-dependent kinase inhibitor flavopiridol induces apoptosis in human leukemia cells (U937) through the mitochondrial rather than the receptor-mediated pathway. *Cell Death and Differentiation, 8*(7), 715-724.

Degrassi, A., Russo, M., Nanni, C., Patton, V., Alzani, R., Giusti, A. M., . . . Texido, G. (2010). Efficacy of PHA-848125, a cyclin-dependent kinase inhibitor, on the K-RasG12DLA2 lung adenocarcinoma transgenic mouse model: Evaluation by multimodality imaging. *Molecular Cancer Therapeutics, 9*(3), 673-681.

Demaria, M., Ohtani, N., Youssef, S., Rodier, F., Toussaint, W., Mitchell, J., . . . Campisi, J. (2014). An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Developmental Cell, 31*(6), 722-733.

DePinto, W., Chu, X., Yin, X., Smith, M., Packman, K., Goelzer, P., . . . Heimbrook, D. (2006). In vitro and in vivo activity of R547: A potent and selective cyclin-dependent kinase inhibitor currently in phase I clinical trials. *Molecular Cancer Therapeutics, 5*(11), 2644-2658.

Desai, B. M., Villanueva, J., Nguyen, T. K., Lioni, M., Xiao, M., Kong, J., . . . Herlyn, M. (2013). The anti-melanoma activity of dinaciclib, a cyclin-dependent kinase inhibitor, is dependent on p53 signaling. *Plos One, 8*(3), e59588.

Desai, R. A., Gao, L., Raghavan, S., Liu, W. F., & Chen, C. S. (2009). Cell polarity triggered by cell-cell adhesion via E-cadherin. *Journal of Cell Science, 122*(7), 905-911.

Dolman, M. E. M., Poon, E., Ebus, M. E., den Hartog, Ilona J. M, van Noesel, Carel J. M, Jamin, Y., . . . Molenaar, J. J. (2015). Cyclin-dependent kinase inhibitor AT7519 as a potential drug for MYCN-dependent neuroblastoma. *Clinical Cancer Research, 21*(22), 5100-5109.

Dunn, J. M., Phillips, R. A., Becker, A. J., & Gallie, B. L. (1988). Identification of germline and somatic mutations affecting the retinoblastoma gene. *Science, 241*(4874), 1797-1800.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. *Genes and Development, 12*(15), 2245-2262.

Ender, L. (2012). 70 immune surveillance of senescent cells-biological significance in cancer- and non-cancer pathologies. *European Journal of Cancer, 48*, S17-S17.

Ekholm, S. V., Zickert, P., Reed, S. I., & Zetterberg, A. (2001). Accumulation of cyclin E is not a prerequisite for passage through the restriction point. *Molecular and Cellular Biology, 21*(9), 3256-3265.

Erlandsson, F., Linnman, C., Ekholm, S., Bengtsson, E., Zetterberg, A., Teknisk-naturvetenskapliga vetenskapsområdet, . . . Datoriserad bildanalys. (2000). A detailed analysis of cyclin A accumulation at the G1/S border in normal and transformed cells. *Experimental Cell Research, 259*(1), 86-95.

Fabre, C., Gobbi, M., Ezzili, C., Zoubir, M., Sablin, M., Small, K., . . . Le Tourneau, C. (2014). Clinical study of the novel cyclin-dependent kinase inhibitor dinaciclib in combination with rituximab in relapsed/refractory chronic lymphocytic leukemia patients. *Cancer Chemotherapy and Pharmacology, 74*(5), 1057-1064.

Fan, W., Chang, J., & Fu, P. (2015). Endocrine therapy resistance in breast cancer: Current status, possible mechanisms and overcoming strategies. *Future Medicinal Chemistry, 7*(12), 1511-1519.

Fang, L., Igarashi, M., Leung, J., Sugrue, M. M., Lee, S. W., & Aaronson, S. A. (1999). p21Waf1/Cip1/Sdi1 induces permanent growth arrest with markers of replicative senescence in human tumor cells lacking functional p53. *Oncogene, 18*(18), 2789-2797.

Feldmann, G., Mishra, A., Bisht, S., Karikari, C., Garrido-Laguna, I., Rasheed, Z., . . . Nelkin, B. D. (2011). Cyclin-dependent kinase inhibitor dinaciclib (SCH727965) inhibits pancreatic cancer growth and progression in murine xenograft models. *Cancer Biology & Therapy, 12*(7), 598-609.

Finn, R. S., Crown, J. P., Ettl, J., Schmidt, M., Bondarenko, I. M., Lang, I., . . . Slamon, D. J. (2016). Efficacy and safety of palbociclib in combination with letrozole as first-line treatment of ER-positive, HER2-negative, advanced breast cancer: Expanded analyses of subgroups from the randomized pivotal trial PALOMA-1/TRIO-18. *Breast Cancer Research : BCR, 18*(1), 67.

Finn, R., Crown, J., Lang, I., Boer, K., Bondarenko, I., Kulyk, S., . . . Slamon, D. (2012). Abstract S1-6: Results of a randomized phase 2 study of PD 0332991, a cyclin-dependent kinase (CDK) 4/6 inhibitor, in combination with letrozole vs letrozole alone for first-line treatment of ER+/HER2− advanced breast cancer (BC). *Cancer Research, 72*(24 Supplement), S1-S1-6.

Fitzgerald, A. L., Osman, A. A., Xie, T. -., Patel, A., Skinner, H., Sandulache, V., & Myers, J. N. (2015). Reactive oxygen species and p21Waf1/Cip1 are both essential for p53-mediated senescence of head and neck cancer cells. *Cell Death and Disease, 6*(3), e1678.

Flaherty, K. T., Lorusso, P. M., Demichele, A., Abramson, V. G., Courtney, R., Randolph, S. S., . . . Schwartz, G. K. (2012). Phase I, dose-escalation trial of the oral cyclin-dependent kinase 4/6 inhibitor PD 0332991, administered using a 21-day schedule in patients with advanced cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 18*(2), 568.

Forrest, N. C., Lindeman, G. J., Gyorki, D. E., Fox, S. B., Brown, M. A., French, J. D., . . . kConFab. (2009). Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nature Medicine, 15*(8), 907-913.

Foulkes, W. D., Smith, I. E., & Reis-Filho, J. S. (2010). Triple-negative breast cancer. *The New England Journal of Medicine, 363*(20), 1938-1948.

Freund, A., Laberge, R., Demaria, M., & Campisi, J. (2012). Lamin B1 loss is a senescence-associated biomarker. *Molecular Biology of the Cell, 23*(11), 2066-2075.

Fry, D. W., Harvey, P. J., Keller, P. R., Elliott, W. L., Meade, M., Trachet, E., . . . Toogood, P. L. (2004). Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. *Molecular Cancer Therapeutics, 3*(11), 1427-1437.

Ghia, P., Scarfo, L., Pathiraja, K., Derosier, M., Small, K., & Patton, N. (2015). A phase 3 study to evaluate the efficacy and safety of dinaciclib compared to ofatumumab in patients with refractory chronic lymphocytic leukemia.*blood , 126*(23), 4171.

Ghoncheh, M., Mahdavifar, N., Darvishi, E., & Salehiniya, H. (2016). Epidemiology, incidence and mortality of breast cancer in asia. *Asian Pacific Journal of Cancer Prevention, 17*(sup3), 47-52.

Giacinti, C., & Giordano, A. (2006). RB and cell cycle progression. *Oncogene, 25*(38), 5220-5227.

Gillett, C., Fantl, V., Smith, R., Fisher, C., Bartek, J., Dickson, C., . . . Peters, G. (1994). Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Research, 54*(7), 1812.

Gillet, C. E. (2000). The molecular basis of cell cycle and growth control. : Stein GS, baserga R, giordano A, et al, eds. (pound45.50.) wiley, 1998. ISBN 0 471 15706 6. *Molecular Pathology, 53*(6), 337-337.

Gojo, I., Sadowska, M., Walker, A., Feldman, E. J., Iyer, S. P., Baer, M. R., . . . Bannerji, R. (2013). Clinical and laboratory studies of the novel cyclin-dependent kinase inhibitor dinaciclib (SCH 727965) in acute leukemias. *Cancer Chemotherapy and Pharmacology, 72*(4), 897-908.

Gui, G. P. H., Wells, C. A., Browne, P. D., Yeomans, P., Jordan, S., Puddefoot, J. R., . . . Carpenter, R. (1995). Integrin expression in primary breast cancer and its relation to axillary nodal status. *Surgery, 117*(1), 102-108.

Gusterson, B. A., & Stein, T. (2012). Human breast development. *Seminars in Cell and Developmental Biology, 23*(5), 567-573.

Gusterson, B. A., Ross, D. T., Heath, V. J., & Stein, T. (2005). Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. *Breast Cancer Research : BCR, 7*(4), 143-148.

Hallstrom, T. C., Mori, S., & Nevins, J. R. (2008). An E2F1-dependent gene expression program that determines the balance between proliferation and cell death. *Cancer Cell, 13*(1), 11-22.

Harper, J. V., & Brooks, G. (2005). The mammalian cell cycle: An overview. *Methods in Molecular Biology (Clifton, N.J.), 296*, 113.

Hartsock, A., & Nelson, W. J. (2008). Adherens and tight junctions: Structure, function and connections to the actin cytoskeleton. *BBA - Biomembranes, 1778*(3), 660-669. He, S., & Sharpless, N. E. (2017). Senescence in health and disease. *Cell, 169*(6), 1000-1011.

Heath, E. I., Bible, K., Martell, R. E., Adelman, D. C., & LoRusso, P. M. (2008). A phase 1 study of SNS-032 (formerly BMS-387032), a potent inhibitor of cyclin-dependent kinases 2, 7 and 9 administered as a single oral dose and weekly infusion in patients with metastatic refractory solid tumors. *Investigational New Drugs, 26*(1), 59-65.

Henley, S. A., & Dick, F. A. (2012). The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. *Cell Division, 7*(1), 10-10.

Herbig, U., Jobling, W. A., Chen, B. P. C., Chen, D. J., & Sedivy, J. M. (2004). Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21CIP1, but not p16INK4a. *Molecular Cell, 14*(4), 501-513.

 Hernando, E., Krizhanovsky, V., Xue, W., Cordon-Cardo, C., Lowe, S. W., Miething, C., . . . Dickins, R. A. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature, 445*(7128), 656-660.

Hervé, J., Bourmeyster, N., & Sarrouilhe, D. (2004). Diversity in protein-protein interactions of connexins: Emerging roles. *Biochimica Et Biophysica Acta, 1662*(1-2), 22.

Hoevel, T., Macek, R., Swisshelm, K., & Kubbies, M. (2004). Reexpression of the TJ protein CLDN1 induces apoptosis in breast tumor spheroids. *International Journal of Cancer, 108*(3), 374-383.

Horiuchi, D., Kusdra, L., Huskey, N. E., Chandriani, S., Lenburg, M. E., Gonzalez-Angulo, A. M., . . . Goga, A. (2012). MYC pathway activation in triple-negative breast cancer is synthetic lethal with CDK inhibition. *Journal of Experimental Medicine, 209*(4), 679-696.

Horning, J. L., Sahoo, S. K., Vijayaraghavalu, S., Dimitrijevic, S., Vasir, J. K., Jain, T. K., . . . Labhasetwar, V. (2008). 3-D tumor model for in vitro evaluation of anticancer drugs. *Molecular Pharmaceutics, 5*(5), 849-862.

Howell, A., Cuzick, J., Baum, M., Buzdar, A., Dowsett, M., Forbes, J., . . . ATAC Trialists' Group. (2005). Results of the ATAC (arimidex, tamoxifen, alone or in combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *The Lancet, 365*(9453), 60-62.

Howlett, A., Bailey, N., Damsky, C., Petersen, O., & Bissell, M. (1995). cellular growth and survival are mediated by beta-1 integrins in normal human breast epithelium but not in breast-carcinoma. *Journal of Cell Science, 108*, 1945-1957.

Howlin, J., McBryan, J., Martin, F., Experimental Pathology, M., Lund University, Experimentell patologi, M., & Lunds universitet. (2006). Pubertal mammary gland development: Insights from mouse models. *Journal of Mammary Gland Biology and Neoplasia, 11*(3), 283-297.

Hu, Z., Fan, C., Oh, D. S., Marron, J. S., He, X., Qaqish, B. F., . . . Perou, C. M. (2006). The molecular portraits of breast tumors are conserved across microarray platforms. *BMC Genomics, 7*(1), 96-96.

Hubackova, S., Krejcikova, K., Bartek, J., & Hodny, Z. (2012). IL1-and TGFβ-Nox4 signaling, oxidative stress and DNA damage response are shared features of replicative, oncogene-induced, and drug-induced paracrine 'bystander senescence'. *Aging, 4*(12), 932-951.

James, M. K., Ray, A., Leznova, D., & Blain, S. W. (2008). Differential modification of p27Kip1 controls its cyclin D-cdk4 inhibitory activity. *Molecular and Cellular Biology, 28*(1), 498-510.

Jeon, J. S., Bersini, S., Gilardi, M., Dubini, G., Charest, J. L., Moretti, M., & Kamma, R. D. (2015). Human 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation. *Proceedings of the National Academy of Sciences of the United States of America, 112*(1), 214-219.

Johmura, Y., Shimada, M., Misaki, T., Naiki-Ito, A., Miyoshi, H., Motoyama, N., . . . Nakanishi, M. (2014). Necessary and sufficient role for a mitosis skip in senescence induction. *Molecular Cell, 55*(1), 73-84.

Jordan, V. C. (2008). The rise of raloxifene and the fall of invasive breast cancer. *Journal of the National Cancer Institute, 100*(12), 831-833.

Kang, D. H., Park, Y. S., & Lee, D. Y. (2018). Senotherapy for attenuation of cellular senescence in aging and organ implantation. *Journal of Industrial and Engineering Chemistry, 60*, 1-8.

Klein, D. K., Hoffmann, S., Ahlskog, J. K., O'Hanlon, K., Quaas, M., Larsen, B. D., . . . Sørensen, C. S. (2015). Cyclin F suppresses B-myb activity to promote cell cycle checkpoint control. *Nature Communications, 6*(1), 5800.

Knudsen, E., Hutcheson, J., Vail, P., & Witkiewicz, A. (2017). Biological specificity of CDK4/6 inhibitors: Dose response relationship, in vivo signaling, and composite response signature. *Oncotarget, 8*(27), 43678-43691.

Kopp, H., Hooper, A. T., Shmelkov, S. V., & Rafii, S. (2007). Beta-galactosidase staining on bone marrow. the osteoclast pitfall. *Histology and Histopathology, 22*(9), 971-976.

 Krishnamurthy, J., Torrice, C., Ramsey, M. R., Kovalev, G. I., Al-Regaiey, K., Su, L., & Sharpless, N. E. (2004). Ink4a/Arf expression is a biomarker of aging. *The Journal of Clinical Investigation, 114*(9), 1299-1307.

Kuilman, T., Michaloglou, C., Mooi, W. J., & Peeper, D. S. (2010). The essence of senescence. *Genes and Development, 24*(22), 2463-2479. Leikam, C., Hufnagel, A. L., Otto, C., Murphy, D. J., Mühling, B., Kneitz, S., . . . Meierjohann, S. (2015). In vitro evidence for senescent multinucleated melanocytes as a source for tumor-initiating cells. *Cell Death and Disease, 6*(4), e1711-e1711.

 Kuilman, T., Michaloglou, C., Vredeveld, L. C. W., Douma, S., van Doorn, R., Desmet, C. J., . . . Peeper, D. S. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell, 133*(6), 1019-1031.

Lallena, M. J., Boehnke, K., Torres, R., Hermoso, A., Amat, J., Calsina, B., . . . Mcnulty, A. (2015). Abstract 3101: In-vitro characterization of abemaciclib pharmacology in ER+ breast cancer cell lines. *Cancer Research, 75*(15 Supplement), 3101-3101.

Langhans, S. (2018). Three-dimensional in vitro cell culture models in drug discovery and drug repositioning. *Frontiers in Pharmacology, 9*.

Lee, B. Y., Han, J. A., Im, J. S., Morrone, A., Johung, K., Goodwin, E. C., . . . Hwang, E. S. (2006). Senescence-associated β-galactosidase is lysosomal β-galactosidase. *Aging Cell, 5*(2), 187-195.

Lehn, S. (2013). Cell cycle deregulation in breast cancer subgroups and effects on proliferation, migration and tamoxifen resistance Department of Laboratory Medicine, Lund University

Lelièvre, S. A. (2010). Tissue polarity-dependent control of mammary epithelial homeostasis and cancer development: An epigenetic perspective. *Journal of Mammary Gland Biology and Neoplasia, 15*(1), 49-63.

Lelievre, S. A., Weaver, V. M., Nickerson, J. A., Larabell, C. A., Bhaumik, A., Petersen, O. W., & Bissell, M. J. (1998). Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus. *Proceedings of the National Academy of Sciences of the United States of America, 95*(25), 14711-14716.

Lew, D. J., Dulić, V., & Reed, S. I. (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (cln) function in yeast. *Cell, 66*(6), 1197-1206.
Li, T., Kon, N., Jiang, L., Tan, M., Ludwig, T., Zhao, Y., . . . Gu, W. (2012). Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. *Cell, 149*(6), 1269-1283.

Liang, J., & Slingerland, J. M. (2003). Multiple roles of the PI3K/PKB (akt) pathway in cell cycle progression. *Cell Cycle, 2*(4), 336-342.

Lim, S., & Kaldis, P. (2013). Cdks, cyclins and CKIs: Roles beyond cell cycle regulation. *Development (Cambridge, England), 140*(15), 3079.

Lin, S., Lin, J., Hsueh, C., Chou, T., & Wong, R. J. (2017). A cyclin-dependent kinase inhibitor, dinaciclib in preclinical treatment models of thyroid cancer. *Plos One, 12*(2), e0172315.

Lodén, M., Stighall, M., Nielsen, N. H., Roos, G., Emdin, S. O., Ostlund, H., . . . Patologi, M. (2002). The cyclin D1 high and cyclin E high subgroups of breast cancer: Separate pathways in tumorogenesis based on pattern of genetic aberrations and inactivation of the pRb node. *Oncogene, 21*(30), 4680-4690.

Logan, J. E., Mostofizadeh, N., Desai, A. J., Von Euw, E., Conklin, D., Konkankit, V., . . . Kabbinavar, F. F. (2013). PD-0332991, a potent and selective inhibitor of cyclin-dependent kinase 4/6, demonstrates inhibition of proliferation in renal cell carcinoma at nanomolar concentrations and molecular markers predict for sensitivity. *Anticancer Research, 33*(8), 2997-3004.

Löschmann, N., Michaelis, M., Rothweiler, F., Zehner, R., Cinatl, J., Voges, Y., . . . Cinatl, J. (2013). Testing of SNS-032 in a panel of human neuroblastoma cell lines with acquired resistance to a broad range of drugs. *Translational Oncology, 6*(6), 685-IN18.

Louie, M. C., McClellan, A., Siewit, C., & Kawabata, L. (2010). Estrogen receptor regulates E2F1 expression to mediate tamoxifen resistance. *Molecular Cancer Research, 8*(3), 343-352.

Lovitt, C. J., Shelper, T. B., & Avery, V. M. (2014). Advanced cell culture techniques for cancer drug discovery. *Biology, 3*(2), 345-367.

Ma, Z., Wu, Y., Jin, J., Yan, J., Kuang, S., Zhou, M., . . . Guo, A. (2013). Phylogenetic analysis reveals the evolution and diversification of cyclins in eukaryotes. *Molecular Phylogenetics and Evolution, 66*(3), 1002-1010.

Martin, F., Labbé, J., Dorée, M., Martinez, A., Afshar, M., & Cavadore, J. (1997). Dual phosphorylation of the T-loop in cdk7: Its role in controlling cyclin H binding and CAK activity. *The EMBO Journal, 16*(2), 343-354.

Martin, T., Watkins, G., Mansel, R., & Jiang, W. (2018). Loss of tight junction plaque molecules in breast cancer tissues is associated with a poor prognosis in patients with breast cancer (vol 40, pg 2717, 2004). *European Journal of Cancer, 88*, 115-116.

Masoud, V., & Pagès, G. (2017). Targeted therapies in breast cancer: New challenges to fight against resistance. *World Journal of Clinical Oncology, 8*(2), 120-134.

Matsen, C. B., & Neumayer, L. A. (2013). Breast cancer: A review for the general surgeon. *JAMA Surgery, 148*(10), 971-979.

Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., . . . Elledge, S. J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 cdk inhibitor family, is a candidate tumor suppressor gene. *Genes & Development, 9*(6), 650-662.

Mazumder, S., DuPree, E. L., & Almasan, A. (2004). A dual role of cyclin E in cell proliferation and apotosis may provide a target for cancer therapy. *Current Cancer Drug Targets, 4*(1), 65-75.

Mercurio, C., dal Zuffo, R., Liontos, M., d'Ario, G., Hahn, W. C., Gorgoulis, V., . . . Botrugno, O. A. (2011). Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. *Nature Cell Biology, 13*(3), 292-302.

Mita, M., Mita, A., Moseley, J., Poon, J., Small, K., Jou, Y., . . . Shapiro, G. (2011). A phase I study of the CDK inhibitor dinaciclib (SCH 727965) administered every 3

weeks in patients (pts) with advanced malignancies: Final results. *Journal of Clinical Oncology, 29*(15) 3080-3080.

Mitic, L. L., Schneeberger, E. E., Fanning, A. S., & Anderson, J. M. (1999). Connexin-occludin chimeras containing the ZO-binding domain of occludin localize at MDCK tight junctions and NRK cell contacts. *The Journal of Cell Biology, 146*(3), 683-693.

Mitri, Z., Karakas, C., Wei, C., Briones, B., Simmons, H., Ibrahim, N., . . . Moulder, S. (2015). A phase 1 study with dose expansion of the CDK inhibitor dinaciclib (SCH 727965) in combination with epirubicin in patients with metastatic triple negative breast cancer. *Investigational New Drugs, 33*(4), 890-894.

Morikawa, A., & Henry, N. L. (2015). Palbociclib for the treatment of estrogen receptor-positive, HER2-negative metastatic breast cancer. *Clinical Cancer Research, 21*(16), 3591-3596.

Muñoz-Espín, D., & Serrano, M. (2014). Cellular senescence: From physiology to pathology. *Nature Reviews Molecular Cell Biology, 15*(7), 482-496.

Muñoz-Espín, D., Cañamero, M., Maraver, A., Gómez-López, G., Contreras, J., Murillo-Cuesta, S., . . . Serrano, M. (2013). Programmed cell senescence during mammalian embryonic development. *Cell, 155*(5), 1104-1118.

Nahta, R., Takahashi, T., Ueno, N.T., Hung, M.C., & Esteva ,F.J. (2004). P27(kip1) downregulation is associated with trastuzumab resistance in breast cancer cells. *Cancer Research*, 64(11), 3981-3986.

Narita, M., Narita, M., Nuñez, S., Heard, E., Lin, A. W., Hearn, S. A., . . . Lowe, S. W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell, 113*(6), 703-716.

Natali, P., Nicotra, M., Botti, C., Mottolese, M., Bigotti, A., & Segatto, O. (1992). changes in expression of alpha-6-beta-4 integrin heterodimer in primary and metastatic breast-cancer. *British Journal of Cancer, 66*(2), 318-322.

Nemunaitis, J. J., Small, K. A., Kirschmeier, P., Zhang, D., Zhu, Y., Jou, Y., . . . Bannerji, R. (2013). A first-in-human, phase 1, dose-escalation study of dinaciclib, a novel cyclin-dependent kinase inhibitor, administered weekly in subjects with advanced malignancies. *Journal of Translational Medicine, 11*(1), 259-259.

Nisticò, P., Di Modugno, F., Spada, S., & Bissell, M. J. (2014). β1 and β4 integrins: From breast development to clinical practice. *Breast Cancer Research: BCR, 16*(5), 459-459.

Novartis Pharmaceutucals. KIQALI (ribociclib) tablets, for oral use: US prescribing Information. 2017. http://www.fda.gov. Accessed 31 Mar 2017.

Oakes, S. R., Hilton, H. N., & Ormandy, C. J. (2006). Key stages in mammary gland development: The alveolar switch: Coordinating the proliferative cues and cell fate decisions that drive the formation of lobuloalveoli from ductal epithelium. *Breast Cancer Research, 8*(2), 207-207.

Ohtani, N., Zebedee, Z., Huot, T. J. G., Stinson, J. A., Sugimoto, M., Ohashi, Y., . . . Hara, E. (2001). Opposing effects of ets and id proteins on p16INK4a expression during cellular senescence. *Nature, 409*(6823), 1067-1070.

Ojakian, G., & Schwimmer, R. (1994). regulation of epithelial-cell surface polarity reversal by beta (1)-integrins. *Journal of Cell Science, 107*, 561-576.

Onder, T. T., Gupta, P. B., Mani, S. A., Yang, J., Lander, E. S., & Weinberg, R. A. (2008). Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Research, 68*(10), 3645-3654.

Pampalona, J., Frías, C., Genescà, A., & Tusell, L. (2012). Progressive telomere dysfunction causes cytokinesis failure and leads to the accumulation of polyploid cells. *PLoS Genetics, 8*(4), 601-611.

Parikh, A. P., Curtis, R. E., Kuhn, I., Becker-Weimann, S., Bissell, M., Xing, E. P., & Wu, W. (2014). Network analysis of breast cancer progression and reversal using a tree-evolving network algorithm. *PLoS Computational Biology, 10*(7), e1003713.

Park, C. C., Zhang, H., Pallavicini, M., Gray, J. W., Baehner, F., Park, C. J., & Bissell, M. J. (2006). β1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. *Cancer Research, 66*(3), 1526-1535.

Parry, D., Guzi, T., Shanahan, F., Davis, N., Prabhavalkar, D., Wiswell, D., . . . Lees, E. M. (2010). Dinaciclib (SCH 727965), a novel and potent cyclin-dependent kinase inhibitor. *Molecular Cancer Therapeutics, 9*(8), 2344-2353.

Patel, P., Asbach, B., Shteyn, E., Gomez, C., Coltoff, A., Bhuyan, S., . . . Blain, S. W. (2015). Brk/Protein tyrosine kinase 6 phosphorylates p27KIP1, regulating the activity of cyclin D-cyclin-dependent kinase 4. *Molecular and Cellular Biology, 35*(9), 1506-1522.

Peppercorn, J., Perou, C. M., & Carey, L. A. (2008). Molecular subtypes in breast cancer evaluation and management: Divide and conquer. *Cancer Investigation, 26*(1), 1-10.

Perna, D., Hydbring, P., Doni, M., Murga, M., Barbacid, M., Campaner, S., . . . Sardella, D. (2010). Cdk2 suppresses cellular senescence induced by the c-myc oncogene. *Nature Cell Biology, 12*(1), 54-59.

Perou, C. M., & Børresen-Dale, A. (2011). Systems biology and genomics of breast cancer. *Cold Spring Harbor Perspectives in Biology, 3*(2), 1-17.

Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., . . . Botstein, D. (2000). *Molecular portraits of human breast tumours.* (). United States: USDOE; National Cancer Institute, Howard Hughes Medical Institute, Life Sciences Research Foundation, Norwegian Cancer Society, Alfred P. Sloan Foundation, Walter and Idun Berry Fellow.

Petersen, O. W., Ronnov-Jessen, L., Howlett, A. R., & Bissell, M. J. (1992). Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America, 89*(19), 9064-9068.

Peyressatre, M., Prével, C., Pellerano, M., & Morris, M. C. (2015). Targeting cyclin-dependent kinases in human cancers: From small molecules to peptide inhibitors. *Cancers, 7*(1), 179-237.

Phase I/Ib dose-escalation of dinaciclib with weekly paclitaxel for advanced solid tumor malignancies & assessment of MYC oncogene overexpression. 2014. Available from: http://clinicaltrials.gov/ show/NCT01676753.

Pines, J., & Hunter, T. (1989). Isolation of a human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. *Cell, 58*(5), 833-846.

Plachot, C., & Lelievre, S. (2004). DNA methylation control of tissu polarity and cellular differentiatin in mammary epithelium. *Experimental Cell Research,* 298,122–132.

Plachot, C., Chaboub, L. S., Adissu, H. A., Wang, L., Urazaev, A., Sturgis, J., . . . Lelièvre, S. A. (2009). Factors necessary to produce basoapical polarity in human glandular epithelium formed in conventional and high-throughput three-dimensional culture: Example of the breast epithelium. *BMC Biology, 7*(1), 77-77.

Polette, M., Gilles, C., Nawrocki-Raby, B., Lohi, J., Hunziker, W., Foidart, J., & Birembaut, P. (2005). Membrane-type 1 matrix metalloproteinase expression is regulated by zonula occludens-1 in human breast cancer cells. *Cancer Research, 65*(17), 7691-7698.

Puyol, M., Martín, A., Dubus, P., Mulero, F., Pizcueta, P., Khan, G., . . . Barbacid, M. (2010). A synthetic lethal interaction between K-ras oncogenes and Cdk4 unveils a therapeutic strategy for non-small cell lung carcinoma. *Cancer Cell, 18*(1), 63-73.

Qian, Y., Zhang, J., Yan, B., & Chen, X. (2008). DEC1, a basic helix-loop-helix transcription factor and a novel target gene of the p53 family, mediates p53-dependent premature senescence. *Journal of Biological Chemistry, 283*(5), 2896-2905.

Qin, G., Xu, F., Qin, T., Zheng, Q., Shi, D., Xia, W., . . . Wang, S. (2015). Palbociclib inhibits epithelial-mesenchymal transition and metastasis in breast cancer via c-Jun/COX-2 signaling pathway. *Oncotarget, 6*(39), 41794-41808.

Rader, J., Russell, M. R., Hart, L. S., Nakazawa, M. S., Belcastro, L. T., Martinez, D., . . . Maris, J. M. (2013). Dual CDK4/CDK6 inhibition induces cell-cycle arrest and senescence in neuroblastoma. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research, 19*(22), 6173-6182.

Raje, N., Hari, P., Landau, H., Richardson, P., Rosenblatt, J., Couture, N., . . . Yule, M. (2013). A phase I/II open-label multicenter study of the cyclin kinase inhibitor AT7519M alone and in combination with bortezomib in patients with previously treated multiple myeloma. *Blood*, *122*(21), 1976.

Rajput, S., Khera, N., Guo, Z., Hoog, J., Li, S., & Ma, C. X. (2016). Inhibition of cyclin dependent kinase 9 by dinaciclib suppresses cyclin B1 expression and tumor growth in triple negative breast cancer. *Oncotarget, 7*(35), 56864-56875.

Rao, S., Stoehr, J., Dokic, D., Wan, L., Decker, J., Konopka, K., . . . Jeruss, J. (2017). Synergistic effect of eribulin and CDK inhibition for the treatment of triple negative breast cancer. *Oncotarget, 8*(48), 83925-83939.

Rizki, A., Weaver, V. M., Lee, S., Rozenberg, G. I., Chin, K., Myers, C. A., . . . Bissell, M. J. (2008). A human breast cell model of preinvasive to invasive transition. *Cancer Research, 68*(5), 1378-1387.

Rocca, A., Farolfi, A., Bravaccini, S., Schirone, A., & Amadori, D. (2014). Palbociclib (PD 0332991): Targeting the cell cycle machinery in breast cancer. *Expert Opinion on Pharmacotherapy, 15*(3), 407-420.

Roskoski, R. (2016). Cyclin-dependent protein kinase inhibitors including palbociclib as anticancer drugs. *Pharmacological Research, 107*, 249-275.

Rossen, K., Dahlstrom, K. K., Mercurio, A. M., & Wewer, U. M. (1994). Expression of the α6β4 integrin by squamous cell carcinomas and basal cell carcinomas: Possible relation to invasive potential? *Acta Dermato-Venereologica, 74*(2), 101-105.

Sagiv, A., Biran, A., Yon, M., Simon, J., Lowe, S. W., & Krizhanovsky, V. (2013). Granule exocytosis mediates immune surveillance of senescent cells. *Oncogene, 32*(15), 1971-1977.

Sangar, M., Genovesi, L., Nakamoto, M., Davis, M., Knobluagh, S., Ji, P., . . . Olson, J. (2017). Inhibition of CDK4/6 by palbociclib significantly extends survival in medulloblastoma patient-derived xenograft mouse models. *Clinical Cancer Research, 23*(19), 5802-5813.

Santo, L., Vallet, S., Hideshima, T., Cirstea, D., Ikeda, H., Pozzi, S., . . . Raje, N. (2010). AT7519, A novel small molecule multi-cyclin-dependent kinase inhibitor, induces apoptosis in multiple myeloma via GSK-3beta activation and RNA polymerase II inhibition. *Oncogene, 29*(16), 2325.

Sasaki, M., Kajiya, H., Ozeki, S., Okabe, K., & Ikebe, T. (2014). Reactive oxygen species promotes cellular senescence in normal human epidermal keratinocytes through epigenetic regulation of p16INK4a. *Biochemical and Biophysical Research Communications, 452*(3), 622-628.

Scaffidi, P., & Misteli, T. (2006). Lamin A-dependent nuclear defects in human aging. *Science, 312*(5776), 1059-1063.

Scaltriti, M., Eichhorn, P. J., Cortés, J., Prudkin, L., Aura, C., Jiménez, J., . . . Arteaga, C. L. (2011). Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2$^+$ breast cancer patients. *Proceedings of the National Academy of Sciences of the United States of America, 108*(9), 3761-3766.

Schmidhauser, C., Schmidhauser, C., Casperson, G. F., Casperson, G. F., Myers, C. A., Myers, C. A., . . . Bissell, M. J. (1992). A novel transcriptional enhancer is involved in the prolactin- and extracellular matrix-dependent regulation of β-casein gene expression. *Molecular Biology of the Cell, 3*(6), 699-709.

Schoenenberger, C., Zuk, A., Zinkl, G. M., Kendall, D., & Matlin, K. S. (1994). Integrin expression and localization in normal MDCK cells and transformed MDCK cells lacking apical polarity. *Journal of Cell Science, 107*(2), 527-541.

Sedlacek, H. H., Czech, J., Naik, R., Kaur, G., Worland, P., Losiewicz, M., . . . Sausville, E. (1996). Flavopiridol (L86 8275; NSC 649890), a new kinase inhibitor for tumor therapy. *International Journal of Oncology, 9*(6), 1143-1168.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., & Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16(INK4a). *Cell, 88*(5), 593-602.

Shimi, T., Butin-Israeli, V., Adam, S. A., Hamanaka, R. B., Goldman, A. E., Lucas, C. A., . . . Goldman, R. D. (2011). The role of nuclear lamin B1 in cell proliferation and senescence. *Genes and Development, 25*(24), 2579-2593.

Simone, C., & Giordano, A. (2001). New insight in cdk9 function: From tat to MyoD. *Frontiers in Bioscience: A Journal and Virtual Library, 6*, D1073.

Singh, R., George, J., & Shukla, Y. (2010). Role of senescence and mitotic catastrophe in cancer therapy. *Cell Division, 5*(1), 4-4.

Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., . . . Børresen-Dale, A. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America, 98*(19), 10869-10874.

Squires, M. S., Feltell, R. E., Wallis, N. G., Lewis, E. J., Smith, D., Cross, D. M., . . . Thompson, N. T. (2009). Biological characterization of AT7519, a small-molecule inhibitor of cyclin-dependent kinases, in human tumor cell lines. *Molecular Cancer Therapeutics, 8*(2), 324-332.

Storer, M., Mas, A., Robert-Moreno, A., Pecoraro, M., Ortells, M. ., Di Giacomo, V., . . . Keyes, W. (2013). Senescence is a developmental mechanism that contributes to embryonic growth and patterning. *Cell, 155*(5), 1119-1130.

Swedish Breast Cancer Group (SweBCG): National Guidelines for Treatment of Breast Cancer. 2011.

Symington, B. E., Takada, Y., & Carter, W. G. (1993). Interaction of integrins α3β1 and α2β1: Potential role in keratinocyte intercellular adhesion. *Journal of Cell Biology, 120*(2), 523-535.

Taddei, I., Faraldo, M. M., Teulière, J., Deugnier, M., Thiery, J. P., & Glukhova, M. A. (2003). Integrins in mammary gland development and differentiation of mammary epithelium. *Journal of Mammary Gland Biology and Neoplasia, 8*(4), 383-394.

Takahashi, A., Ohtani, N., Yamakoshi, K., Iida, S., Tahara, H., Nakayama, K., . . . Hara, E. (2006). Mitogenic signalling and the p16INK4a-rb pathway cooperate to enforce irreversible cellular senescence. *Nature Cell Biology, 8*(11), 1291-1297.

Talhouk, R. S., Mroue, R., Mokalled, M., Abi-Mosleh, L., Nehme, R., Ismail, A., . . . El-Sabban, M. E. (2008). Heterocellular interaction enhances recruitment of α and β-catenins and ZO-2 into functional gap-junction complexes and induces gap junction-dependant differentiation of mammary epithelial cells. *Experimental Cell Research, 314*(18), 3275-3291.

Tarasewicz, E., Rivas, L., Hamdan, R., Dokic, D., Parimi, V., Bernabe, B. P., . . . Jeruss, J. S. (2014). Inhibition of CDK-mediated phosphorylation of Smad3 results in decreased oncogenesis in triple negative breast cancer cells. *Cell Cycle, 13*(20), 3191-3201.

Thomas, A. L., Lind, H., Hong, A., Dokic, D., Oppat, K., Rosenthal, E., . . . Jeruss, J. S. (2017). Inhibition of CDK-mediated Smad3 phosphorylation reduces the Pin1-Smad3

interaction and aggressiveness of triple negative breast cancer cells. *Cell Cycle, 16*(15), 1453-1464.

Tong, W., Chen, R., Plunkett, W., Siegel, D., Sinha, R., Harvey, R. D., . . . Wierda, W. G. (2010). Phase I and pharmacologic study of SNS-032, a potent and selective Cdk2, 7, and 9 inhibitor, in patients with advanced chronic lymphocytic leukemia and multiple myeloma. *Journal of Clinical Oncology, 28*(18), 3015-3022.

Toogood, P. L., Harvey, P. J., Repine, J. T., Sheehan, D. J., VanderWel, S. N., Zhou, H., . . . Fry, D. W. (2005). Discovery of a potent and selective inhibitor of cyclin-dependent kinase 4/6. *Journal of Medicinal Chemistry, 48*(7), 2388-2406.

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J., & Jemal, A. (2015). Global cancer   statistics, 2012. *CA: A Cancer Journal for Clinicians, 65*(2), 87-108.

Tzu, J., & Marinkovich, M. P. (2008). Bridging structure with function: Structural, regulatory, and developmental role of laminins. *International Journal of Biochemistry and Cell Biology, 40*(2), 199-214.

Utz, H., Wei, W., Annie, D., Wendy. A, J., & John.M, S. (2003). Real-time imaging of transcriptional activation in live cells reveals rapid up-regulation of the cyclin-dependent kinase inhibitor gene CDKN1A in replicative cellular senescence. *Aging Cell, 2*(6), 295-304.

Veliparib and dinaciclib with or without carboplatin in treating patients with advanced solid tumors. 2014. Available from: http://clinicaltrials.gov/ show/NCT01434316. Vermeulen, K., Van Bockstaele, D.R., &Berneman, Z.N. (2003). The cell cycle: A review of regulation, deregulation and therapeutic targets in cancer. *Cell Proliferation, 36*(3), 131-149.

Vidi, P., Bissell, M. J., & Lelièvre, S. A. (2013). Three-dimensional culture of human breast epithelial cells: The how and the why. *Methods in Molecular Biology, 945,* 193-219.

Vidi, P., Maleki, T., Ochoa, M., Wang, L., Clark, S. M., Leary, J. F., & Lelièvre, S. A. (2013;2014;). Disease-on-a-chip: Mimicry of tumor growth in mammary ducts. *Lab on a Chip, 14*(1), 172-177.

Wahba, H., EI-Hadaad. H., (2015). Current approaches in treatment of triple-negative breast cancer. *Cancer Biol Med, 12*(2), 106-116.

Walker, A. J., Wedam, S., Amiri-Kordestani, L., Bloomquist, E., Tang, S., Sridhara, R., . . . Pazdur, R. (2016). FDA approval of palbociclib in combination with fulvestrant for the treatment of hormone receptor-positive, HER2-negative metastatic breast cancer. *Clinical Cancer Research, 22*(20), 4968-4972.

Walsby, E., Lazenby, M., Pepper, C., & Burnett, A. K. (2011). The cyclin-dependent kinase inhibitor SNS-032 has single agent activity in AML cells and is highly synergistic with cytarabine. *Leukemia, 25*(3), 411-419.

Wang, D., Sun, Y., Li, W., Ye, F., Zhang, Y., Guo, Y., . . . Suo, J. (2018). Antiproliferative effects of the CDK6 inhibitor PD0332991 and its effect on signaling networks in gastric cancer cells. *International Journal of Molecular Medicine, 41*(5), 2473-2484.

Wang, F., Hansen, R. K., Radisky, D., Yoneda, T., Barcellos-Hoff, M. H., Petersen, O. W., . . . Bissell, M. J. (2002). Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. *Journal of the National Cancer Institute, 94*(19), 1494-1503.

Wang, F., Weaver, V. M., Petersen, O. W., Larabell, C. A., Dedhar, S., Briand, P., . . . Bissell, M. J. (1998). Reciprocal interactions between β1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: A different perspective in epithelial biology. *Proceedings of the National Academy of Sciences of the United States of America, 95*(25), 14821-14826.

Watt, F. M., Kubler, M. -., Hotchin, N. A., Nicholson, L. J., & Adams, J. C. (1993). Regulation of keratinocyte terminal differentiation by integrin-extracellular matrix interactions. *Journal of Cell Science, 106*(1), 175-182.

Weaver, V. M., Petersen, O. W., Wang, F., Larabell, C. A., Briand, P., Damsky, C., & Bissell, M. J. (1997). Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *The Journal of Cell Biology, 137*(1), 231-245.

Weaver, V., Lelievre, S., Lakins, J., Chrenek, M., Jones, J., Giancotti, F., . . . Bissell, M. (2002). beta 4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. *Cancer Cell, 2*(3), 205-216.

Weigelt, B., Lo, A. T., Park, C. C., Gray, J. W., & Bissell, M. J. (2010). HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment. *Breast Cancer Research and Treatment, 122*(1), 35-43.

Wolff, A. C., & Davidson, N. E. (2001). Use of SERMs for the adjuvant therapy of Early-Stage breast cancer. *Annals of the New York Academy of Sciences, 949*(1), 80-88.

Wong, E. S. M., Le Guezennec, X., Demidov, O. N., Marshall, N. T., Wang, S. T., Krishnamurthy, J., . . . Bulavin, D. V. (2009). p38MAPK controls expression of

multiple cell cycle inhibitors and islet proliferation with advancing age. *Developmental Cell, 17*(1), 142-149.

Xu, R., Spencer, V. A., & Bissell, M. J. (2007). Extracellular matrix-regulated gene expression requires cooperation of SWI/SNF and transcription factors. *Journal of Biological Chemistry, 282*(20), 14992-14999.

Yang, N., & Hu, M. (2005). The limitations and validities of senescence associated-β-galactosidase activity as an aging marker for human foreskin fibroblast Hs68 cells. *Experimental Gerontology, 40*(10), 813-819.

Yao, H., He, G., Yan, S., Chen, C., Song, L., Rosol, T. J., & Deng, X. (2017). Triple-negative breast cancer: Is there a treatment on the horizon? *Oncotarget, 8*(1), 1913-1924.

Yuan, N., Meng, M., Liu, C., Feng, L., Hou, L., Ning, Q., . . . Zhao, X. (2014). Clinical characteristics and prognostic analysis of triple-negative breast cancer patients. *Molecular and Clinical Oncology, 2*(2), 245-251.

Yun, M. H., Davaapil, H., & Brockes, J. P. (2015). Recurrent turnover of senescent cells during regeneration of a complex structure. *Elife, 4*(MAY).

Zalzali, H., Harajly, M., Abdul-Latif, L., El-Chaar, N., Dbaibo, G., Skapek, S. X., & Saab, R. (2012). Temporally distinct roles for tumor suppressor pathways in cell cycle arrest and cellular senescence in cyclin D1-driven tumor. *Molecular Cancer, 11*(1), 28-28.

Zalzali, H., Nasr, B., Harajly, M., Basma, H., Ghamloush, F., Ghayad, S., . . . Saab, R. (2015). CDK2 transcriptional repression is an essential effector in p53-dependent cellular senescence-implications for therapeutic intervention. *Molecular Cancer Research: MCR, 13*(1), 29-40.

Zhang, R., Chen, W., & Adams, P. D. (2007). Molecular dissection of formation of senescence-associated heterochromatin foci. *Molecular and Cellular Biology, 27*(6), 2343-2358.

Zhou, J., Ng, A. Y. N., Tymms, M. J., Jermiin, L. S., Seth, A. K., Thomas, R. S., & Kola, I. (1998). A novel transcription factor, ELF5, belongs to the ELF subfamily of ETS genes and maps to human chromosome 11p13-15, a region subject to LOH and rearrangement in human carcinoma cell lines. *Oncogene, 17*(21), 2719-2732.

Zihni, C., Balda, M. S., & Matter, K. (2014). Signalling at tight junctions during epithelial differentiation and microbial pathogenesis. *Journal of Cell Science, 127*(Pt 16), 3401.