THE ROLES OF PROTEIN KINASE D2 IN CHEMORESISTANT BREAST CANCER CELL LINES

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ABSTRACT

THE ROLES OF PROTEIN KINASE D2 IN CHEMORESISTANT BREAST CANCER CELL LINES

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Even though chemotherapy keeps its position as the most preferred and potent strategy of cancer treatment, resistance of tumor to the anti-neoplastic drug poses an obstacle for chemotherapy success. Multidrug resistance (MDR) is a phenomenon that is defined as the intrinsic or acquired resistance against structurally and functionally unrelated drugs. Acquisition of multidrug resistance can be through several distinct mechanisms such as increased drug efflux by ABC transporters, increased drug detoxification through phase I and II enzymes, altered death pathways and increased damage repair, making MDR a multifaceted problem that remodel many regulatory or metabolic circuits. MDR phenotype has also been linked to increased aggressiveness marked by mobility and invasiveness or vice versa.

Protein kinase D2 (PKD2) is one of the isoforms in three-membered serine/threonine kinase family, PKD. PKD family members can possess redundant as well as specific roles on proliferation, survival, angiogenesis and motility, the events that are relevant to cancer. In glioblastoma, leukemia, colorectal, pancreas and breast cancer, tumor promoting and suppressing roles of PKD members have been shown. In particular,

breast cancer, the most common cancer type in women, PKD2 and PKD3 appear to have oncogenic roles while PKD1 possesses tumor-suppressive functions. Specifically, PKD2 seems to be ubiquitous in many breast cancer types, while PKD1 and PKD3 are not.

To this end, we aimed to characterize the ubiquitous member, PKD2, in a panel of breast cancer cell lines. We found that the expression of PKD2 does not differ between cell lines, whereas its basal level activity is higher in chemoresistant MCF7 derivatives compared to parental MCF7 cell line, implying that PKD2 may have role in drug resistance and associated phenotypes. Cell proliferation assay showed that PKD2 downregulation does not affect the drug resistance in MCF7/DOX cells. PKD2 knockdown also does not significantly change the expression of potential PKD targets that are implicated in MDR and apoptosis. MCF7/DOX cells are phenotypically different from parental cell line such that they have higher expression of epithelial to mesenchymal transition markers, higher mobility and invasive characteristics. Since PKD2 is also implicated in motility we checked whether PKD2 downregulation influences the migration of MCF7/DOX cells towards a chemoattractant. The migration assay showed that PKD2 downregulation suppresses the migration of MCF7/DOX cells.

The data implied that under this experimental setup PKD2 did not alter the drug resistance whereas it changes the migration potential of doxorubicin resistant MCF7 cell line. Further research is needed to uncover the roles of other isoforms PKD1 and PKD3 as well as upstream regulators of PKD members in chemoresistance.

Keywords: Cancer, drug resistance, protein kinase D

ÖZ

PROTEİN KİNAZ D2'NIN İLAÇ DİRENÇLİ MEME KANSERİ HÜCRE HATLARINDAKİ İŞLEVLERİ

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Kemoterapi, kanser tedavisinde en etkin ve en çok başvurulan yöntemdir. Ancak, tümörün kemoterapi ilaçlarına karşı gösterdiği direnç, başarılı kemoterapi uygulamalarının önündeki en önemli engellerden biridir. Çoklu ilaç dirençliliği yapısal ve işlevsel olarak birbirinden farklı olan ilaçlara karşı, baştan beri varolan ya da sonradan edinilen dirençlilik olarak tanımlanır. Çoklu ilaç dirençliliği çeşitli mekanizmalarla gerçekleşebilir. Bunlardan en yaygın olanları; ABC sınıfı taşıyıcı proteinler vasıtasıyla ilacın hücre dışına pompalanması, ilacın metabolize edilerek etkisiz hale gelmesi, ilacın toksik etkisinin ölümle sonuçlanmaması, ilacın yol açtığı DNA hasarının çabuk giderilmesi şeklinde sıralanabilir. Sözkonusu mekanizmalara bakıldığında çoklu ilaç dirençliliği probleminin hücrenin metabolik ve diğer düzenleyici yolakları ile yakından ilişkili olan çok yönlü bir problem olduğu göze çarpmaktadır. Tüm bunların yanında çoklu ilaç dirençliliğinin agresif tümörlerde gözlenen invazyon ve motilite ile bağlantılı olduğu gösterilmiştir.

Protein kinaz D2, üç üyeden oluşan protein kinaz D ailesinin bir üyesidir. PKD ailesinin üyeleri, benzer işlevler üstlenebildikleri gibi herbirinin kendilerine özgü işlevleri de

olabilir. PKD üyeleri hücre çoğalması, sağkalımı, anjiyojenez, hücre hareketliliği gibi hücre için gerekli ve aynı zamanda kanser ile bağlantılı olaylarda rol alırlar. Gliyoblastom, lösemi, kalın bağırsak kanseri, pankreas kanseri ve meme kanseri gibi kanser türlerinde PKD üyelerinin zaman zaman benzer zaman zaman da birbirine karşıt roller üstlendikleri gözlenmiştir. Özellikle meme kanserinde PKD2 ve PKD3 enzimlerinin tümör destekleyici, PKD1'in ise tümör baskılayıcı roller üstlenebildikleri düşünülmektedir.

Bu çalışmamızda, çoğu meme kanseri alt türünde yaygın olarak gözlenen PKD üyesi PKD2 ilaç dirençli hücrelerde karakterizasyonu yapılmıştır. PKD2'nin; reseptör statüsü farklı olan meme kanseri hücre hatlarında ve MCF7 hücre hattının ilaç dirençli türevlerinde ifade edildiği saptanmıştır. Bazal PKD2 aktivitesinin ise hücre hatları arasında farklılık gösterdiği; aktif PKD2 düzeyinin ilaç dirençli MCF7 türevlerinde, MCF7 hattına göre daha fazla olduğu tespit edilmiştir. Buradan, yüksek PKD2 aktivitesinin ilaç dirençliliği ya da ilaç dirençliliğinin yol açtığı diğer fenotipler ile ilişkili olabileceği çıkarımı yapılmıştır. Hücre çoğalma analizi, PKD2'nin susturulduğu MCF7/DOX türevinin doksorubisin dirençliliğinde bir değişim olmadığını göstermiştir. Ayrıca, PKD2'nin susturulması, MCF7/DOX hücrelerinde apoptoz ve çoklu ilaç dirençliliği ile ilişkili kimi genlerin ifadelerinde değişikliğe yol açmamıştır. MCF7/DOX hücreleri, MCF7 hücrelerinden farklı olarak, yüksek oranda epitelyal-mezenşimal geçiş markörleri ifade eden motil ve invazif karakterde hücrelerdir. PKD2 susturulduğunda MCF7/DOX hücrelerinin yönlendirilmiş migrasyonunda düşüş saptanmıştır.

Sonuçlar, PKD2'nin geçici susturulmasının PKD2 ifadesinin önemli ölçüde azalttığını ancak bu etkinin MCF7/DOX hücrelerinin ilaç dirençliliğini etkilemediğini; yönlendirilmiş migrasyonu ise azalttığını göstermiştir.

Anahtar Sözcükler: Kanser, ilaç dirençliliği, protein kinaz D

To Ekin and Kerem,

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ACTB	β-actin (encoding gene)
AP-1	Activator protein 1
APS	Ammonium persulfate
BCRP	Breast cancer resistance protein
BRCA	Breast cancer susceptibility protein
cFLIP	Cellular FLICE-like inhibitory protein
CRD	Cysteine-rich domain
CYP	Cytochrome P450
DAG	Diacyl glycerol
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sufoxide
ECL	Enhanced chemiluminescence substrate
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-to-mesenchymal transition
FBS	Fetal bovine serum
GPCR	G-protein coupled receptor
HDAC	Histone deactylase
IC_{50}	Inhibitory concentration-50
LPA	Lysophosphatidic acid
MCF7/DOX	1000 nM Doxorubicin-resistant MCF7 cell line
MCF7/ETO	1000 nM Etoposide-resistant MCF7 cell line
MCF7/ZOL	8 μM Zoledronic acid-resistant MCF7 cell line

MDR	Multidrug resistance
MDR1	Multidrug resistance protein 1
MRP1	Multidrug resistance-associated protein 1
NF-κB	Nuclear factor-kappa B
PBS	Phosphate-buffered saline
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PUMA	p53 up-regulated modulator of apoptosis
qRT-PCR	Quantitative real time-polymerase chain reaction
RIPA buffer	Radioimmunoprecipitation buffer
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S1P	Sphingosine-1-phosphate
SDS	Sodium dodecyl-sulfate
SSH1L	Slingshot-like protein 1
TBS	Tris-buffered saline
TBST	0.1% Tween 20 in TBS
TEMED	N,N,N',N'-Tetramethylethylenediamine
TMD	Transmembrane domain
TP53	Tumor suppressor p53
XRCC	X-ray repair cross complementing protein
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

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CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a general term for the diseases marked by uncontrolled growth of normal cells in any part of the body. Uncontrolled cell growth may have several outcomes: The mass of cells can invade the neighboring tissue; can pass to the bloodstream or lymph and get access to distant sites of the body, which is called metastasis. Or simply, the punch of cells that does not leave the site of origin can put pressure on body structures. The former cases exemplify malignant tumors while the latter is an example of benign tumor (Becker, Kleinsmith, & Hardin, 2006; Cancer Research UK, 2014b)

GLOBOCAN Project, conducted by International Agency for Research on Cancer aims to supply estimates of incidence, prevalence and mortality of different types of cancer from 184 countries (International Agency for Research on Cancer, 2014). Based on 2012 output of this project, World Health Organization stated that cancer is of the leading causes of deaths worldwide with 8.2 million cases. In addition, there are 14.1 million new cancer cases emerged in 2012 (World Health Organization, 2014). The most prevalent causes of cancer deaths independent of sex are lung, liver, stomach, colorectal and breast cancers. However, the frequency of those cancer types differs between males and females. For example, lung cancer is the most frequent type of cancer observed in both sexes combined; while breast cancer is the most common type of cancer in female.

Cancer can be grouped with respect to type of origin (Becker *et.al.,* 2006): Carcinomas constitutes the vast majority of the cancer cases. It refers to cancer of epithelial cells.

Breast, colon, lung cancers are examples of carcinoma. Sarcoma, on the other hand, refers to supporting tissue cancers such as bone, cartilage, connective tissue. Lymphomas and leukemias are the cancers originated in blood- and lymp- originated cells (Becker *et.al.*, 2006).

Independent of the subgroup, any cancer types have certain characteristics as reviewed wisely in Hanahan, Weinberg, & Francisco, 2000 and Hanahan & Weinberg, 2011: Those characteristics involve proliferation independent of growth signals, resistance to anti-proliferative signals, escape from apoptosis, induction of invasion and metastasis, sustained angiogenesis and unlimited replication capacity. It seems that there are a number of hallmarks yet to be defined. Hanahan and Weinberg suggested two additional characteristics to their list: It appears that cancer cells rewire energy metabolism in order to maximize the neoplastic growth capacity. Moreover, tumor cells disable anti-tumor activities of immune cells like CD8+ T cells, NK cells through soluble factors or via recruitment of suppressive immune cells such as Tregs. A cancer type may not carry all the characteristics; however, the "rules" governing the carcinogenesis are very much defined in the context of these hallmarks.

1.2 Breast cancer

Breast cancer is the most frequent type of carcinoma observed in women. It has been noticed that breast cancer does not refer to a single disease; rather, there is a huge heterogeneity among breast cancer cases (Esebua, 2013), each with characteristic clinical and molecular features and morphology. More than 95% of the breast cancers are adenocarcinoma arising in the epithelial cells in the form of in situ carcinomas and invasive carcinomas (Esebua, 2013). Depending on the origin, most common types of breast adenocarcinoma are ductal carcinoma, which arises in the linings of milk ducts and lobular adenocarcinomas, which arises in the milk glands (National Cancer Institute,

2014a) (Figure 1.1). In situ carcinoma that emerges in ducts and lobules refers to over-proliferation within the borders of site of origin, while invasive carcinoma indicates the neoplastic growth crossing the basal membrane and entering the stroma. In the latter form, transformed cells can enter the bloodstream or lymphatic system and carried to distant sites of the body.

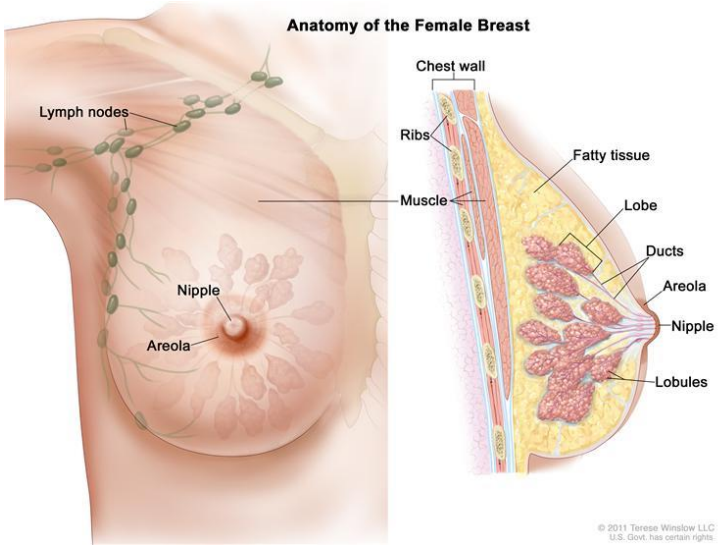

Figure 1.1:Anatomy of female breast. Ducts and lobules are the main origin sites of neoplasm. Retrieved from http://www.cancer.gov/cancertopics/pdq/treatment/breast/Patient

Breast cancer epidemiology has accumulated plenty of data on the risk factors. Race has been regarded as an important factor for breast cancer mortality. In United States, black women appears to develop more aggressive breast cancers relative to white women (Dunn *et.al.*, 2010). Early menarche, parity, late first-time pregnancy and late menopause are also considered as breast cancer risk factors through their effects on sex hormone levels, persistent remodeling of breast tissue (Coughlin & Cypel, 2013). In addition, extended period of breast feeding is associated with reduced risk of breast cancer (Coughlin & Cypel, 2013). Similar to other cancer types, diet and physical activity are important components of breast cancer risk factors. For instance, increased

consumption of alcohol, red meat, fats as well as reduced consumption of calcium, vitamins C, D and E; carotenoids are regarded as risk factors for breast cancer. Physical activity – probably through its effects on body mass composition and hormone levels – reduces the risk of breast cancer (Coughlin & Cypel, 2013). Apart from these, genetic background is a significant determinant of breast cancer susceptibility. First of all, around 20% of breast cancer patients have first degree relatives that have developed breast cancer, implying that family history of the disease could be a risk factor (Coughlin & Cypel, 2013). Single nucleotide polymorphisms (SNPs) in *XRCC2* and *XRCC3* genes as well as mutations in *BRCA1, BRCA2, TP53, PTEN, STK1* are convincingly associated with breast cancer (Coughlin & Cypel, 2013).

1.3 Options in breast cancer treatment

Currently, breast cancer can be cured by surgery, chemotherapy, radiotherapy, hormone therapy and targeted therapy (National Cancer Institute, 2014b).

1.3.1 Surgery

Most breast cancer patients are treated by removal of tumor by surgery. The extent of surgery, i.e. either removal of just tumor site together with neighboring tissue or complete removal of breast (total mastectomy) depends on aggressiveness, size of tumor etc. In each case, neighboring lymph nodes can also be removed if cancerous cells are observed around them.

1.3.2 Hormone therapy

Certain breast cancer subtypes are dependent on hormones like estrogen in order to grow. In such cases, inhibiting the action of hormones either by preventing the receptor-hormone interactions or by blocking hormone synthesis could be promising strategy.

Tamoxifen usage for the former case and the aromatase inhibitors for the latter case are the most common applications of hormone therapy.

1.3.3 Radiotherapy

Radiotherapy is the use of ionizing radiation to damage DNA of tumor tissue, thereby inducing cell death. It can be applied either alone or with other therapy options, i.e. after surgery to prevent recurrence, and together with chemotherapy.

1.3.4 Chemotherapy

In basic means, chemotherapy is the application of chemical cytotoxic reagents (anti-neoplastic drugs) in order to kill cancerous cells, to prevent their growth or hinder metastasis. In breast cancer treatment, a wide variety of drugs can be used alone or in combinations depending on the type of tumor, its grade, chemotherapy history (Dönmez & Gündüz, 2011; Kaplan & Gündüz, 2012). The drugs that are used in breast cancer treatment are cyclophosphamide, epirubicin, 5-fluorouracil, mitomycin, mitozantrone, doxorubicin, gemcitabine and docetaxel, etoposide, gemcitabine (Cancer Research UK, 2014a). Those drugs directly target the cell proliferation by interfering with nucleic acid metabolism, DNA polymerization or microtubule dynamics. Some drugs that are not anti-cancer agents by themselves are used in combinations with the chemotherapy agents in order to cope with secondary outcomes. For example, bone tissue is a host to metastatic breast cancer; for treatment of bone metastases zoledronic acid can be applied.

1.4 Multidrug resistance

Chemotherapy acts mainly on rapidly-dividing cancerous cells; thus in principle, it is expected to stop cell proliferation and eventually to shrink the tumor. However, chemotherapy may fail even though the well-stated side effects were overcome

somehow. Major cause of chemotherapy failure is the intrinsic or acquired resistance to structurally and functionally distinct drugs, which is regarded as multidrug resistance (MDR) (Choi, 2005; Gillet & Gottesman, 2010). Emergence of MDR could render patient refractory to different chemotherapy regimens; as the definition implies, MDR phenotype may occur in the beginning- without any exposure to drug, which is the case in many lung and rectal cancer cases (Choi, 2005) or following the drug treatment.

1.4.1 Mechanisms of multidrug resistance

Cellular multidrug resistance mechanisms can be categorized in two broad means: Firstly, the drug does not reach the critical concentration inside the cell in order to exert its cytotoxic effect. Secondly, even though active drug compound can be internalized and accumulated, the eventual outcome, such as induction of apoptosis, cessation of proliferation, does not come up (Baguley, 2010; Lage, 2008).

The alterations in mechanisms by which the drug is internalized and by which the drug is metabolized determine the intracellular concentration of drug. Drugs can cross the lipid bilayer either through passive diffusion –in case of lipophilic drugs- or via transporters – in case of hydrophilic compounds like nucleoside analogs and prevention of drug entry may result in drug resistance. For instance, one important mechanism for uptake of antifolates like methotrexate is through folate carrier. Decreased expression of reduced-folate carrier or polymorphisms on it makes the patients unresponsive to the drug (Assaraf, 2007). Another mechanism to prevent intracellular drug accumulation is the enhanced efflux of the drug. This is the most pronounced way of multidrug resistance and mediated mainly by a highly conserved membrane transport proteins, ATP-binding cassette (ABC) family of transporters (Gillet & Gottesman, 2010). ABC transporters are a group of widely-expressed active transporters that can excrete xenotoxins, food components, thereby protecting tissues –specifically toxin-vulnerable tissues like brain, cerebrospinal fluid, testes (Borst & Elferink, 2002; Choi, 2005).

However, those transporters are mostly remembered in their roles on cancer multidrug resistance. ABC transporters are membrane-localized "efflux pumps" that can translocate a wide spectrum of chemotherapy agents out of the cell in ATP dependent manner. The family is categorized in 7 subfamilies (named ABCA to ABCG) and general structure of ABC transporters includes two nucleotide-binding domains where ATP binds to be hydrolyzed, and 12 transmembrane alpha helices, 6 of which comprise a transmembrane domain (TMD). The best known drug-effluxing ABC transporters are P-glycoprotein (ABCB1 or MDR1), MRP1 (ABCC1), BCRP (ABCG2) (Szak *et.al.*, 2009). Many classical chemotherapy compounds are effluxed through those proteins. A DNA intercalating, topoisomerase II inhibitor, doxorubicin, is exported mainly by ABCB1 and ABCC1 (Choi, 2005; Gillet & Gottesman, 2010; Lage, 2008). Etoposide, that is a topoisomerase II poison, is also exported by ABCB1 and ABCC1 (Choi, 2005; Gillet & Gottesman, 2010; Lage, 2008).

Table 1.1:Major ABC transporters and their endogenous and exogenous substrates. Adapted from (Choi, 2005)

Name	Endogenous substrate	Exogenous cytotoxic substance
MDR1	Estrogen glucuronide conjugates (estradiol, estriol), endorphin, glutamate, steroids (cortisol, aldosterone, corticosterone), beta-amyloid, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (generically platelet-activating factor, PAF)	Anthracyclines (doxorubucin, daunorubicin, epirubicin), actinomycin D, colchicine, podophyllotoxin (etoposide, teniposide), methotrexate (only in carrier-deficient cells), mitomycin C, mitoxantrone, taxenes (paclitaxel, docetaxel), vinca alkaloids (vincristine, vinblastine)
MRP1	Estradiol-17beta(beta-D-glucuronide) glutathione, glutathione S-conjugate leukoetriene C4, glucuronosyl bilirubin	Anthracyclines, cochicine, etoposide, heavy metals (arsenite, arsenate, antimonials), vincristine, vinblastine, paclitaxel
MRP2	Estradiol-17beta(beta-D-glucuronide), glutathione, glutathione S-conjugate Leukoetriene C4, glucuronosyl bilirubin,	Cisplatin, CPT-11, doxorubicin, etoposide, methotrexate, SN-38, vincristine, vinblastine
MRP3	S-(2,4-dinitrophenyl)glutathione	Cisplatin, doxorubicin, etoposide, methotrexate, teniopside, vincristine,
MRP4	Glucuronide and glutathione conjugates	Methotrexate, nucleotide analogs, PMEA*
MRP5	Glutamate and phosphate conjugates	Doxorubicin, methotrexate, nucleotide analogs, topotecan,
MRP6	Cyclic nucleotides (cAMP, cGMP), glutathione conjugate	Doxorubicin, etoposide, teniposide
MRP7	?	?
MRP8	17beta-estradiol-(17-beta-D-glucuronide), leukotriene C4, cyclic nucleotides	5'-Fluorouracil, 5'-fluoro-2'-deoxyuridine, 5'-fluoro-5'-deoxyuridine, PMEA*
BCRP	Heme or porphyrin	Anthracyclines, bisantrene, camptothecin, epirubicin, flavopiridol, mitoxantrone, S-38, topotecan

Drug effluxing pumps, particularly ABCB1, are subjected to tight regulations especially in transcription level (Scotto, 2003). It was shown that ABCB1 promoter can be

controlled by well-known signaling cascades. For example, “Guardian of genome”, p53, was shown to suppressed ABCB1 expression through a novel binding site on its promoter (Johnson *et.al.,* 2001). Activator protein-1 (AP-1) is a transcription factor that regulates life, death, differentiation and transformation. Acting as a dimers of Jun, Fos, Maf and ATF family proteins, When active AP-1 binds to certain response elements on the promoter regions with or without interacting with other factors and regulate the gene expression (Shaulian & Karin, 2002). They are activated by several stimuli like cytokines, growth factors, cell-matrix interactions, which is mediated mainly by ERK. The dimers are also activated in stress conditions like genotoxic stress; this is mediated by stress MAPK JNK and p38. It was shown that ABCB1 and ABCC1 promoters contain AP-1 consensus site. Even though there are extensive study on relationship between AP-1 and ABCB1 expression, the findings are controversial. Overexpression of JNK downregulates ABCB1 in mRNA level by enhancing the binding of AP-1 to the ABCB1 promoter in MDR derivative gastric and pancreas cell lines (Zhou et al., 2006). In another report, it was observed that JNK is activated by chemotherapy agents like doxorubicin, vinblastine and etoposide, which in turn activates AP-1. However, this time it follows the increased expression of ABCB1, at transcription level indeed (Osborn & Chambers, 1996). This implies the involvement of other factors in ABCB1 gene regulation and its dependence on the context. Ras/Raf signaling is also involved in ABCB1 regulation through Sp1 and Sp3 transcription factors (Scotto, 2003).

In addition to reduced drug uptake and increased drug efflux, rate of drug metabolism plays significant role on drug bioavailability, thus drug effectiveness. Drug detoxification is carried out by Phase I enzymes and prepared for excretion by Phase II enzymes. Cyctochrome P450 (CYP) enzymes, also called Phase I enzymes, oxidize the drug and make it ready for conjugation. Although they are mainly expressed in liver, they are still present in healthy and malignant tissue. Phase II enzymes performs glutathionylation, glucuronidation or sulfation of readily oxidized, -inactive but reactive-

drug into hydrophilic compound. These conjugates are excreted through ABCC type transporters (Gillet & Gottesman, 2010). Increased expression of these types of enzymes in tumor can lead to rapid detoxification and efflux of drug, thereby making the treatment ineffective.

Second line of multidrug resistance involves the dysregulation of death-survival-proliferation pathways. Indeed, altered death pathways were already defined as a cancer hallmark (Hanahan *et.al.*, 2000); thus, it is not weird to consider that chemotherapy, which exerts its cytotoxicity through sensors and effectors within the death machinery, is strongly dependent on the functional death mechanisms (Lage, 2008).

1.4.1.1 Apoptosis and its relevance to multidrug resistance

As emphasized several times in this section, eventual aim in chemotherapy is to activate death pathways- in both apoptotic and non-apoptotic means such as necrosis, mitotic catastrophe, autophagy (Saraswathy & Gong, 2013). Therefore, dysregulation in these pathways have the potential to render cells refractory to the drug. Particularly, apoptosis is believed to be the major route of drug-induced death and is maybe the best characterized of all other mechanisms. Apoptosis takes place in separate but interconnected pathways- namely extrinsic and intrinsic apoptotic pathways (Fulda & Debatin, 2006). As explained in detail in Fulda 2006, in extrinsic pathway, death ligands such as tumor necrosis factor (TNF), TNF-related apoptosis inducing ligand (TRAIL), FasL engage with the corresponding so-called “death receptors”, causing the recruitment and activation of caspase 8. Active caspase-8 then activates the executioner caspases (caspase 3, caspase 7). In intrinsic pathway, mitochondria play the central role. Release of cytochrome c from mitochondria is the main trigger for apoptosome formation, the complex that is responsible for autoactivation of caspase 9. Activated caspase 9 then cleaves and activates executioner caspases. Indeed, extrinsic and intrinsic pathways converge with the activation of main caspases, although they are also interconnected in

upper parts of the pathways. Boost of active caspase is an indication of ongoing apoptosis; however, till caspase activation both extrinsic and intrinsic pathways are subjected to massive positive and negative regulations in every step. For instance, activation of death receptors by receptor-ligand interaction may not end up with caspase 8 activation, where cFLIP blocks caspase 8 activation. cFLIP was found overexpressed in particular tumors (Fulda & Debatin, 2006) and it was one of the anti-apoptotic proteins that was also implicated in MDR. For example, resistance to certain anti-neoplastic drugs that make use of extrinsic pathways was reported to be circumvented by cFLIP knockdown (Longley *et. al.*, 2006; Rogers *et. al.*, 2007). Intrinsic pathway, on the other hand, is regulated in the level of mitochondrial membrane permeability. Increased outer membrane permeability causes the release of death-related factors including cytochrome c. Cytochrome c binds to Apaf-1 and enables its association with dATP, which then oligomerize and leads to caspase 9 activation. Main actors controlling the membrane permeability are anti- and pro-apoptotic Bcl-2 family members that are grouped in three categories (Fernandez-Luna, 2008; Pommier, *et. al.*, 2004):

- Anti-apoptotic members that contain four Bcl-2 homology domains (BH1, BH2, BH3, BH4): Bcl-2, Bcl-x_L, Bcl-w, Mcl-1;
- Pro-apoptotic members that possess BH1, BH2, BH3 domains: Bax, Bak, Bok, Bcl-rambo;
- Pro-apoptotic BH3-only proteins that have only BH3 domain: Bad, Noxa, Puma, Harakiri (Hrk), Bid, Bim, Bik, Bmf

In principle, anti-apoptotic members hinder binding of pro-apoptotic proteins to the mitochondria and cytochrome c release from the mitochondria. Upon binding to mitochondria membrane multi-BH proapoptotic members can form pores through which cyctochrome c and other apoptotic proteins are effluxed to the cytosol. BH3-only proteins, however, have role in sensing apoptotic stimuli and activating multi-BH pro-

apoptotic proteins. Activities of each of these proteins are regulated by major signaling cascades at levels of transcription (e.g. p53 upregulates Noxa, Puma) and post-translation (e.g. Bid is cleaved by caspase 8; Bid is dephosphorylated, thereby relieved from 14-3-3 sequestration) (Fernandez-Luna, 2008; Fulda & Debatin, 2006). Again, dysregulations in those proteins can be related to multidrug resistance.

Apoptosis is also regulated beyond cytochrome c release and activation of executioner caspases: *Inhibitor of apoptosis proteins* (IAP) are a group of proteins that negatively regulates caspase activity through direct binding to them. IAP family comprises proteins like survivin, XIAP, cIAP1, cIAP2, livin, Apollon. Overexpression of IAP family proteins are also implicated in MDR: 5-fluouracil resistance in oral squamous cell carcinoma was overcome by downregulation of cIAP2, which increased caspase activation upon drug treatment (Nagata *et.al.*, 2011). Another IAP, survivin, has been implicated in drug-resistance: Inhibition of survivin was shown to reverse the drug resistance in lyphoblastic leukemia (Park *et.al.*, 2011). Some studies have revealed that IAPs may mediate drug resistance by directly regulating the drug-efflux pumps. For example, overexpression of survivin in MCF7 cells enhanced resistance to doxorubicin 64-fold (Liu *et.al.,* 2007). The study showed that survivin downregulation increased drug accumulation by inhibiting the P-gp, possibly via P-gp sorting or turnover. In another study, it was revealed that survivin controlled the expression of BCRP in 5-fluorouracil resistant MCF7 cells through NF-κB pathway (Wang *et.al.,* 2013). In addition to survivin, X-linked inhibitor of apoptosis protein, XIAP downregulation in chemoresistant ovarian cancer cell lines was shown to reverse cisplatin resistance in p53-dependent manner (Sasaki *et.al.*, 2000). Similarly, in MDA-MB-231 cells, downregulation of XIAP through RNAi increased the sensitivity towards TRAIL and taxanes (McManus *et.al.*, 2004).

Second line of caspase inhibition that is brought by IAPs can be reverted by mitochondrial proteins that are released together cytochrome c in apoptosis. Those proteins include SMAC/DIABLO, HtrA2/Omi (Fulda & Debatin, 2006). By direcly interacting with caspase-bound IAP (like survivin, XIAP, cIAP1, cIAP2, Apollon), SMAC/DIABLO set the IAP-sequestered caspases free. Omi/HtrA2 acts in similar manner to activate caspases; being a protease it can govern apoptosis by itself-independent of caspases. The effects of these "secondary activators" on chemotherapy response seemed inconclusive. Still, there are reports that stated SMAC / DIABLO overexpression can reduce the resistance to certain drugs, while its absence did not affect the drug response (Zhao *et.al.*, 2006).

1.4.1.2 DNA repair and multidrug resistance

Many classical chemotherapy agents target DNA replication and they are efficacious as long as they can induce damage in DNA. In normal conditions, DNA may face replication stress and also it is subjected to physically- and chemically-induced damages. All these problems, firstly, are sensed by damage sensing factors such as ATM, ATR, CHK1, CHK2 which eventually cause cell cycle arrest (Jackson & Bartek, 2009; Yang *et.al.*, 2004). The arrest saves time for DNA damage response, and is important to prevent replication of damaged DNA or mitosis with damaged DNA. DNA damage is fixed by different repair systems depending on type of damage and its extent: Base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), non-homologous end joining (NHRJ) or homologous recombination (HR) (Jackson & Bartek, 2009; Lage, 2008) are significant components of DNA damage repair system. If DNA damage cannot be repaired cells undergo apoptosis or enter senescence.

In tumor cells being treated with radiotherapy and /or chemotherapy, status of DNA damage response is of high importance. In theory, over-active DNA damage repair could eventually fix the majority of damage induced by the therapy, rendering the therapy

inefficacious. For example, loss of MMR was associated with the resistance to topoisomerase inhibitors (doxorubicin, etoposide), platinum-containing drugs (cisplatin), DNA alkylator (procarbazin) and some other drugs (Fedier *et.al.*, 2001; Fink *et.al*, 1998). Also, it was shown that acquired etoposide resistance may be accompanied by downregulation of MMR proteins (Kaplan & Gündüz, 2012).

Changes in the expression of target proteins; mutations or extensive post-translational modifications on proteins that could prevent drug-protein interactions; or altered subcellular localization of proteins can also end up with drug resistance.

Besides the cellular mechanisms of multidrug resistance there are some other mechanisms that can prevent systemically administered drug from coming near the tumor cells and causing an ineffective chemotherapy. Those mechanisms involve lowered tumor vasculature, hypoxia, enhanced binding to the plasma proteins (Baguley, 2010).

1.4.1.3 Other phenotypes related with multidrug resistance

There are striking evidences that associate multidrug resistance and cancer invasiveness. Cells exposed to conventional chemotherapeutic agents become resistant to those drugs; meanwhile they start to express epithelial-to-mesenchymal transition (EMT) markers (Saxena *et.al.*, 2011; Zhang *et.al.*, 2012). EMT is a process that "immobilized" epithelial cells undergo in order to "get move" to invade basal membrane, enter the neighboring tissue and eventually, reach the lymphatic system or bloodstream to metastasize. Normally, epithelial cells are held in contact with each other through proteins like E-cadherin. Epithelial cells going through EMT downregulate E-cadherin while upregulating N-cadherin, vimentin, fibronectin, which reconstruct the cytoskeleton suitable for migration and invasion (Zhang *et.al.*, 2012).

There are several molecular cues that support coregulation of MDR and EMT: Certain EMT-related transcription factors directly regulate the expression of ABC transporters, namely ABCB1. Cell culture-based in vitro studies showed that transcription factors that initiate EMT can also trigger multidrug resistance via upregulation of ABC transporters (Saxena *et.al.*, 2011). In another study, when Snail is knocked down in 5-fluorouracil resistant MCF7 cells, cells not only reverse EMT but also become sensitive to the drug, suggesting that there are common mechanisms regulating both EMT and MDR (Zhang *et.al.*, 2012). Depletion of another factor, Twist-1, has been shown to reduce both invasiveness and MDR in doxorubicin-exposed MCF7 cells (Li *et.al.*, 2009).

1.5 Protein kinase D (PKD) family

Protein kinase D family comprises three serine/threonine kinases that are named PKD1, PKD2 and PKD3. Due to high homology of their catalytic domains with the calcium/calmodulin-regulated (CAM) kinase superfamily they are categorized under this superfamily (Rozengurt *et.al.*, 2005; Storz, 2012). Like classical and novel protein kinase C isoforms, PKD is diacylglycerol (DAG) effectors. Indeed, they share a similar structural backbone with PKC members that are responsive to DAG and phorbol esters, which makes the founder member, PKD1 (formerly PKCμ) be included in PKC family (Rozengurt *et.al.*, 2005). Later it was seen that PKD activation is dependent on phosphorylation by novel PKCs (η, δ, ε, θ) or classical PKCs (α, βI, βII) (Li *et.al.*, 2004; Rozengurt *et.al.*, 2005).

1.5.1 PKD structure and activation models

DAG generation is the major stimulus for PKC and PKD signaling. Receptor tyrosine kinase (RTK) or G-protein coupled receptor signaling (GPCR) that is initiated by growth factors, hormones, neurotransmitters activates phospholipase C (PLC) isozymes (PLCγ

by RTKs, PLCβ by GPCRs). PLC activity is the major source for membrane DAG (Griner & Kazanietz, 2007).

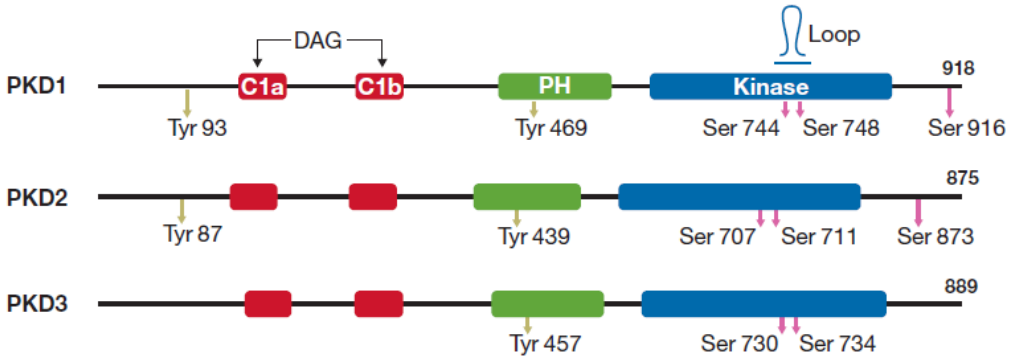

Figure 1.2:Organization of PKD izozymes Retrieved from Fu & Rubin, 2011

PKD family members are noticed in their domain distributions (Figure 1.2). Proximal to N-terminus, there are two zinc-finger-like cysteine rich motifs (C1a and C1b in the figure) that constitute the so-called cysteine-rich domain (CRD). This domain is common to other DAG/phorbol ester responsive proteins and is required for localization to golgi, nucleus or membrane and autoinhibition of PKD catalytic activity. Pleckstrin homology (PH) domain serves as an interphase for protein-protein interactions. Also, like CRD, it shades the catalytic domain, thereby rendering the protein inactive (Fu & Rubin, 2011; Rozengurt *et.al.*, 2005; Storz, 2012). Kinase catalytic domain is responsible for substrate phosphorylation. For PKD1, the most studied member, the phosphorylation motif is L/I-X-R-X-X-S/T (Ubersax & Ferrell, 2007).

Protein kinase D, together with protein kinase C, is translocated to the plasma membrane upon DAG generation (Figure 1.3). In one model, novel PKC or classical PKC transphosphorylates PKD from critical serine residues in the catalytic domain. Active PKD breaks off the membrane and localizes back to cytosol or is shuttled to the nucleus and interacts with its partners and/or substrates.

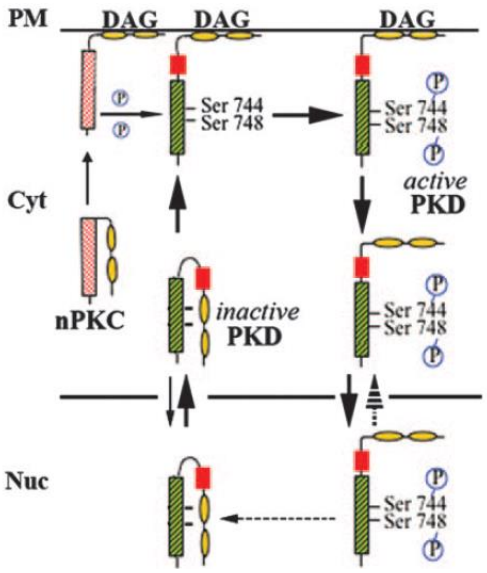

Figure 1.3:PKC-mediated activation of PKD-a model. Retrieved from Rozengurt *et.al.*, 2005

In addition to this classical PKC-dependent activation model, it was shown generation of reactive oxygen species (ROS) may activate PKD through tyrosine phosphorylations by Src and Abl (Steinberg, 2012; Storz, 2012) and subsequent PKCδ phosphorylation. Proteolytic cleavage of PKD1 by caspase 3 was also shown; however, there are still debates on the use of proteolytically activated PKD1 and its regulation (Steinberg, 2012).

1.5.2 Signaling cascades mediated by PKD family members

Growth factor receptors functioning as RTKs -like platelet-derived growth factor (PDGF), epithelial growth factor (EGF), vascular endothelial growth factor (VEGF) can activate PKD (Storz & Toker, 2003; Storz, 2012; Van Lint, 1998). For example, activation of PKD1 by PDGF follows the first activation model where PLCγ and PKC lie upstream of PKD (Van Lint, 1998). PKD members are also involved in transduction of mitogenic signals like bombesin, endothelin, bradykinin, vasopressin as well as

signaling lipid derivatives such as lysophosphatydic acid and spingosine-1-phosphate (Figure 1.4) that act through GPCRs (Storz, 2012; Xiang *et.al.*, 2013). Some of these signals converge on DAG generation through different phospholipase type (PLC isoforms or PLD) depending on the G_{α} subtype. For example, in cardiomyocytes LPA and S1P activate $G_{\alpha 12/13}$ which subsequently activates RhoA through RhoGEF (that replaces GDP bound to inactive RhoA with GTP, thereby activating RhoA). RhoA then stimulates phospholipases PLCε and PLD1 and leads to formation of DAG, which, in turn, activates nPKC and eventually promotes the activation of PKD (Xiang *et.al.*, 2013).

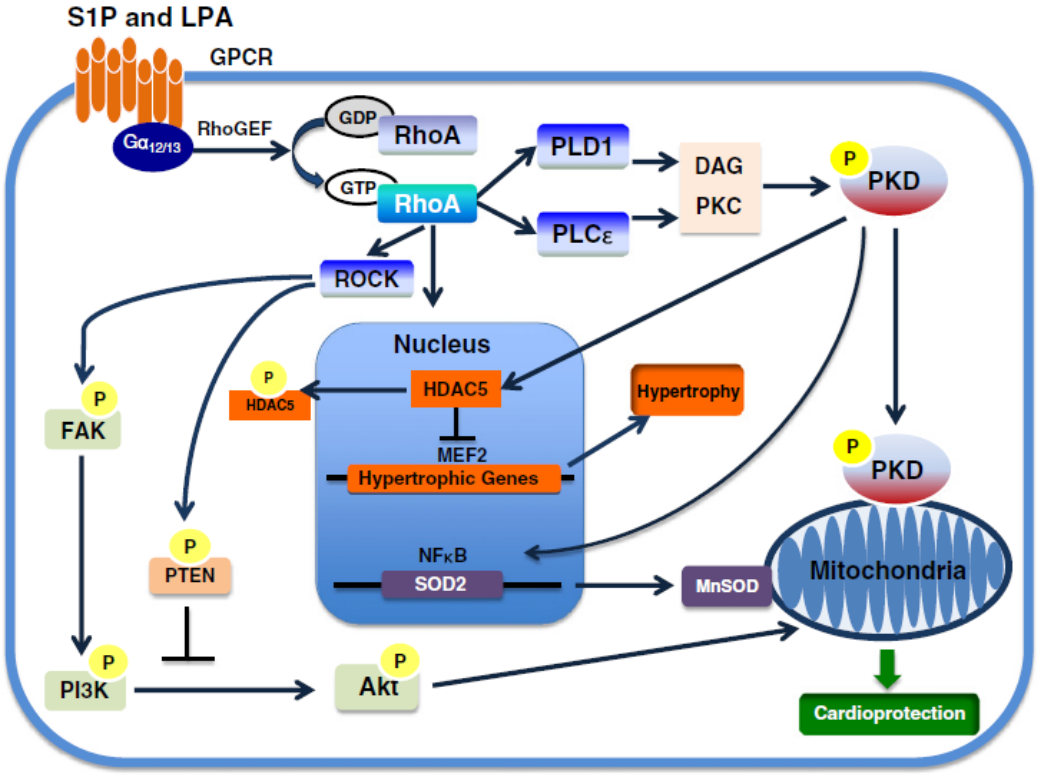

Figure 1.4:S1P and LPA-mediated signaling cascades, including PKD activation. Retrieved from Xiang *et.al.*, 2013

1.5.3 Physiological roles of PKD family

PKD family members have a wide spectrum of functions in normal cell physiology (Fu & Rubin, 2011). They, particularly PKD1, regulate survival pathways through NF-κB upon oxidative stress. They function in cell motility through F-actin reorganization and via distinct substrates. PKD members are involved in vesicular transport –especially in the vesicular fission on the way from trans-golgi network to plasma membrane. PKD members contribute to regulation of gene expression through histone deacetylases (HDAC4, HDAC5, HDAC7 and HDAC9). Those HDACs generally have repressive roles in cell-specific manner; phosphorylation by PKD causes dissociation of these proteins from promoter regions and maybe promotes their association with 14-3-3 proteins and sequestration in cytoplasm (Rozengurt, 2011). PKD members are also required for berberine induced insulin receptor transcription (Zhang *et.al.*, 2010). PKD was also implicated in cytokine synthesis: PKD2 is required for LPA-induced IL-8 production through activation of NF-κB (Chiu *et.al.*, 2007). PKD members are also implicated in cardiac homeostasis: They can regulate contraction, hypertrophy, proliferation and death in heart (Avkiran *et.al.*, 2008). In addition, PKD2 was reported to mediate secretion of platelet granule and promote thrombus formation through classical PKC (Konopatskaya *et.al.*, 2011).

1.5.4 PKD members in malignancy

PKD members functions in the key events in cellular physiology; thus, it is not surprising for them to have roles in cancer development. Many studies associated PKD members with malignancy. It has been shown that PKD1 can trigger tumor cell survival by inducing insensitivity against Fas-mediated death, upregulating anti-apoptotic cFLIP and survivin; and promote proliferation in pancreas adenocarcinoma (Trauzold *et.al.*, 2003). Moreover, PKD was implicated in NF-kB-driven cell survival through upregulation of TRAF1 and cIAP2 (Johannes *et.al.*, 1998). In chronic myeloid leukemia,

PKD2 activated by Bcl-Abl induced NF-kB (Mihailovic *et.al.,* 2004). It was observed that elevated PKD3 expression and nuclear sorting correlated with prostate cancer grade and aggressiveness (Chen, *et.al.,* 2008). It was shown that overexpression of PKD3 in LNCaP prostate cancer cells having low PKD3 expression speeded up S phase entry, activated the key survival signaling, AKT signaling and ERK1/2 signaling (Chen *et.al.*, 2008).

Even though the role of PKD in chemoresistance is yet to be verified, there are indications that PKD members modulate chemoresistance via distinct mechanisms (Storz, 2012). In tumors with high reactive oxygen species (ROS) as well as in tumors treated with chemotherapeutic agents that generate ROS to kill; ROS-driven, NF-kB and FOXO3a-mediated upregulation of scavenger genes contribute to drug resistance (Storz, 2012). Under oxidative stress Src and Abl activated PKD1, which then activate NF-kB (Storz & Toker, 2003). Moreover, PKD-mediated accumulation of sphingosine kinase 2 (SPHK2) in cytosol confers resistance to sodium butyrate in HCT116 colon cancer cell line (Xiao *et.al.,* 2012). All these cases exemplify that PKD can be involved in chemoresistance in multiple aspects.

It appears that PKD has function in cell migration and invasion; however different members can have not only distinct but also contradictory roles in context-dependent manner. It has been shown that PKD inhibition by structural analogs of a well-known PKD inhibitor, CID755673 successfully reduced the rate of wound closure and suppressed the invasion of matrigel (Lavalle *et.al.*, 2010). siRNA-mediated knockdown of PKD2 as well as its pharmacological inhibition lowered the migratory and invasive potential of U87MG glioblastoma cell line probably partly through modulating the expressions of matrix metalloproteinases and integrins (Bernhart *et.al.*, 2013). In addition, PKD1 promotes invasiveness and angiogenesis in pancreatic ductal adenocarcinoma (Ochi *et.al.*, 2011). Besides, in BON endocrine cells, PKD2 worked in

favor of increased invasion of matrigel media where siRNA mediated PKD2 knockdown decreased and ectopic expression of PDK2 increased the migration (Jackson *et.al.,* 2006). Still, reports on breast cancer cases implies the variability of PKD functions depending on the context such that PKD members may function oppositely to common notion, which is discussed in the following section.

1.5.4.1 PKD in breast cancer

Several in vivo and in vitro studies have attempted to elucidate the roles of PKD members in breast cancer. Despite the high homology between members and functional redundancy observed in certain cases, in breast cancer, PKD1 appears to behave more like a tumor suppressor while PKD2 and PKD3 have oncogenic roles (Borges & Storz, 2013) although there are several reports contradictory to this deduction. In one study, PKD1 expression is not detected in invasive breast cancer cell lines MDA-MB-231, SKBR-3 while it is expressed in immortalized non-transformed MCF10A and non-invasive BT-474 and MCF7 cell lines (Eiseler *et.al.,* 2009). It should be noted that T47D that was shown to lack PKD1 expression was categorized as invasive in that study while it is accepted as non-invasive in the literature (Holliday & Speirs, 2011). PKD2, on the other hand, expressed –in varying levels- in all the lines tested independent of the invasiveness and there is no significant correlation with receptor status either. In an another study, PKD2 and PKD3 were shown to be the major isozymes expressed in invasive cell lines like MDA-MB-468, HCC1806, which do not express PKD1. Indeed, it was seen that PKD1 and PKD3 expressions changed dramatically between the cell lines while PKD2 seemed more constitutive (Hao *et.al.,* 2013), supporting the observation in the study of Eiseler (2009).

Some mechanistic studies give molecular cues for the differential effects of these isoforms. For example, downregulation or pharmalogical inhibition of PKD2 and PKD3 cause reduced cell growth in HCC1806 triple-negative breast cancer cell line (Hao *et.al.*,

2013). In another study, overexpression of PKD1 in MCF7 cell line increases cell proliferation through accelerated G1-to-S passage and reduces the serum- and anchorage dependence for growth (Karam *et.al.*, 2012). Although this study did not take cell motility and invasiveness into account as a measure of "aggressiveness" the findings are not compatible with a protein regarded as "tumor suppressor".

PKD members are also involved in motility and invasion. siRNA-mediated knockdown of PKD1 and PKD2 was shown to promote invasion of MDA-MB-231 and MCF7 cell lines (Peterburs *et.al.*, 2009), indicating that both members are negative regulators of cell migration. In the same study, it was proved that SSH1L, a phosphatase that renders cofilin in dephosphorylated active form, is phosphorylated by PKD1 and PKD2 and then sequestered by 14-3-3 proteins. Inhibition of PKD1/2 sets SSH1L active; it can interact with F-actin and cofilin, creating local dephosphorylated cofilin pool that promotes cell migration. One study that related MDR and PKD family demonstrated that in MDA-MB-231 cell line paclitaxel treatment stimulates PKD2 activity in a time-dependent manner in parallel to ABCB1 induction. Downregulation of PKD2 led to decreased ABCB1 expression when treated with paclitaxel for 72 hours such that this sensitized MDA-MB-231 cells to paclitaxel, which is effluxed by ABCB1 (Chen *et.al.*, 2011).

1.6 Scope of the study

PKD signals are related with major cellular events like proliferation, survival, motility, apoptosis, and invasiveness. Multidrug resistance, as a multifaceted phenotype, is strongly interconnected with these events. However, upstream control mechanisms responsible for MDR are yet to be understood, necessitating testing new alternatives. To this end, we aimed to study the involvement of the ubiquitously expressed isoform in breast cancer, PKD2, in multidrug resistant breast cancer cell lines. Our main goals are listed as

- To screen the basal activity and expression of PKD2 in a panel of breast cancer cell lines
- To elucidate the potential roles of PKD2 in chemoresistance of MDR sublines by RNAi mechanism
- To check the involvement of PKD2 in motility and invasiveness of multidrug resistant sublines

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell lines and culture conditions

MCF-7 cell line was purchased from ŞAP Institüte, Ankara. 1000 nM doxorubicin-resistant (abbreviated as MCF7/DOX), 1000 nM etoposide resistant (abbreviated as MCF7/ETO) and 8 μM zoledronic acid resistant (abbreviated as MCF7/ZOL) MCF7 variants were previously developed by the members of our laboratory (Kaplan & Gündüz, 2012; Kars *et.al.*, 2006; Kars *et.al.*, 2007). MDA-MB-231 cell line was a kind gift from Ferit Avcu, Gülhane Military Medical Academy, Ankara. SK-BR-3 cell line was from Regül Çetin-Atalay, Bilkent University, Ankara. BT-474 cell line was from Bala Gür Dedeoğlu, Ankara University, Ankara. All cell lines except BT-474 were maintained in RPMI 1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (Biochrom AG, Germany). Drug resistant sublines were maintained at their reported drug concentrations. BT-474 cell line was cultured in RPMI 1640 medium supplemented with 10% FBS, 0.02 mg/mL human recombinant insulin (Biological Industries, Israel), 100U/mL penicillin and 100μg/mL streptomycin (HyClone, USA). All cells were plated on 25 cm^2 or 75 cm^2 culture flasks or multi-well plates depending on the experiment and were incubated at 37^oC humidified atmosphere with 5% carbon dioxide.

All cell types are subcultured at various ratios when they reached 80% confluency. Briefly, culture media was removed. Then the surface of the culture plate onto which cells were adhered was gently washed with phosphate buffered saline (pH 7.2) twice.

Cells were detached from the surface by adding 0.25% Trypsin-EDTA solution (Biological Industries, Israel) and incubating for five minutes at 37^{o}C. MCF7/DOX and SK-BR-3 cells were hardly detached from the surface; they were incubated longer (up to ten minutes). To stop trypsinization, culture medium was added onto flasks and clumps of cells are dissolved by pipetting. For culture continuation cells were transferred to new flask after an appropriate dilution. For any other downstream applications cells were pelleted by centrifugation at 200g for 4°C, 5 minutes and resuspended in culture media.

2.1.1 Freezing and thawing cells

After pelleting the cells, culture media was completely removed. 1ml of cold freezing medium [9:1 ratio of FBS: DMSO (Applichem, Germany)] was used to resuspend the pellet and suspension was immediately transferred to pre-chilled cryovial. The vial was incubated for 1 hour at -20°C then transferred to -80°C. After 1-month period, the vials were taken to liquid nitrogen tank (at vapor phase). In order to thaw the cells, the vial was incubated at water bath at 37°C. After complete thawing the suspension was quickly transferred onto 9 mL complete medium and cells were pelleted. Cells were resuspended in medium and cultured in flask.

2.1.2 Cell counting

Viable cell count was performed using hemacytometer and tyrphan blue (Biological Industries, Israel). Tryphan blue is a dye that is excluded from the viable cells as it cannot cross the lipid bilayer; whereas dead cells whose membrane is disrupted can internalize tryphan blue and appear blue. Briefly, an aliquot of cell suspension to be counted was taken into a microfuge tube and mixed with tryphan blue at a ratio of 9:1 (suspension: tryphan blue). At the same time a coverslip was adhered onto the counting chamber. 10 mL of the mixture was loaded into the tiny space between chamber and

coverslip. The cells falling inside 16 squares (256 small squares) were counted; blue cells that take up tryphan blue were ignored. Cell concentration is calculated as follows: When a coverslip was stick on counting chamber the volume that was formed between the coverslip and the smallest square is 1/4 000 000 of one milliliter. There are 256 small squares when all big squares are counted. Also, there is a dilution on cell number owing to tryphan blue addition. Overall, the cell concentration was calculated using the formula:

$$\text{Cell number/mL} = \frac{Number\ of\ cells\ counted\ on\ 16\ squares}{256} \times 4 \times 10^6 \times \frac{10}{9}$$

2.2 Gene expression analysis

2.2.1 RNA isolation

Total RNA was extracted from cells using TriPure (Roche, Germany). All disposals used in RNA extraction such as pipette tips, microfuge tubes were DEPC-treated. For RNA isolation from culture flask, cells were collected by trypsinization, pelleted, washed with ice-cold PBS and pelleted once again. PBS was completely removed and up to 2 million of cells 1 mL of TriPure reagent was used. Cells are lysed by pipetting up and down until viscous lysate disappeared. The lysate was transferred into an 2-mL microfuge tube and incubated for 5 minutes at room temperature. Then 0.2 mL chloroform (Sigma Aldrich, Germany) was added onto the TriPure lysate and mixed by inversions for 15 seconds. Then tubes are incubated on ice for 5 minutes. The mixtures were centrifuged at 12 000g, 4°C for 15 minutes. Upper, colorless aqueous phase was transferred to a 1.5 mL microfuge tube containing 0.5 mL 2-propanol (Sigma-Aldrich, Germany). The mixture was mixed by inversions and tubes were incubated for 20 minutes at -20°C. Then the tubes were centrifuged at 12 000g, 4°C for 10 minutes. The supernatant was discarded and RNA pellet was washed in 1 mL 75% ethanol and

centrifuged at 7500g for 5 minutes. The wash step was repeated once again. After complete removal of ethanol pellet was air-dried and resuspended in appropriate volume of nuclease free water (HyClone, USA). In order to completely dissolve the pellet and open up any secondary structures of RNA the solution was incubated at 55°C for 10 minutes. RNA was stored at -80^{o}C in several aliquots.

If RNA was to be isolated from cells on 6-well plates, the steps above were still valid with some minor modifications. Firstly, cells were directly lysed on plate such that the media was removed, the cells were washed with ice-cold PBS and 1 mL TriPure was added per well. The plate was rocked at room temperature for complete lysis and lysate was collected into microfuge tube. Secondly, since 6-well plates accommodated fewer cells, RNA yield could be lower. In order to increase the yield and make the pellet visible a carrier like glycogen could be used. Glycogen is water-soluble and in 2-propanol it co-precipitates with RNA, increasing the yield from dilute solution. Thus, during isolation from 6-well plates, 20 μg/mL glycogen was used in 2-propanol step.

Quantity and purity of RNA extract were checked using NanoDrop Spectrometer (Thermo Scientific, USA). As purity parameters, NanoDrop offered A260/A280 and A260/A230 ratios. The former is the indicator of significant protein contamination if it is lower than 1.8. RNA preparation lacking protein contamination should have a ratio around 2.0. The latter ratio is the indicator of organic solvent contamination such as phenol if it is low. Recent reports have shown that glycogen could be responsible for a low A260/A230 ratio (http://www.nanodrop.com/Library/T042-NanoDrop-Spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf). RNA integrity was another measure of extract quality. It was checked by running RNA in 1% agarose gel prestained with 0.5 μg/mL ethidium bromide (Sigma Aldrich, Germany). RNA solution was mixed 1:1 ratio with 2X RNA loading buffer (Thermo Scientific, USA). Then the mixture was loaded on agarose gel and run at 80 V for 1 hour. Image was taken in Vilber Lourmat

UV imager. Major RNA bands (28S rRNA, 18S rRNA, 5S rRNA) and –if any- gDNA band were observed. Intact RNA should have an intensity ratio of 2:1 between 28S and 18S rRNA bands. Degraded RNA could be seen as a smear between 28S and 18S rRNA bands or as black lane. gDNA could be observed as band stacked on wells or just below the wells.

2.2.2 DNase I treatment

Every RNA preparations can contain residual genomic DNA- even if it seems clear of gDNA in agarose gel. In order to prevent their amplification in subsequent polymerase chain reactions that can obscure the expression analysis RNA solutions were treated with DNase I prior to cDNA synthesis. To this end, RapidOut DNA removal kit (Thermo Scientific, USA) was used to remove DNA contamination. Two μg RNA was mixed with 2 mL 10X DNase buffer and 1U DNase I. The volume was completed to 20 mL with nuclease free water. The mixture was incubated at 37°C for 30 minutes. 2 mL DNase removal reagent was added to DNase reaction and mixture was incubated for 2 minutes at room temperature with occasional vortexing. DNase removal reagent is bead slurry that can bind DNase I and precipitate upon centrifugation. To remove DNase removal reagent (DRR) and bound DNase I, mixture was centrifuged at 10 000g for 90 seconds and supernatant was transferred to a new tube.

2.2.3 cDNA synthesis

Twenty mL RNA was recovered in each time after removal of DRR, containing 2 μg RNA with 100ng/mL. cDNA was synthesized using 500ng RNA and RevertAid reverse transcriptase (Thermo Scientific, USA). cDNA was made by preparing a master mix containing 6.5 mL nuclease free water, 4 mL 5X reverse transcriptase buffer, 2 mL 10mM dNTP mix, 1 mL of 100 μM random primer, 0.5 mL 40U/mL Ribolock RNase

inhibitor, 1 mL of 200U/mL RevertAid reverse transcriptase per well. Master mix was distributed into the individual tubes and 5 mL of DNase I treated RNA (500ng) was added on each tube.

After preparing the mixture the tube was incubated sequentially at 25°C for 10 minutes, at 42°C for 1 hour for synthesis, at 70°C for 10 minutes for the enzyme inactivation. In each time a "No RT control" that did not contain reverse transcriptase was prepared to detect any gDNA carryover contamination in RNA preparation. Finally, cDNAs were diluted 1:20 in nuclease-free water and stored at -20^{o}C.

2.2.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

Expression analysis was finalized by qRT-PCR analysis of cDNAs. To this end, 10 mL of 2x FastStart SYBR Green Master Mix (Roche, Germany) that contained reaction buffer, hot-start *Taq* polymerase, dNTP mix, $MgCl_2$ and SYBR Green was mixed with predetermined amount of forward and reverse primers and cDNA. Total reaction volume was completed to 20 mL by nuclease-free water. The reactions were performed on Rotor Gene Q (Qiagen, Germany) real-time thermal cycler. The amounts of reaction components and cycling conditions were shown in Table 2.1

Table 2.1: The amounts of reaction components and cycling conditions for each amplicon

Component	*PKD2*	*ACTB*	*MDR1*	*VIMENTIN*	*cFLIP*	*SURVIVIN*
Master mix (µl)	10	10	10	10	10	10
Forward primer 10µM (µl)	0.4	0.5	0.2	0.5	0.5	0.2
Reverse primer 10µM (µl)	0.4	0.5	0.2	0.5	0.5	0.2
Nuclease-free water (µl)	5.2	5	5.6	5	5	5.6
cDNA (1:20 diluted)	4	4	4	4	4	4
Initial denaturation	95°C; 10 min	95°C; 10 min	95°C; 10 min	95°C; 10 min	95°C; 10 min	95°C; 10 min
Denaturation	95°C; 15 s	95°C; 20 s	95°C; 15 s	95°C; 15 s	95°C; 15 s	95°C; 15 s
Annealing temperature	60°C; 60 s	55°C; 45 s	60°C; 60 s	60°C; 60 s	60°C; 60 s	60°C; 60 s
Extension temperature		72°C; 45 s				
Cycle number	35	32	37	40	40	36

The primers were either designed using PrimerBlast and Primer 3 or retrieved from literature. The list of primers were displayed in Table 2.2.

Table 2.2: List of primers

Primer	Sequence 5' - 3'	Amplicon size (bp)	Reference
PKD2 forward	AGTTCCTCATCACCCAGATCC	146	NCBI Primer Blast
PKD2 reverse	GATGCGAGCAAAGCCAAAGT		
ABCB1 forward	GGGAGCTTAACACCCGACTTA	154	Harward PrimerBank, ID: 318037598c2
ABCB1 reverse	GCCAAAATCACAAGGGTTAGCTT		
VIMENTIN forward	ATCTGGATTCACTCCCTCTGGTTG	161	Tezcan, 2013
VIMENTIN reverse	TGCTGGTAATATATTGCTGCACTG		
cFLIP forward	GAACATCCACAGAATAGACC	262	Bangert et.al, 2012
cFLIP reverse	GTATCTCTCTTCAGGTATGC		
SURVIVIN forward	AGCCAGATGACGACCCCATAGAGG	60	NCBI Primer Blast
SURVIVIN reverse	AAAGGAAAGCGCAACCGGACGA		
ATCB forward	CCAACCGCGAGAAGATGA	97	Kaplan, 2012
ATCB reverse	CCAGAGGCGTACAGGGATAG		

2.2.4.1 Prepresentation of qRT-PCR data

Quantification of qRT-PCR data was done according to Livak & Schmittgen, 2001. The method is a relative quantification method that eventually expresses the fold change in the gene expression with respect to untreated control group. Briefly, for treated and untreated groups, cycle threshold of test amplicon (C_{tT}) was normalized to cycle threshold an internal control (C_{ti}) that is, a housekeeping gene whose expression is not affected by the treatment.

$\Delta C_{t"Treated"} = (C_{tT})_{Treated} - (C_{ti})_{Treated}$

$\Delta C_{t"Untreated"} = (C_{tT})_{Untreated} - (C_{ti})_{Untreated}$

To find normalized cycle number difference normalized threshold value of untreated group ($\Delta C_{t"untreated"}$) was subtracted from that of treated group ($\Delta C_{t"Treated"}$).

$\Delta\Delta C_t = \Delta C_{t"Treated} - \Delta C_{t"Untreated"}$

The cycle number change was represented as fold change as $2^{-\Delta\Delta Ct}$.

2.3 Protein expression analysis

2.3.1 Protein isolation

Total protein isolation was performed by radioimmunoprecipitation (RIPA) buffer (Santa Cruz, USA). Cells were seeded on 6-well plates and allowed to adhere. Then, the culture medium was removed, the surface was washed twice in ice-cold PBS. Next, 100X protease inhibitor cocktail and 100X phosphatase inhibitor cocktail (Pierce, USA) were added into ice-cold RIPA to 1X dilution. Then 150 mL of complete RIPA was added per well and the plate was shaken on ice for 5-min to facilitate lysis. Next, the lysate was collected to a corner of well using a scraper and aggregates were dissolved by

pipetting through a gel loading tip. The lysate was transferred into a pre-chilled 1.5 mL microfuge tube and sonicated for 30 seconds. After that, extracts were centrifuged for 15 minutes at 14 000 g at cold. Cellular debris and other insoluble materials were pelleted; the supernatant was taken into a fresh tube and aliquoted for protein quantitation. The remaining part was stored at -20°C.

2.3.2 Protein quantitation by Bradford Assay

Bradford assay was performed on 96-well plate format. Various concentrations of 100 mL bovine serum albumin solution were prepared on distilled water and RIPA such that in each standard tube final RIPA concentration was 10%. Test samples that were already in RIPA were diluted 1:10 in distilled water. 5 mL of each standard and diluted test sample was spotted in quadruplicate. 250 mL of 1X Bradford reagent that was previously equilibrated at room temperature was added into each well. Wells containing only 10% RIPA was taken as blank. The assay plate was incubated at dark for 5 minutes, shaken for 15 seconds and the absorbance was measured at 595 nm. A calibration curve was constructed using the standard concentrations and absorbance values. From the curve equation, the concentration of test samples was calculated and averaged.

2.3.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

2.3.3.1 Gel preparation

Vertical gel casting system (Owl, Thermo, USA) was used to prepare PAGE. To separate 100kDa PKD2 and 34kDa GAPDH proteins 8% gel was prepared. For 30 mL gel mixture 14.2 mL distilled water, 7.5 mL 4X separating gel buffer, 8ml 30% acrylamide-bisacrylamide (37.5:1) solution (Amresco, USA), 0.3 mL 10% ammonium

persulfate (APS), 12 mL N,N,N',N'-Tetramethylethylenediamine (TEMED) were mixed and loaded into the glass sandwich up to a predetermined level. The upper surface of the gel mixture was covered with 2-propanol to avoid oxygen-driven inhibition of gel polymerization. After complete polymerization, 2-propanol was removed completely and 4% stacking gel was prepared: For 10 mL, 6 mL distilled water, 2.5 mL 4X stacking gel buffer, 1.33 mL 30% acrylamide-bisacrylamide solution, 100 mL 10% APS, 5 mL TEMED were mixed and loaded above separating gel. Comb was placed on stacking gel and the gel was allowed to dry completely.

2.3.3.2 Sample preparation

The volume that contains 20 μg protein was mixed with 4x sample loading buffer in 3:1 ratio. The mixture was then vortexed and incubated for 5 minutes at 95°C. for complete denaturation. Heated samples were vortexed again.

2.3.3.3 Gel loading and running

When the polymerization was complete the gel sandwich was placed on gel system and the electrophoresis tank was filled with Tris-Glycine-SDS running buffer. The wells were washed with running buffer in order to remove any unpolymerized gel remainings. Protein mixture and pre-stained protein marker (Pierce, USA) were loaded on the wells. Electrophoresis was performed at 120 V in the stacking gel and at 150 V in the separating gel. When the bromophenol blue reached the bottom of the gel, run was ceased.

2.3.3.4 Wet transfer of proteins

The proteins were transferred onto nitrocellulose membrane to enable antibody probing. To this end, gel was incubated on transfer buffer for 15 minutes. Pre-cut nitrocellulose

membrane, foam pads, filter papers were wetted on transfer buffer for 5 minutes. After incubation, gel and membrane was sandwiched between filter papers and foam pads and placed on transfer tank (Biorad, Germany). The sandwich was placed such that gel should be close to cathode and membrane should be close to anode. The tank was completely filled with transfer buffer. Transfer was performed at 4°C for 8 hours at 200mA. Complete transfer could be checked by monitoring pre-stained marker.

2.3.3.5 Blocking

After complete transfer the membrane was washed once in tris-buffered saline (TBS) for removal of residual SDS and methanol on membrane. Then, the membrane was blocked in 5% skimmed milk prepared with 1X tris-buffered-saline with Tween 20 (TBST) for 1 hour at room temperature. Next, the blocking buffer was removed and membrane was washed three times in TBST for 5 minutes.

2.3.3.6 Primary and secondary antibody treatments

Primary and secondary antibodies were from Abcam, USA. Primary antibody against GAPDH and PKD2 phosphorylated at Ser876 (p-PKD2) had rabbit origin, while that against PKD2 had mouse origin. Horseradish peroxidase linked anti-rabbit IgG secondary antibody and anti-mouse secondary antibody were from goat. Antibody treatments were performed at dilutions, temperature and periods specified at Table 2.3:

Table 2.3: Antibody dilutions and incubation conditions

Target protein	1° antibody dilution	Period	2° antibody dilution	Period
P-PKD2	1:1000	o/n 4°C	1:8 000	1hr RT
PKD2	1:1000	o/n 4°C	1:20 000	1hr RT
GAPDH	1:10000	o/n 4°C	1:20 000	1hr RT

Antibodies were diluted in fresh TBST and primary antibodies were reused several times. After incubation in primary antibody, membrane was washed three times in TBST and probed with corresponding secondary antibody. Next, the secondary antibody was removed and membrane was washed three times in TBST.

2.3.3.7 Image development

After serial washes, membrane was incubated in enhanced chemiluminescence substrate (ECL) kit (Pierce, USA) for 1 minute and an X-ray film (Thermo Scientific, USA) was exposed to membrane for 1-5 minutes. The film was developed on Kodak X-ray Processor, Germany. Image analysis was performed using ImageJ. PKD2 band intensities were normalized to those of GAPDH and expressed relative to first lane in each blot. Phosphoryation index was calculated by taking ratio of p-PKD2 to PKD2 band intensities (Chen *et. al*., 2011).

2.3.3.8 Stripping and reprobing

In order to reprobe the membrane for a different protein (e.g. loading control), excess ECL substrate and bound antibodies were removed by membrane stripping. After chemiluminescent detection membrane was washed in TBS for four times and incubated in stripping buffer with gentle agitation for 30 minutes at 50°C. The buffer was removed and membrane was washed four times in TBST. Membrane was then blocked in 5% skimmed milk once again and probed against second target protein.

2.4 siRNA Transfection

Small interfering RNA targeting specifically the protein kinase D2 encoding mRNA was purchased from Qiagen, Germany and used to transiently silence *PRKD2* gene (Table 2.4). siRNA that did not have a target in human transcriptome was used as control. It

was bought from Santa Cruz, USA. siRNA delivery was managed by HiPerfect Transfection Reagent (Qiagen, Germany).

Table 2.4: Sequences of *PKD2* mRNA targeting siRNA (Flexitube Hs_PRKD2_6, Qiagen, Germany)

siRNA	Sequence (5'-3')
PKD2 Sense	GGG UGG UUC AUU ACA GCA ATT
PKD2 Antisense	UUG CUG UAA UGA ACC ACC CAA

2.4.1 Optimization of transfection efficiency

Transfection efficiency is the fraction of the cultured cells that take up the nucleic acid complexed with the transfection reagent. Depending on the culture conditions and cell type transcription efficiency may vary. Low transfection efficiency, however, may hinder the detection of phenotypic effect arisen from specific gene knockdown unless they are selected. In order to avoid variations between transfections, transfection conditions needed to be optimized. First of all, internalization of siRNA and the percent of cells transfected was validated using Alexa Fluor® 488 conjugated negative siRNA (Qiagen, Germany). To this end, cells were seeded at a density of 200 000 cells in 2.3ml complete culture medium into 6-well plates. Various amounts of siRNA at final concentrations of 1nM, 2nM, 5nM, 10nM and 20nM were diluted in serum-free culture medium and mixed with 12 mL transfection reagent. As a control, only-transfection reagent containing mixture was made. After 10-minute incubation at room temperature and dark to allow formation of transfection complex, 100 mL of transfection mix was added dropwise onto the cells. After 20 hours of transfection, cells were collected by trpsinization, washed in PBS, pelleted and resuspended in PBS and transferred onto ice.

Transfection efficiency for each siRNA concentration was determined by flow cytomety (Accuri, BD, USA) at FL-1 channel (for green fluorescence).

2.4.2 Transfection at 6-well plates and validation of knockdown

MCF-7 or its drug resistant variants were cultured at 6-well plates at a density of 200 000 cells in 2.3 mL medium. *PRKD2* and control siRNA were diluted in serum free culture medium to a final concentration of 10nM (when seeded on cells) and 12 mL of transfection reagent was added. After 10 minute incubation, cells were treated either with control siRNA or *PRKD2* siRNA. For each cell line, one well was left untransfected and taken up only serum free culture medium. Cells were incubated in normal culture conditions for 24, 48 and 72 hours. At specific time points, cells were lysed in TriPure reagent for RNA isolation or in RIPA buffer for protein extraction. Silencing was verified by qRT-PCR at mRNA level and by Western blotting at protein level.

In certain instances, successive transfections could yield higher knockdown efficiency. In that case, after first transfection cells were harvested by trypsinization, counted and seeded again at the same density. Cells were treated with freshly prepared transfection complexes and incubated further.

2.4.3 Transfection at 96-well plate

Two pmol siRNA was diluted on 50 mL serum-free medium and mixed with 0.75 mL transfection reagent. Transfection mixture was incubated at room temperature for 10 minutes. At the same time, cells were harvested by trypsinization, pelleted, counted. Ten-thousand cells were cultured per well at 150 mL complete medium. Transfection complexes were added on cells dropwise and incubated for 72 hours.

2.5 Cell viability assay using XTT reagent

Cell viability after drug treatment was assayed using XTT reagent. XTT is a tetrazolium salt that is converted into colored formazan dyes in viable cells whose mitochondria were active. Intensity of color developed is proportional to relative viability.

Cells were seeded and transfected, when necessary. After 72 hours following the transfection, medium was discarded and washed in complete medium once. For treatment of MCF7/DOX cells, highest drug concentration- containing medium (250 μM doxorubicin) was applied into second column in 100 mL while all other wells received 100 mL complete medium. Next, 200 mL highest drug concentration-containing medium was added on 3rd column that already had 100 mL medium, dropping the final drug dose to 2/3 of highest drug dose. After gentle pipetting, 200 mL from 3rd column was transferred to 4th column. Serial dilutions between successive columns were repeated till 12th column. First column was left untreated and no cells were seeded on 1st and 8th rows to be taken as blank since doxorubicin itself gave absorbance at 492nm. After 48 hours of treatment 50 mL of activated XTT reagent were added on each well and incubated for 4 hours at cell culture incubator.

Absorbance at 492 nm was measured and absorbance of doxorubicin in cell-free wells (1st and 8th wells in each column) was substracted from the absorbance reading of each test sample at that specific column. 100% proliferation was assigned to untreated cells and the rest was calculated accordingly.

2.6 Migration assay

In order to see the effect of PKD2 knockdown on motility of MCF7/DOX cells, migration of the cells towards serum-containing medium across a porous membrane was

assayed (Figure 2.1: Migration assay overview. Adapted from Kramer *et.al.*, 2013Figure 2.1).

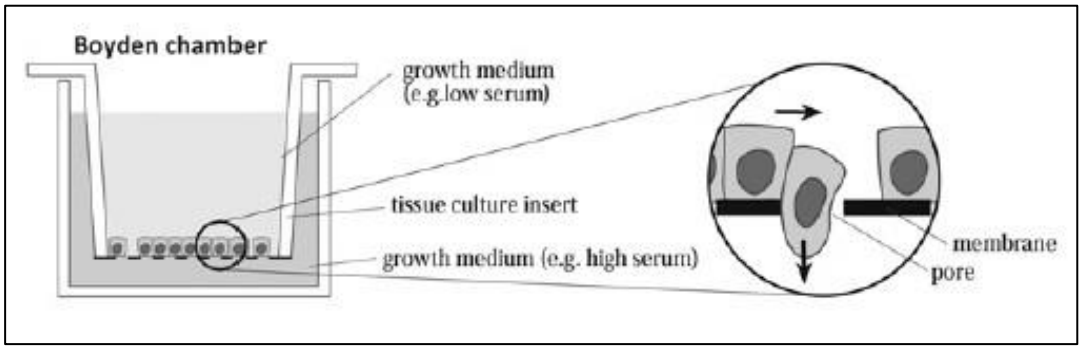

Figure 2.1: Migration assay overview. Adapted from Kramer *et.al.*, 2013

Migration assay procedure was adapted from Cimen *et.al.*, 2009; Tezcan & Gündüz, 2014 with some modifications. Briefly, cells are transfected with control siRNA or PKD2 siRNA for 72 hours and incubated in 0.5% FBS containing starvation medium 12 hours before the assay. Cells are harvested in 0.05% Trypsin EDTA and washed twice in 0.5% FBS containing starvation medium; then cells are resuspended in starvation medium and counted. 50 000 cells were seeded in 200 mL starvation medium per culture insert having 8 μm pore size (Greiner, Germany). Culture inserts were then placed on 10% FBS medium, which serves as chemoattractant, and incubated for 24 hours at culture incubator. Migrated cells were then visualized and counted as follows: Media inside the insert was removed and inserts were washed in PBS twice. Next, migrated cells that were stuck on membrane were fixed in ice-cold 100% methanol for 10 minutes at room temperature. Methanol was removed and inserts were washed twice in PBS. In order to visualize the cells, the inserts were treated with Giemsa stain (Merck, Germany) for 2 minutes at dark. Excess dye was removed by serial washes in PBS. Using a cotton swab non-migrated cells were cleared off the membrane surface. Membrane was separated from the insert by lancet; mounted on coverslides with a drop of mineral oil

and visualized under microscope with 100X and 200X total magnifications. At least three distinct areas from the inserts were chosen for count.

2.7 Wound healing assay

Two hundred cells were transfected in duplicates with either control siRNA or PKD2 siRNA for 72 hours on 6-well plates, reaching approximately 100% confluency. In each well four wounds were generated using sterile yellow pipette tips in the same geometries. Wounds were imaged just after wounding. Fifteen hours after wound induction, wound closure was imaged. Average wound closures were calculated in ImageJ by measuring four horizontal edge-to-edge distances (arrows, Figure 3.12) in each wound. Wound closure was expressed as average percent closure from two experiments.

2.8 Statistical analysis

Results gathered from at least two independent experiments are expressed as mean ± SEM and were analyzed using GraphPad Prism Version 5.01 with one-way ANOVA followed by Tukey's Test or Student's t-test.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 PKD2 is expressed in a variety of breast cancer cell lines and drug-resistant derivatives.

Western blot analysis demonstrated that parental breast cancer cell lines with different phenotypic features (MCF7, SKBR3, BT474, MDA-MB-231) as well as drug-resistant MCF7 variants (MCF7/DOX, MCF7/ETO, MCF7/ZOL) expressed PKD2 at comparable levels (Figure 3.1). Band intensities were reported relative to MCF7 lane. Values below PKD2 lane indicated the normalized PKD2 expression (ratio of relative band intensity of PKD2 to that of GAPDH). Values below p-PKD2 lane implied a so-called "phosphorylation index" parameter that was calculated as indicated in Materials and Methods 2.3.3.7.

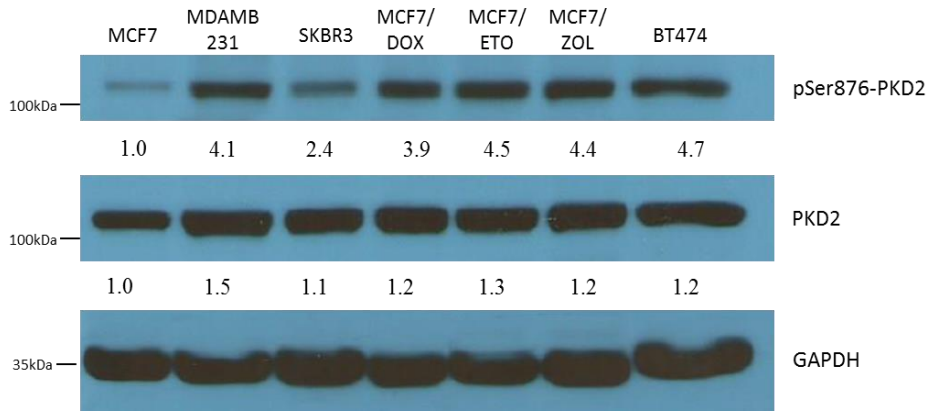

Figure 3.1: PKD2 expression in breast cancer cell lines.

It was observed that PKD2 protein levels did not vary among the cell lines. However, as a signaling molecule, the basal level activity of PDK2 may have equal importance with its expression. Various sources identified serine 876 of PKD2 as an autophosphorylation site whose phosphorylation correlates with activity (Chen *et.al.,* 2011; Sturany *et.al.*, 2001). Therefore, we also checked the levels of PKD2 phosphorylated from Ser876 (pSer876-PKD2) within total lysate. To this end, we used an antibody that specifically reacts with a linear epitope containing pSer876. According to the results, basal level activity showed differences between cell lines. Notably, drug resistant derivatives of MCF7 had higher basal level Ser876 phosphorylation than the parental cell line as demonstrated in the phosphorylation indexes (Figure 3.1). This finding directed us to speculate that increased PKD2 activity could be associated with drug resistance.

It should be noted that phosphorylations and other post-translational modifications can cause band shifts in SDS-PAGE (http://www.abcam.com/Protein-Kinase-D2-phospho-S876-antibody-EP1496Y-ab51251.html#description_images_1). It seemed that this shift is not dramatic for PKD2 (Sturany *et.al.*, 2001; Figure 6A) and strongly dependent on resolution of SDS-PAGE. Since we aimed simultaneous detection of 34kDa protein (GAPDH, as loading control) and 100kDa (PKD2) on the same gel, our resolution was limited around high molecular weights (above 100 kDa), disabling complete separation of PKD2 and p-PKD2.

In order to study the effects of PKD2 on chemoresistance, several strategies can be followed. Pharmacological modulation of the activity can be one alternative. Currently, there are several PKD inhibitors in the market such as Kb-NM142-70 (Hao *et.al.*, 2013; Lavalle *et.al.*, 2010), Gö6976 (Hao *et.al.*, 2013), CRT0066101 (Harikumar *et.al.*, 2010). However, the major problem with those inhibitors is their selectivity and potency: They are not isoform-specific and they can also inhibit related group of kinases like PKCs and MAP kinases (Fu & Rubin, 2011). Therefore, they are not compatible with the

mechanistic studies to unravel the functions of specific subtypes even though they could be successful in clinical means. Since PKD family can be activated by phorbol esters, one may think using them to activate PKD. However, classical and novel PKC members can also be activated by phorbol esters and may transduce signals though as well as independent of PKD; thereby scrambling the results. All these drawbacks directed us to consider RNAi mediated knockdown of PKD2 by using siRNA. In that way, PKD2 would be specifically downregulated in drug-resistant sublines.

3.2 Determination of siRNA transfection efficiency

Transfection efficiency could be determined visually or phenotypically (http://www.qiagen.com/resources/molecular-biology-methods/transfection/#Performing appropriate RNAi control experiments). Here MCF7/DOX cells were transfected with fluorescently-labeled control siRNA and analyzed the uptake by flow cytometry (Figure 3.2).

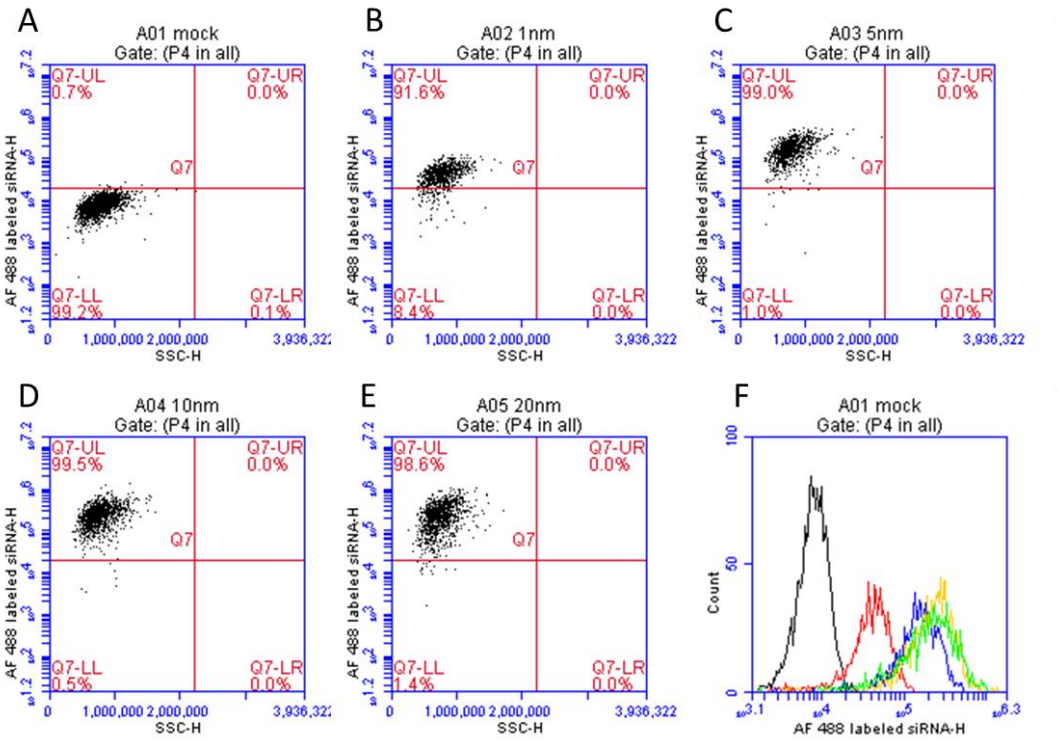

Figure 3.2: Optimization of transfection efficiency. MCF7/DOX cells were transfected with transfection reagent alone (A), 1nM siRNA (B), 5nM siRNA (C), 10nM siRNA (D) and 20nM siRNA (E). Mean fluorescence intensity from each treatment is shown in F (black-mock; red-1nM; blue-5nM; yellow-10nM; green-20nM).

Upper left quarter of each quadrands (Figure 3.2A-E) was assigned to have siRNA positive cells after setting a threshold at FL-1 channel readings, while lower left quarter has siRNA-negative cells. Percents at each quarter showed the percent of the cells located within that quarter. (Even at 1nM siRNA, more than 90% of the cell population was transfected. This ratio increased up to 99.5% at 10 nM and did not increase futher at 20 nM.

Figure 3.2F and Figure 3.3 displayed the mean fluorescence intensity, a parameter that measures internalized siRNA.

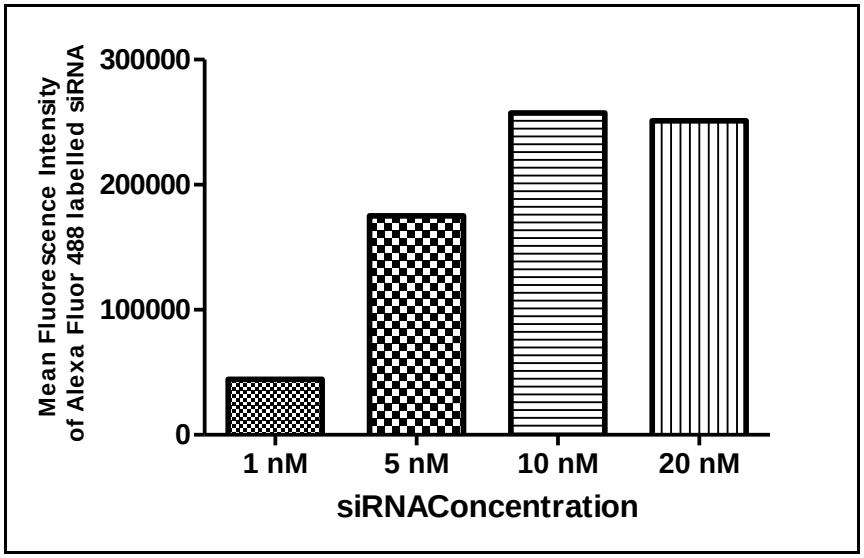

Figure 3.3: Graphical representation of mean fluorescence intensity of internalized siRNA (Figure 3.2F)

Since mean fluorescence intensity is the highest at 10nM, we did not select a higher siRNA concentration for downstream experiments. Indeed, there is a trade-off between silencing efficiency-siRNA internalization and off-target effect which arises due to incomplete complementarity between mRNAs and siRNA or immune response generated against siRNA entry. Using low siRNA dose may protect from off-target effects; however, it may not yield effective silencing, preventing the evaluation of the role of protein. On the other hand, using too much siRNA can cause off-target effects such as nonspecific silencing and induction of immune response (Caffrey *et.al.*, 2011). Actually, Caffrey (2011) reported that siRNA concentration as low as 1 nM changed the expression of immune-related genes –despite being to a lesser extent compared to 25 nM- while 25 nM siRNA can dramatically affect the expression of non-targeted genes. In some other reports the cut-off concentration was accepted as 100 nM while it was demonstrated that 20 nM siRNA was safe to avoid off-target effects (Persengiev *et.al.*, 2003). These parallel but quantitatively-distinct reports suggested that there is no fixed "safe" siRNA concentration; rather it is dependent on the type of siRNA, efficiency of

transfection (that in turn depends on the properties of the transfection reagent). In addition to siRNA concentration, there are other factors that can influence off-target effects. Firstly, siRNA modifications such as 2'-O-methyl modifications can reduce non-specific effects without affecting the specific silencing efficiency (Jackson *et.al.*, 2006). Secondly, design of siRNA is not only important for strength of silencing but also for selectivity. siRNA should be designed accordingly to prevent strong siRNA-nonspecific target interactions i.e. by allowing at least several mismatches with unintended targets (Semizarov *et.al.*, 2003). Since genome-wide screen for each and every siRNA to check the off-targets is cumbersome; one should select the "minimum" effective siRNA concentration –if necessary with modifications- to escape from these unwanted effects. In our case, since the persistence of silencing and its extend are desirable 10 nM concentration was selected.

3.3 Validation of *PKD2* knockdown

PKD2 expression was analyzed in both mRNA and protein levels using 10nM *PRKD2* targeting siRNA. qRT-PCR analysis showed that *PRKD2* transcription reduced by 80% compared to untransfected controls from 24h to 72h after transfection while control siRNA did not significantly alter PDK2 expression (Figure 3.4).

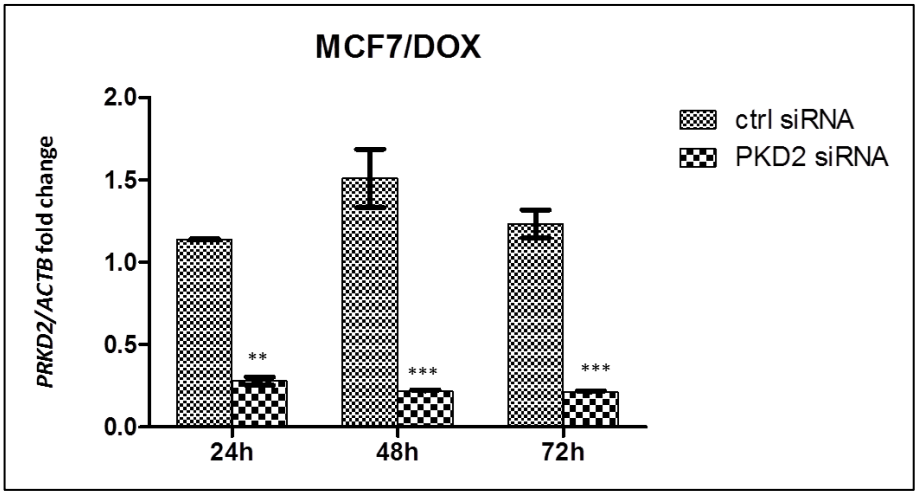

Figure 3.4: PKD2 expression following 24 hours, 48 hours, 72 hours post-transfection. Experiment was repeated twice. Data was presented as mean ± SEM and analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test using GraphPad Prism. Stars showed the significance of change between control siRNA and PKD2 siRNA treatment within a given time period (for p<0.01; *** for p<0.001)**

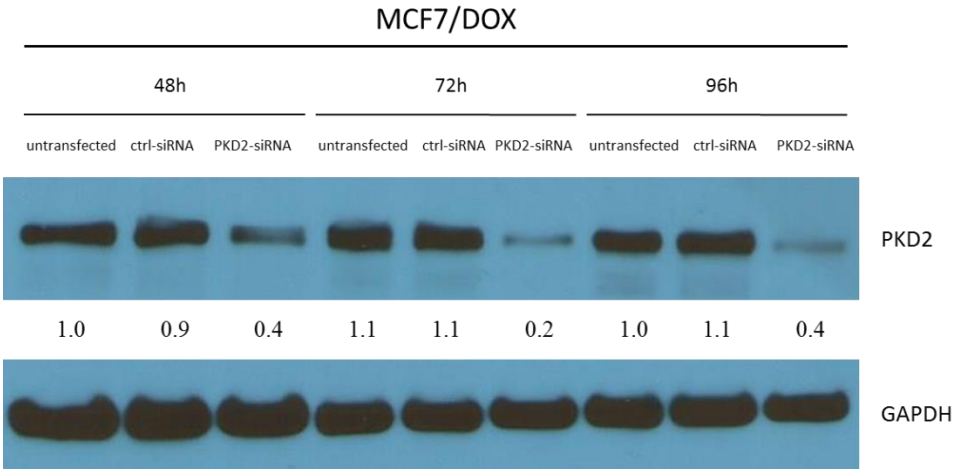

Figure 3.5: Validation of PKD2 knockdown at protein level. Experiment was repeated twice and values informed PKD2/GAPDH densitometric ratio normalized to MCF7. PKD2 expression significantly reduced in 72 hours following transfection and remains low at 4th day.

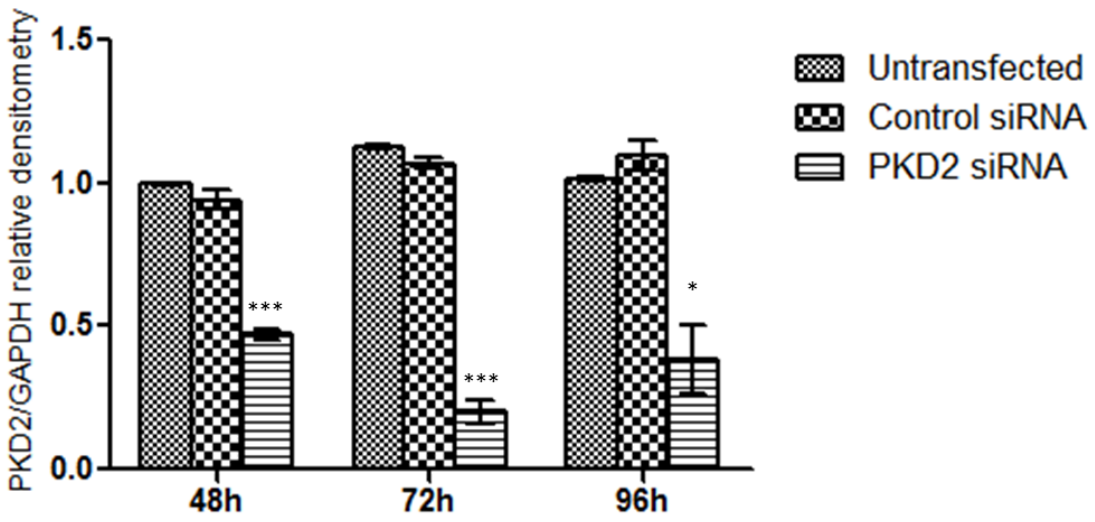

Figure 3.6: PKD2 silencing was quantified by densitometry analysis in MCF7/DOX. Experiment was repeated twice. Data was presented as mean ± SEM and analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test using GraphPad Prism. Stars showed the significance of change between control siRNA and PKD2 siRNA treatment for each time point (* for $p<0.05$; * for $p<0.001$).**

Results demonstrated that mRNA silencing endured at least for 72 hours and protein level started to decrease at 24th hour lasting at least till 96th hour (Figure 3.5; Figure 3.6). Indeed, PKD2 expression in PKD2-knockdown cells was still 40% of the expression of control-siRNA treated cells at sixth day after transfection (experimented once, data not shown). Also, 10 nM siRNA yielded most uniform transfection (99.5% transfection efficiency, with highest siRNA internalization). Another point is that MCF7/DOX cells grew very fast; they quadrupled in 72 hours: Based on calculations on 6-wells, when 200 000 cells were seeded per 6 well, after 72 hours it yielded 900 000 cells, meaning cells divide twice. This might dilute internalized siRNA dramatically and may cause ineffective gene silencing after a while. Using 10 nM siRNA instead of 5 nM may overcome this obstacle to some extent. Under these circumstances, functional assays were decided to be carried out with 10 nM siRNA concentration.

3.4 Effect of PKD2 silencing on drug-resistance of MCF7/DOX subline

We observed a higher basal activity of PKD2 assessed by Ser876 phosphorylation in MCF7/DOX cell line where dramatic upregulation of ABCB1 was principle mechanism of drug resistance (Dönmez & Gündüz, 2011). Also, Chen (2011) reported that paclitaxel treatment increased the phospho-PKD2 levels in time-dependent manner in MDA-MB-231 cells and this was relevant to drug resistance such that PKD2 knockdown sensitized the cells to paclitaxel and blocked the rise in the expression of ABCB1, which was normally upregulated by paclitaxel treatment (Chen *et.al.*, 2011). In the light of these observations we speculated that PKD2 knockdown might reverse chemoresistance in MCF7/DOX cells. XTT based cell proliferation analysis demonstrated that PKD2 expression was not important for doxorubicin resistance in that derivative cell line (Figure 3.7). We observed that in a variety of drug doses scanned (4-250 μM), there was no statistically significant difference in proliferation among untransfected, control siRNA transfected and PKD2 siRNA transfected cells.

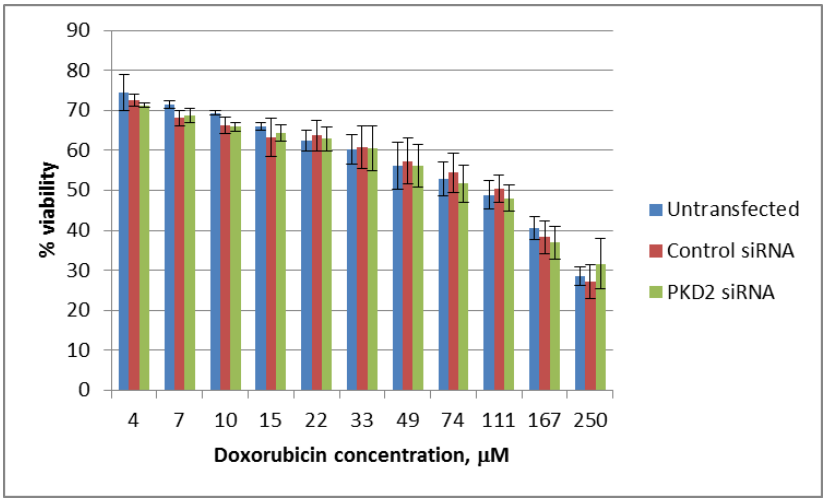

Figure 3.7: Silencing of PKD2 does not alter doxorubicin resistance in MCF7/DOX subline. Results were obtained from three independent experiments, represented as mean ± SEM.

There might be several explanations for this observation: First of all, PKD2 might not have a significant role in drug resistance in MCF7/DOX: It was reported that cell lines selected for drug resistance most often exhibit constitutive overexpression of drug transporters including ABCB1 through mechanisms like gene amplification (Scotto, 2003) implying that regulation of ABCB1 expression in drug-sensitive cell lines might be distinct from the drug-selected cell lines. Although we did not compare regulation of ABCB1 gene expression in parental and doxorubicin resistant cell lines, we observed that PKD2 knockdown did not affect neither the intrinsic resistance of MCF7 parental cell line towards doxorubicin nor its ABCB1 expression, indirectly suggesting that there was no significant involvement of PKD2 in doxorubicin resistance in MCF7 cells. Comparing with the literature, it appeared that the role of PKD2 on chemoresistance was context- and maybe drug-dependent.

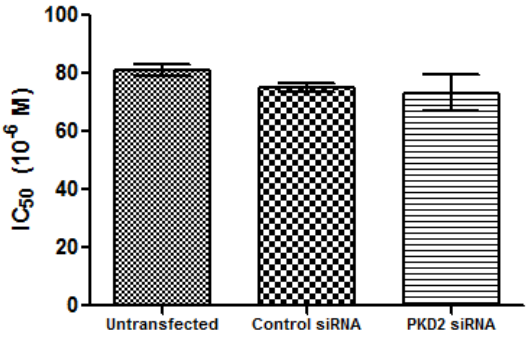

Figure 3.8: IC_{50} value of doxorubicin did not change significantly upon PKD2 knockdown in MCF7/DOX cells. IC_{50} values were calculated from logarithmic trendline expression after viability-dose curves (Figure 3.7) were constructed.

3.5 Effect of PKD2 silencing on MDR-, apoptosis- related gene expression

We tried to further verify the cell proliferation data that showed the ineffectiveness of PKD2 silencing in reversal of doxorubicin resistance. To this end, we screened the expression of ABCB1, cFLIP(L) and Survivin in PKD2-knockdown cells. qRT-PCR

analysis showed that there was no significant difference in neither of the genes screened, further suggesting that PKD2 knockdown did not affect the particular doxorubicin-induced death pathways (Figure 3.9).

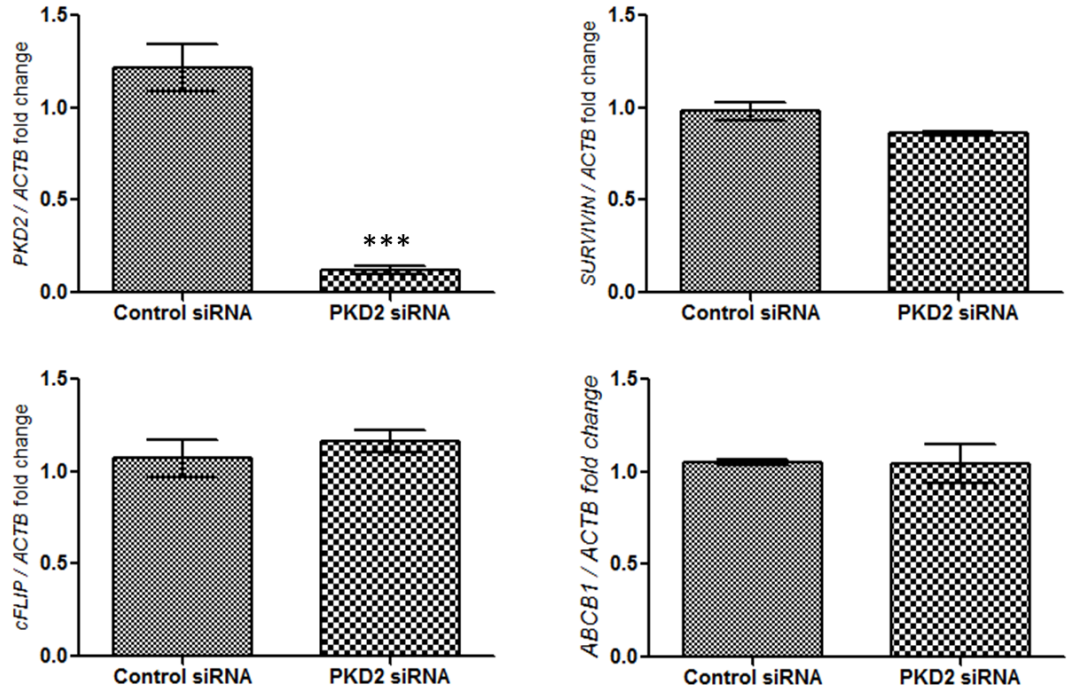

Figure 3.9: Silencing PKD2 did not change the mRNA expressions of apoptosis and multidrug resistance-related genes that were implicated in doxorubicin resistance. MCF7/DOX cells were transfected and lysed for RNA isolation 72 hour-post transfection. Expression analysis was performed as indicated in Chapter 2.

ABCB1, the major ABC transporter upregulated in MCF7/DOX cell line (Dönmez & Gündüz, 2011; Kars *et. al.*, 2006) and its expression was controlled by PKD2 (Chen *et.al.*, 2011) in another system. Anti-apoptotic genes Survivin and long form of cFLIP (cFLIP (L) were upregulated by overexpression of PKD1 (Trauzold *et.al.*, 2003). Also, cFLIP silencing sensitized MDA-MB-468, MDA-MB-231, MCF-7 breast cancer cells to doxorubicin- induced death (Rogers *et.al.*, 2007). In addition, pharmacological inhibition of PKD resulted in increased cell death via reduced expression of anti-

apoptotic proteins Survivn, Bcl-2, Bcl-X_L, cIAP1 in pancreas cancer tumor explants (Harikumar *et.al.*, 2010). All these data let us speculate they could also role in resistance of MCF7/DOX cells. As expected from the XTT cell proliferation assay results, PKD2 knockdown did not alter the gene expression in MCF7/DOX cells, suggesting that PKD2 did not affect certain doxorubicin related death pathways-at least at gene expression level- to a functional consequence.

3.6 Effect of PKD2 silencing on directed migration

Doxorubicin resistant MCF7 derivative is highly invasive and motile compared to parental cell line, as determined in chemotactic migration assay (Tezcan & Gündüz, 2014). We next checked whether PKD2 expression is associated with this phenotype.

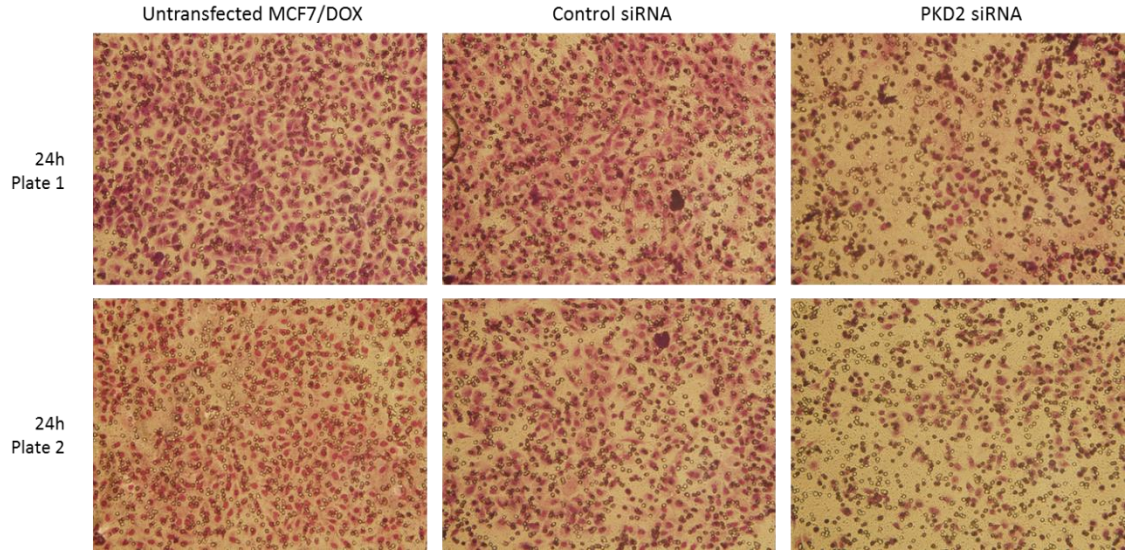

Figure 3.10: PKD2 silencing suppressed the migration of MCF7/DOX cells through transwell culture insert towards high-FBS medium.

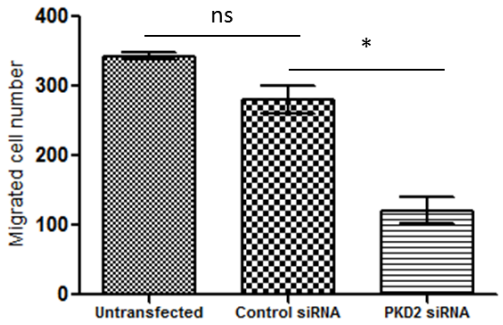

Figure 3.11: PKD2 knockdown declined the number of migrating cells. Experiments were repeated twice and data was expressed as mean ± SEM. One-way ANOVA followed by Tukey's post test indicated change in migrating cell number was significant (*, $p<0.05$) between control- and PKD2 siRNA treated cells while difference between untransfected and control siRNA treated cells was not statistically significant (ns).

Cell migration assay demonstrated that PKD2 silencing inhibited migration towards 10% FBS containing medium. This may imply PKD2 can drive cell motility in MCF7/DOX cells (Figure 3.10; Figure 3.11).

In order to provide additional proof for the association of PKD2 with motility of MCF7/DOX cells wound healing assay was performed.

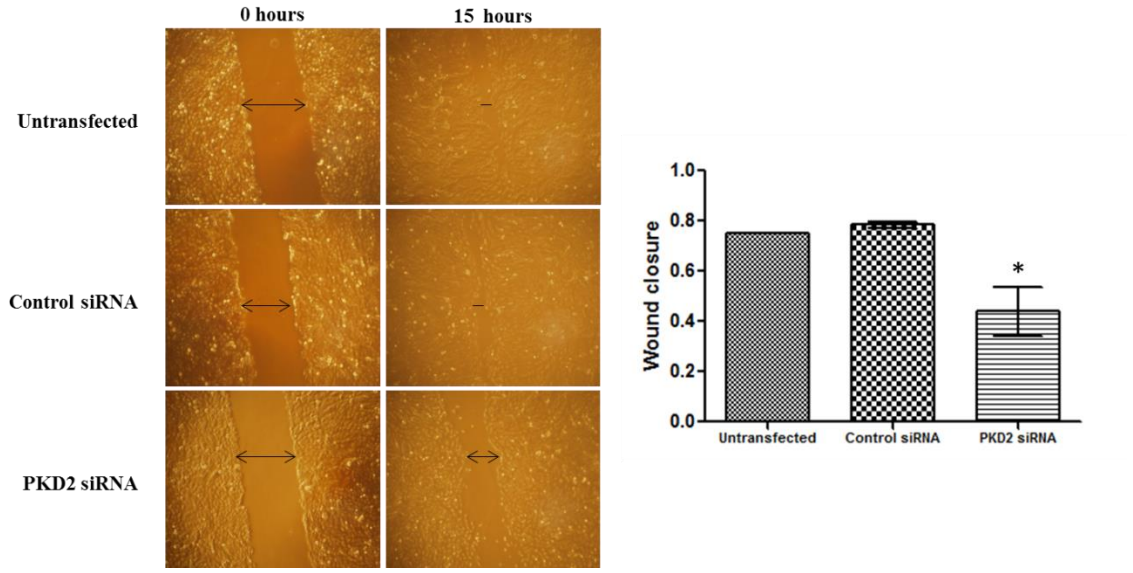

Figure 3.12:(*Left*) Wound healing assay demonstrated that motility was reduced by PKD2 knockdown. (*Right*) A smaller fraction of the gap was filled by PKD2-silenced MCF7/DOX cells compared to that filled by control siRNA treated cells (* for $p<0.05$).

Fifteen hours following wound induction, approximately 80% of the wound was closed in control siRNA-transfected MCF7/DOX cells while around 50% of the gap was closed in PKD2-knockdown MCF7/DOX cells (Figure 3.12), providing another evidence for the conclusion that PKD2 knockdown reduced the motility of MCF7/DOX cells.

Actually, there are several reports showing that PKD members have roles in cell migration and invasion although the functions of PKD members vary in cancer types. For instance, siRNA mediated knockdown of PKD2 in glioblastoma cell line U87MG suppressed migration in uncoated and matrigel coated transwells towards 10% FBS medium (Bernhart *et.al.*, 2013). Mechanistically, PKD2 knockdown was shown to affect the expression of migration- and invasion-related genes important to glioblastoma such as integrin alpha 2, integrin alpha 4, plasminogen activator urokinase (uPA) and its receptor (uPAR). Similar to this study, knockdown of PKD3 and PKD2 were shown to

reduced migration and invasion in prostate cancer cell lines by regulating the uPA and a class of matrix metalloproteinase proteins (Zou *et.al.*, 2012). However, in MCF7 and MDA-MB-231 cells siRNA mediated knockdown of PKD1 and PKD2 both increased the motility of the cells (Peterburs *et.al.*, 2009). Indeed, PKD literature is rich in confusing results due to assignement of even the same isoform to both migration-promoting and migration-suppressing roles (Döppler *et.al.*, 2014). It was speculated that cofilin, a protein that is involved in actin remodeling and directional migration, is the key in breast cancer metastasis (Wang *et.al.*, 2007); and several reports revealed that PKD mediated regulation of cell migration went over cofilin pathway in breast cancer (Döppler *et.al.*, 2014; Peterburs *et.al.*, 2009).

For migration to take place, a functional cofilin cycle involving spatial and temporal phosphorylations and dephosphorylations of cofilin is required. It was shown that both phosphorylation and dephoshorylation of cofilin are under control of PKD-mediated signaling pathways (Figure 3.13) (Döppler *et.al.*, 2014). In HeLa and MDA-MB-468 cells expressing predominantly PKD3 and PKD2, under normal growth conditions, cell migration can take place with a functional cofilin cycle. In that context, PKD3 and PKD2 mediated activation of PAK4-LIMK axis phosphorylates cofilin while SSH1L was also active to reverse this, altogether creating a functional cycle. Downregulation of PKD3 reduced the migration, as it did not feed further the PAK4 and LIMK activity to phosphorylate cofilin, leading to accumulation of dephosphorylated cofilin. However, when constitutively active PKD2 and PKD3 expressed in these cell lines, migration was also reduced. This was explained as follows: Under basal PKD3-PKD2 signaling PAK4 is maximally active such that constitutively active PKD3 and PKD2 did not further increase PAK4 activity to drive more LIMK activation, thus cofilin phosphorylation. Rather, SSH1L was phosphorylated by constitutively active PKD2 and become inactive. This, in turn, caused accumulation of phospho-cofilin, thereby disrupting the cofilin cycle.

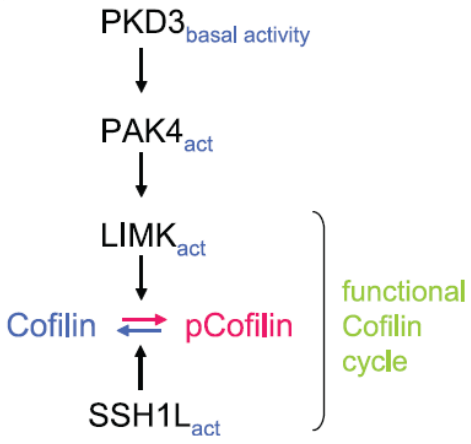

Figure 3.13: The principle cascade regulating the function cofilin cycle. Retrieved from, Döppler *et.al.*, 2014

It seems that choice of experimental tools may also be confusing at such a complex regulatory circuit. Some groups assigned migration-inhibitory roles to PKD members by expressing constitutively active mutants in different cell lines including HeLa, NMuMG (mouse mammary gland epithelial cells), HuMEC (human mammary epithelial cells), MDA-MB-468 (Döppler *et.al.*, 2014; Spratley *et.al.*, 2011) while knockdown of the members can also inhibit the migration (Bernhart *et.al.*, 2013; Döppler *et.al.*, 2014). Considering that PKD can modulate both positive and negative regulators of cofilin cycles, this can make sense-in case each and every component of the cofilin cycle and its regulators are investigated de novo without assigning a general role for these proteins. For instance, level of cofilin expression itself had effect on migration or invasion potential; in some cancers high cofilin expression counteracts the invasion while in some others its downregulation has pro-migratory effects (Wang *et.al.*, 2007). Overexpression of LIMK, which inactivates cofilin by phosphorylation, together with cofilin was also increased migration and invasiveness while overexpression of LIMK alone led to suppression of migration (Wang *et.al.*, 2007). Thus, expression and activity of each component of cofilin cycle –PAK4 LIMK, cofilin, SSH1L and the other redundant

proteins- may alter the response to constitutive activation, inhibition or silencing of PKD members at a given context. It looks wise to consider each context individually to assign a role for a given PKD member.

Besides cofilin cycle, another substrate as well as interaction partner of PKD2, calcium and integrin binding protein 1a (CIB1a) was shown to increase rate of cell migration when phosphorylated at S118 position by PKD2 (Armacki *et.al.*, 2014; Armacki, 2010). When HeLa cells were forced to express phosphomimetic mutant form of CIB1a (S118E), its non-phosphorylable form (S118A) or wild type protein, it was seen that phoshomimetic form caused increased migration (Armacki, 2010). Interestingly, Armacki found out that in HeLa and U87MG cell lines, when phosphorylated by PKD2, CIB1a colocolized with vimentin (Armacki, 2010), an intermediate filament that was highly expressed in invasive and metastatic breast cancers (Tezcan & Gündüz, 2014). Moreover, independent of its phosphorylation status CIB1a interacted with vimentin (Milena Armacki, 2010). This implied that involvement of CIB1a and vimentin might add further complexity to PKD-driven cell migration through actin remodeling, focal adhesion formation. We should note that vimentin was significantly upregulated in MCF7/DOX cell compared to MCF7 cell line, which is normally vimentin negative (Işeri *et.al.*, 2011; Tezcan & Gündüz, 2014). Since we had a molecular cue for vimentin-CIB1a-PKD2 axis for migration, differential responses of MCF7 and MCF7/DOX cells to PKD2 knockdown might be associated with vimentin status. To remind, the response of MCF7/DOX is more similar to that of U87MG glioblastoma cell line, where both cell lines are vimentin-positive. Still, this hypothesis needs to be verified experimentally. One immediate experimental approach might be co-transfection of MCF7/DOX cells with vimentin and PKD2 siRNAs and comparing it with only-vimentin knockdown and only-PKD2 knockdown cases. We should keep in mind that vimentin downregulation also decreased migration and invasion in MCF7/DOX cell lines (Işeri *et.al.*, 2011; Tezcan & Gündüz, 2014).

It has been shown that expression of EMT markers including vimentin can be contolled by PKC members in breast cancer cell lines (Morse-Gaudio *et.al.,* 1998) and ectopic expression of classical PKC, PKC-α, was shown to cause loss of epithelial character with increased vimentin expression in MCF7 cell line (Ways *et.al.,* 1995). Being a downstream effector of PKC members we thought that the effect of PKD2 ablation on migration might be mediated by lowered vimentin expression. Nevertheless, we observed that PKD2 knockdown did not affect the vimentin expression, suggesting that reduction in migration did not occur through PKD2-mediated decline in vimentin expression (Figure 3.14). Still, this did not invalidate a possible vimentin-PKD axis in migration of MCF7/DOX cells; rather, it simply eliminated PKD2-mediated expressional control of vimentin to be the cause here.

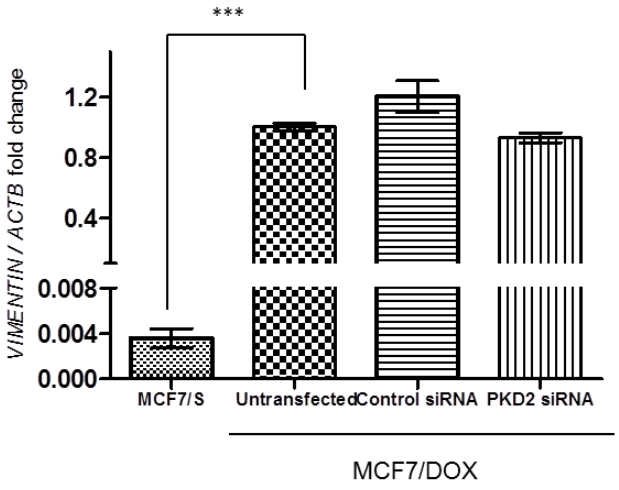

Figure 3.14: PKD2 silencing did not affect the vimentin expression. A portion of transfected and untransfected cells that were used in migration assay were lysed in TriPure reagent for RNA isolation. Expression analysis was performed as mentioned in Chapter 2. There was around 280-fold upregulation in vimentin expression in MCF7/DOX cells compared to parental MCF7 cells.

To sum up, PKD isoforms can undertake apparently contradictory roles in different contexts or even within the same context. To adapt into our case, different responses of

parental and doxorubicin resistant derivative cell line can have different signal dynamics downstream of the PKD2. Therefore, each context could be investigated individually to figure out the dynamic regulation of cell migration by PKD members. Here, we showed that PKD2 silencing might reduce the chemotactic migration in highly invasive MCF7/DOX subline, whereas it needs detailed investigation for the mechanism.

CHAPTER 4

CONCLUSION

1. PKD2 was expressed in MCF7, MDA-MB-231, SK-BR-3, BT-474 breast cancer cell lines as well as doxorubicin, etoposide and zoledronic acid resistant MCF7 derivatives. Basal level PKD2 activity is higher in drug-resistant sublines of MCF7 compared to parental cell line.

2. In MCF7/DOX cells, at 10 nM siRNA concentration, 99.5% transfection efficiency was achieved, with the highest siRNA internalization.

3. *PKD2* mRNA was reduced by 80% starting from 24th hour lasting at least to 72nd hour post-transfection. At protein level PKD2 remained silenced significantly at 96th hour.

4. PKD2 silencing did not alter the doxorubicin resistance of MCF7/DOX cell line as XTT cell proliferation assay indicated. PKD2 downregulation did not change the expressions of *ABCB1* and apoptosis-related genes like *SURVIVIN*, *cFLIP*, *PUMA*.

5. PKD2 downregulation significantly suppressed the migration of MCF7/DOX cells.

This was the first study to characterize the function of a protein kinase D member on acquired drug-resistance model. The data simply suggested that transient silencing of PKD2 did not affect the doxorubicin resistance while it reduced the motility of MCF7/DOX cells. Still, there are several questions remained unanswered. One immediate step should be assaying the involvement of PKD2 in invasiveness of MCF7/DOX cell line. Also, the prevailing mechanism by which PKD2 modulates the migration of MCF7/DOX cells needs to be found out. Moreover, function of increased basal level PKD2 activity in other MCF7 derivatives and cell lines remains to be elucidated. Next, it could be wise to screen the expression of other isoforms, PKD1 and PKD3 and study their involvement in chemoresistance and aggressiveness of drug-resistant cell lines. This project might serve as a starting point to study the involvement of PKD members in chemotherapy resistance and increased aggressiveness promoted by chemotherapy.

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APPENDIX A

BUFFERS AND SOLUTIONS

A.1. Freezing medium

9ml FBS (Biochrome, Germany)

1ml DMSO (Applichem, Germany)

Mixed and stored at +4°C

A.2. Phosphate buffered saline (pH 7.2)

1 PBS tablet (Sigma, Germany) in 200ml distilled water.

After the tablet dissolved PBS is autoclaved at 121°C for 20 minutes.

A.3. SDS-PAGE Gel Mixtures

Table A. 1: SDS-PAGE gel receipts

Component	8% Separating gel		4% Stacking gel	
	30 ml	60 ml	10 ml	20 ml
dH2O	14.2 ml	28.4 ml	6 ml	12 ml
30% Acrylamide (Amresco)	8 ml	16 ml	1.33 ml	2.66 ml
4X separating buffer	7.5 ml	15 ml	-	-
4X stacking buffer	-	-	2.5 ml	5 ml
10% APS (Applichem)	0.3 ml	0.6 ml	0.1 ml	0.2 ml
TEMED (Applichem)	12 µl	24 µl	5µl	10µl

4X separating buffer

91 g Tris base (Bioshop)

2 g SDS (Applichem)

Volume is completed to 500 mL with distilled water, after adjusting pH to 8.8.

4X stacking buffer

30.35 g Tris base (Bioshop)

2 g SDS (Applichem)

Volume is completed to 500 mL with distilled water, after adjusting pH 6.8.

Running buffer

100 mL Tris-Glycine buffer (10X)

890 mL distilled water

10 mL 10% SDS

A.4. 4X Sample loading buffer (10 mL)

2.0 mL 1M Tris-HCl, pH 6.8
0.8 g SDS
4.0 mL 100% glycerol
1.0 mL 0.5 M EDTA
8.0 mg bromophenol blue
2.6 mL H_2O

After preparation the buffer is aliquoted in 96 mL and stored at -20°C at dark. Before using 4 mL of 17.4 M beta-merchaptoethanol (Amresco) is added per aliquot.

A.5. Bradford reagent (5X)

100 mg Coomassie G-250 (Serva)
47 mL methanol
100 mL 85% phosphoric acid (Riedel-de Haen)
Volume is completed to 200 mL with distilled water and stored at 4°C, at dark.

A.6. TBS (10X)

24,23 g Tris.HCl

80,06 g NaCl

Volume is completed to 1 L with distilled water, after adjusting pH 7.6. TBS is autoclaved at 121°C for 20 minutes

A.7. TBST (0.1% Tween 20)

100 mL TBS 10X

900 mL distilled water

1 mL Tween 20 (Amresco)

A.8. Blocking buffer (5% skimmed milk in TBST)

5 g skimmed milk (Amresco) is dissolved in TBST.

A.9. Tris-Glycine Buffer (10X)

30.3 g Tris base

144.1 g Glycine (Bioshop)

Volume is completed to 1 L with distilled water, after adjusting pH around 8.3.

A.10. Transfer buffer

400 mL 10X Tris-Glycin buffer (10X)

20 mL 10% SDS

800 mL Methanol (Sigma)

Volume is completed to 4 L with distilled water.

A.11. Stripping buffer

0.76 g Tris base

2 g SDS

700 mL beta-merchaptoethanol

Volume is completed to 1 L with distilled water, after adjusting pH to 6.8.

APPENDIX B

RNA QUALITY AND QUANTITY

B.1. Representative RNA gel images and Nanodrop Spectrometry results

a) RNA from MCF7 cells transfected and treated with etoposide and doxorubicin.

Sample ID	Nucleic Acid Conc.	Unit	260/280	260/230	Sample Type
4d-untr	344.7	ng/µl	2.07	2.23	RNA
4d-untr	340	ng/µl	2.07	2.25	RNA
4d-ctrl	395.2	ng/µl	2.1	1.76	RNA
4d-ctrl	361.6	ng/µl	2.07	1.92	RNA
4d-pkd2	379.7	ng/µl	2.09	1.97	RNA
4d-pkd2	378.9	ng/µl	2.08	1.92	RNA
untr + dox	131.4	ng/µl	2.09	1.73	RNA
untr + dox	128.2	ng/µl	2.04	2.06	RNA
ctrl+ dox	113.7	ng/µl	2.07	2.09	RNA
ctrl+ dox	115.6	ng/µl	2.04	2.11	RNA
pkd2+ dox	132.1	ng/µl	2.09	1.67	RNA
pkd2+ dox	130.3	ng/µl	2.05	1.79	RNA
untr + eto	240.4	ng/µl	2.09	1.58	RNA
untr + eto	244.1	ng/µl	2.03	1.86	RNA
ctrl + eto	295.2	ng/µl	2.09	2.17	RNA
ctrl + eto	279.6	ng/µl	2.09	2.12	RNA
pkd2 + eto	252.7	ng/µl	2.09	2.23	RNA
pkd2 + eto	256.8	ng/µl	2.05	2.1	RNA
5d-untr	453.7	ng/µl	2.07	2.12	RNA
5d-untr	411.9	ng/µl	2.1	2.12	RNA
5d-ctrl	338.1	ng/µl	2.08	2.09	RNA
5d-ctrl	340.5	ng/µl	2.09	2	RNA
5d-pkd2	324.4	ng/µl	2.09	2.06	RNA
5d-pkd2	332.9	ng/µl	2.08	2.15	RNA

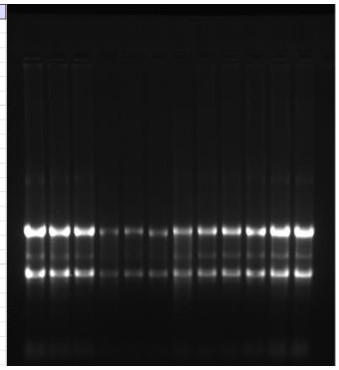


Figure B. 1: RNA quality and quantity. (left) Nanodrop results, showing RNA concentration, A260/A280 and A260/A230 ratios. (Right) Image of RNA gel (1% agarose, 80 V) showing major RNA bands and no detectable gDNA contamination

b) RNA from MCF7/DOX cells transfected and treated with IC50 dose of doxorubicin

Sample ID	Nucleic Acid Conc.	Unit	260/280	260/230	Sample Type
untransfec-dox	207.8	ng/µl	2.04	1.92	RNA
untransfec-dox	205	ng/µl	2.05	1.87	RNA
ctrl-dox	284	ng/µl	2.03	2.09	RNA
ctrl-dox	280.9	ng/µl	2.04	2.09	RNA
pkd-dox	368.2	ng/µl	2.03	2.08	RNA
pkd-dox	370.3	ng/µl	2.04	2.09	RNA
untransfec+dox	113.9	ng/µl	1.99	1.97	RNA
untransfec+dox	111.5	ng/µl	2.01	2.03	RNA
ctrl+dox	82.3	ng/µl	2.06	1.44	RNA
ctrl+dox	86.3	ng/µl	2.07	1.51	RNA
pkd2+dox	186.6	ng/µl	2.04	1.97	RNA
pkd2+dox	187.6	ng/µl	2.05	1.96	RNA

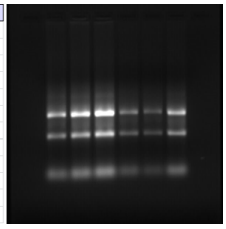


Figure B. 2: RNA quality and quantity. (left) Nanodrop results, showing RNA concentration, A260/A280 and A260/A230 ratios. (Right) Image of RNA gel (1% agarose, 80 V) showing major RNA bands and no detectable gDNA contamination

APPENDIX C

AMPLIFICATION CURVES, MELT CURVES, STANDARD CURVES AND AGAROSE GEL IMAGES

C.1. *PKD2*

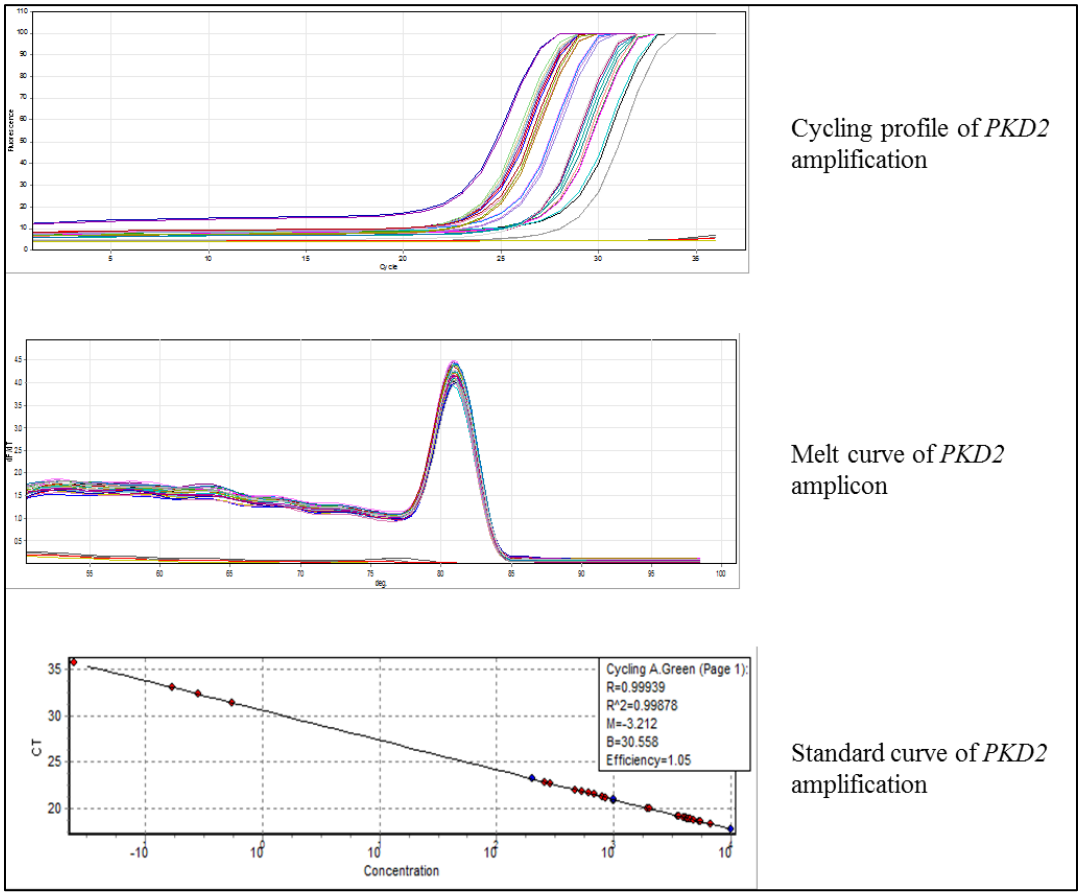


Figure C. 1: Cycling profile (upper), melt curve (middle) and standard curve (lower) for *PKD2* amplicon.

C.2. β-Actin (*ACTB*)

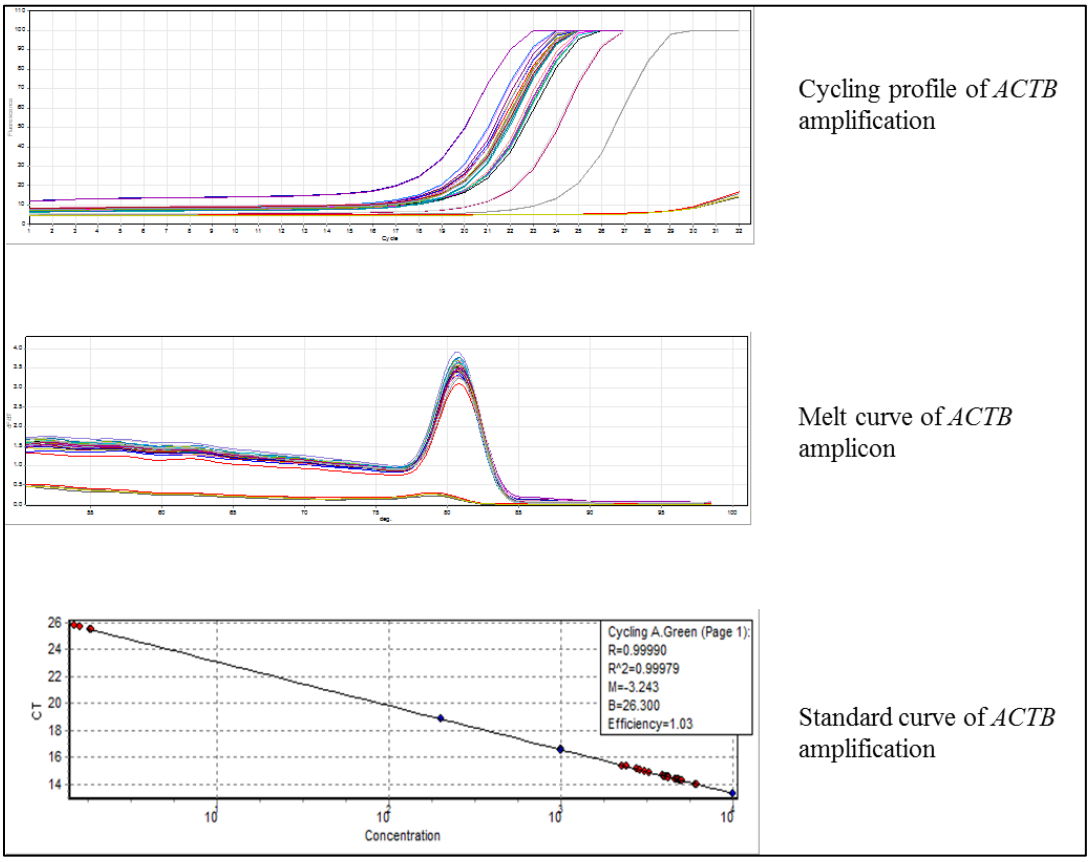


Figure C. 2:Cycling profile (upper), melt curve (middle) and standard curve (lower) for *ACTB* amplicon.

C.3. ABCB1

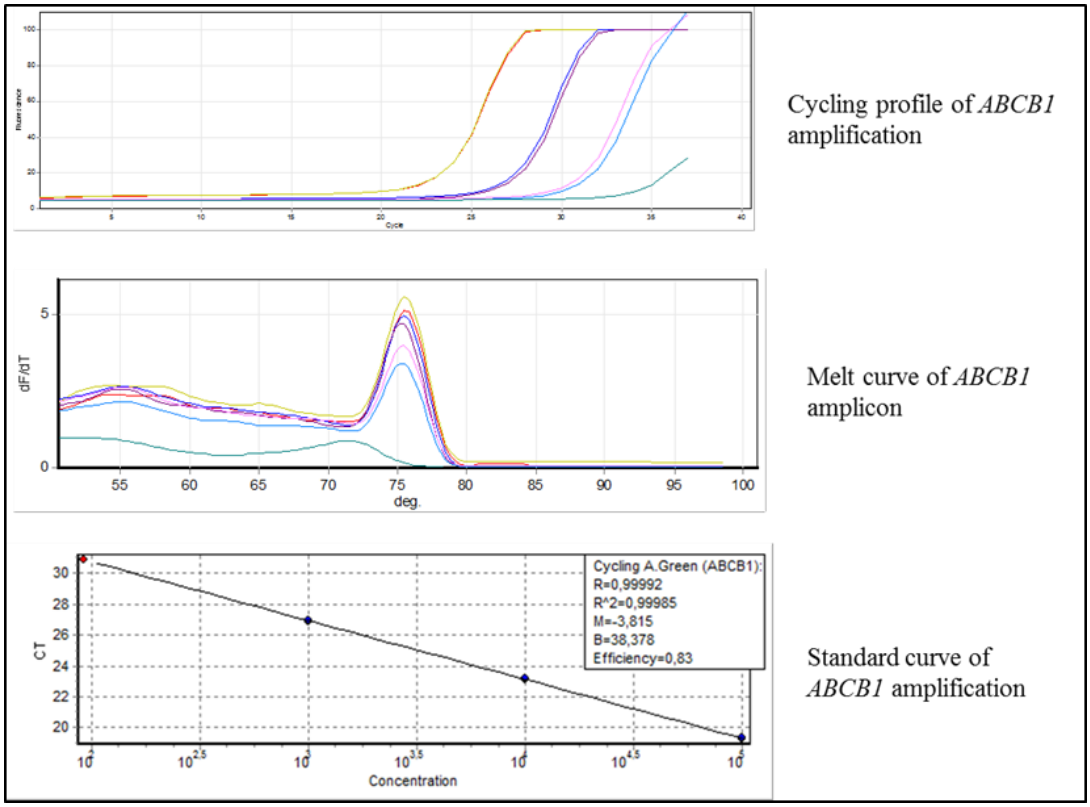


Figure C. 3:Cycling profile (upper), melt curve (middle) and standard curve (lower) for *ABCB1* amplicon.

C.4. *SURVIVIN*

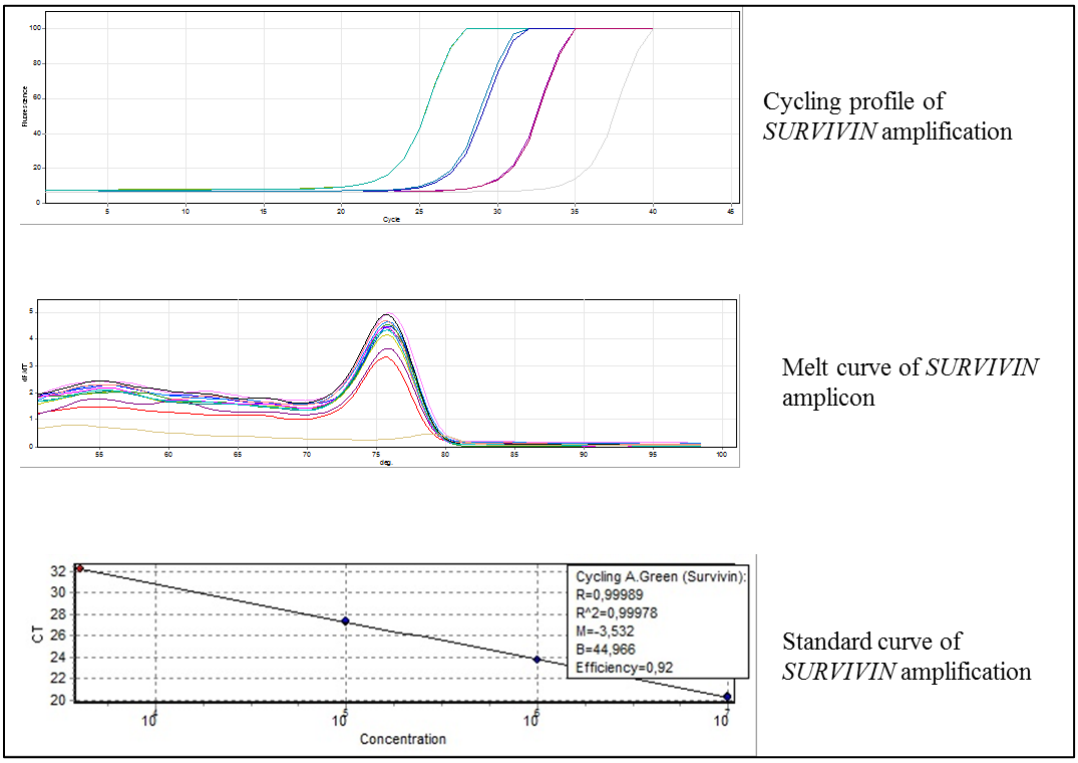


Figure C. 4: Cycling profile (upper), melt curve (middle) and standard curve (lower) for *SURVIVIN* amplicon.

C.5. *cFLIP* (long)

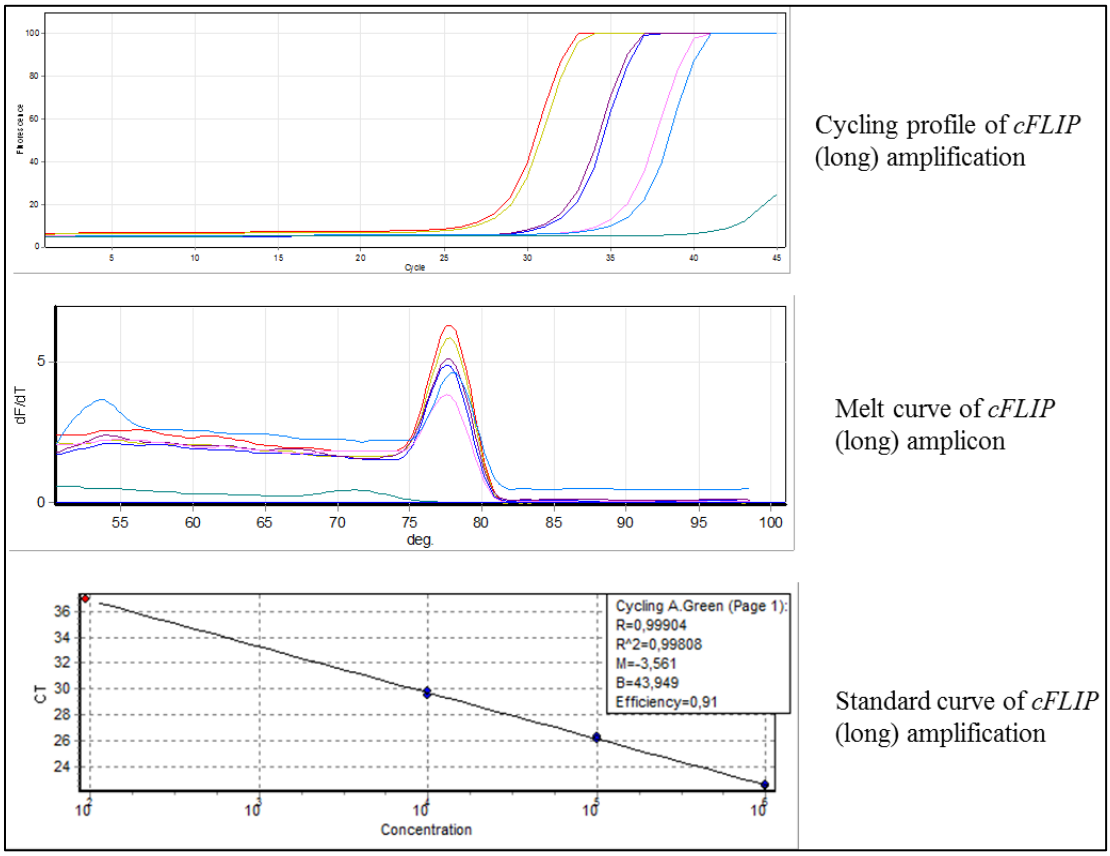
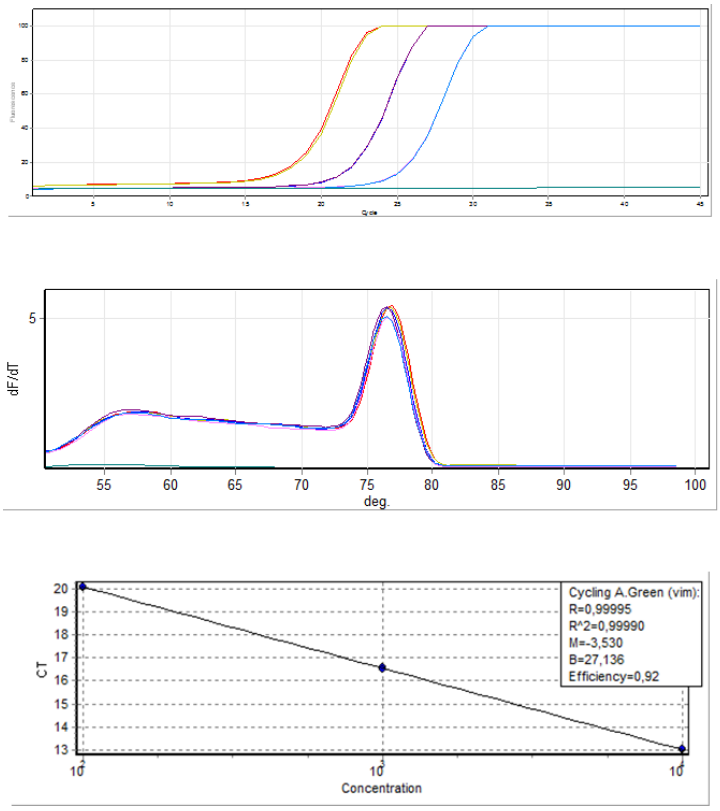


Figure C. 5: Cycling profile (upper), melt curve (middle) and standard curve (lower) for cFLIP amplicon.

C.6. *VIMENTIN*



Cycling profile of *VIMENTIN* amplification

Melt curve of *VIMENTIN* amplicon

Standard curve of *VIMENTIN* amplification

Figure C. 6: Cycling profile (upper), melt curve (middle) and standard curve (lower) for VIMENTIN amplicon.

C.7. Representative agarose gel images for each amplicon

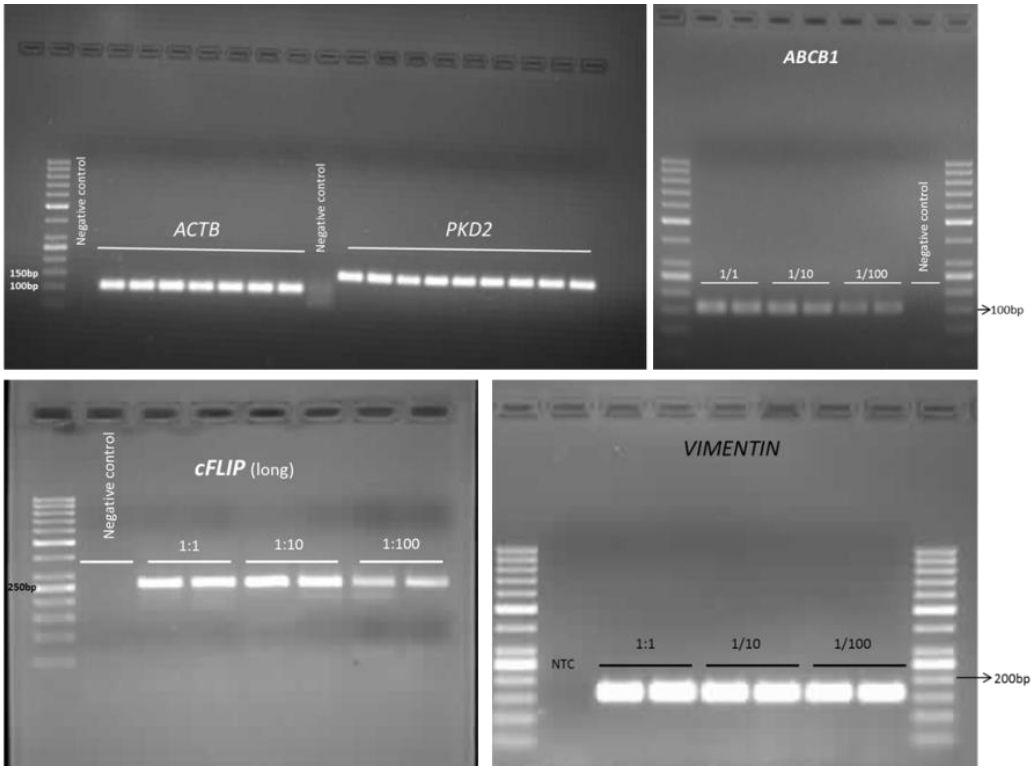


Figure C. 7: Representative gel images for amplicons *ACTB* and *PKD2* (upper left), *ABCB1* (upper right), *cFLIP* (lower left), *VIMENTIN* (lower right). *ABCB1*, *cFLIP* and *VIMENTIN* amplifications were tested in different cDNA dilutions (undiluted to 1/100 dilutions) described on corresponding picture.

APPENDIX D

REPRESENTATIVE CALIBRATION CURVE FOR BRADFORD ASSAY

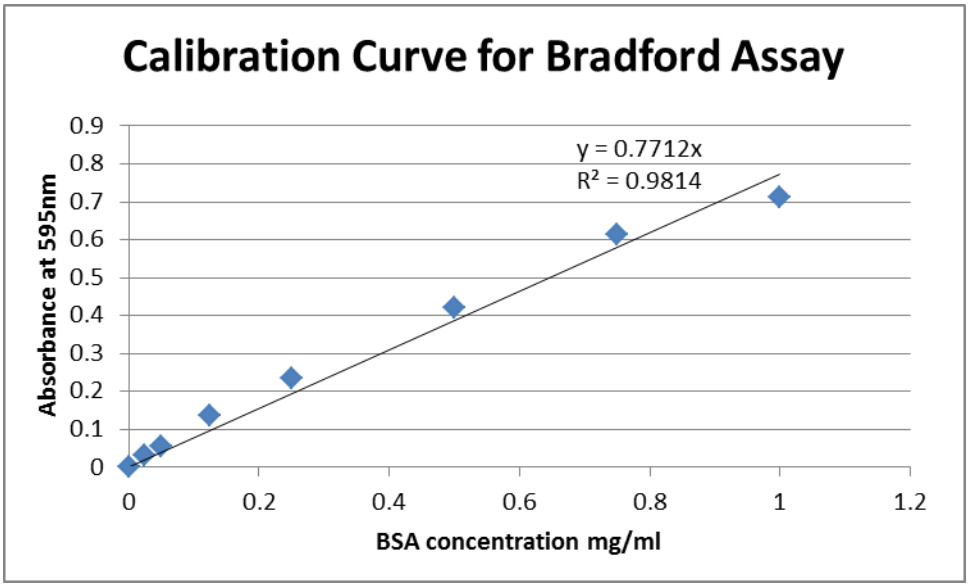


Figure D. 1: Representative calibration curve for Bradford assay

APPENDIX E

EXPRESSIONS OF *SURVIVIN*, *CFLIP* AND *ABCB1* IN DOXORUBICIN-TREATED, PKD2-KNOCKDOWN MCF7/DOX CELL LINE

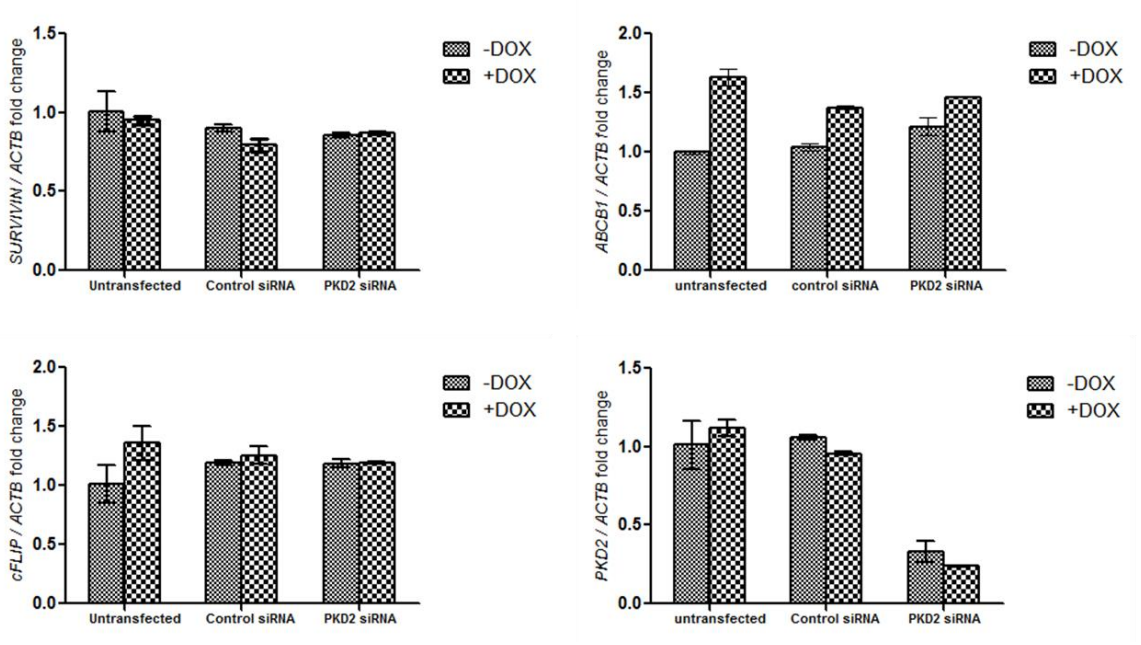


Figure E. 1: Expressions of target genes were not affected by PKD2 knockdown or doxorubicin treatment in MCF7/DOX cell line. *SURVIVIN* expression (upper left), *ABCB1* expression (upper right), *cFLIP* expression (lower left), *PKD2* expression (lower right).

Briefly, MCF7/DOX cell line was transfected for 72 hours and then left untreated or treated with IC_{50} dose (80 μM) of doxorubicin for 24 hours. Results showed that treatment of doxorubicin as well as PKD2 knockdown did not affect the gene expression although PKD2 silencing was verified.