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THE NOVEL ROLE OF BRIP1 IN BREAST TUMOR DEVELOPMENT AND PROGRESSION

BY

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ABSTRACT

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Title: The Novel Role of BRIP1 in Breast Tumor Development and Progression.

Supervisors of Dissertation: Allal, Ouhtit and Said, Sif.

Breast cancer (BC) is the most common malignancy and the leading cause of death in women worldwide. Only 5-10% of mutations in the *BRCA* genes are attributed to familial breast tumors in Eastern countries, suggesting the contribution of other genes to be identified. Pursuant to this goal, our literature search led to the following observations: **1)** in a recent study of my supervisor's team using microarray gene expression profiling of BC in Omani population identified *BRIP1* (5 fold upregulation) as a potential gene associated with BC progression; **2)** *BRIP1* is a tumor suppressor that inhibits cell growth and controls DNA repair mechanisms. Despite its role as a tumor suppressor, the precise role of *BRIP1* in breast tumor cell progression has not been explored yet; this prompted us to hypothesize that *BRIP1* is upregulated during breast tumorigenesis to promote breast tumor cell proliferation and invasion. Using a combination of cellular and molecular approaches, our results of structural validation experiments showed differential over-expression of *BRIP1* in different BC cell lines. Functional assays confirmed the novel role of BRIP1 in malignant phenotype. siRNA Down-regulation of *BRIP1* attenuated cell proliferation significantly and induced cell cycle arrest in G1/S phase. Furthermore, siRNA-mediated *BRIP1* knockdown significantly reduced both cell migration and invasion by targeting a number of potential cell motility-associated genes. Altogether, our

investigation is the first to validate the novel function of *BRIP1* in promoting breast tumor cell invasion, and identifying a unique set of pro-invasive genes to predict the mechanisms that underpin *BRIP1*-promoting BC progression. Ongoing/future experiments combining bioinformatics analysis and functional cell approaches aim to validate the relevance of these genes in BC progression. This is in order to better understand the exact molecular mechanisms that underpin *BRIP1*-promoting cell invasion, and validate the genes mediating *BRIP1* function in cell proliferation and invasion as biomarkers and/or targets to guide the design of appropriate BC targeted therapies.

DEDICATION

I dedicate this work to my dear husband and beloved boys; Ayham and Tameem who have had to endure so much stress even at such tender ages just for me.

My love for you can never be quantified.

To my parents and family for endless love and care…

To my beloved country Qatar, that deserves the best ….

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APS	Ammonium Persulfate
AR	Amphiregulin
ATCC	American Type Cell Collection
ATM	Ataxia telangiectasia mutated
BACH1	BRCA1 Associated C-terminal Helicase 1
BAP1	BRCA1 Asociated Protein 1
BARD1	BRCA1 Associated RING Domain 1
BC	Breast cancer
BCA	Bicinchoninic Acid
BLM	Bloom Syndrome
BPE	Bovine Pituitary Extract
BRCA 1	Breast Cancer Associated gene 1
BRCA 2	Breast Cancer Associated gene 2
BRCT domain	BRCA1 C Terminus domian
BRIP1	BRCA1 Interacting C-terminal helicase Protein 1
BSA	Bovine Serum Albumin
Ca2+	Calcium ion
CCND1	Cyclin D1

CDH1	Cadherin-1
CDK1	Cyclin-dependent kinase 1
cDNA	Complementary DNA
CGM	Complete Growth medium
CHEK2	Checkpoint Kinase 2
CLDN	Claudins
Ct	Cycle Threshold
CtIP	CtBP-Interacting Protein
CXCL12	C-X-C motif chemokine 12
DDR	DNA Damage Repair
DEPC water	Diethyl Pyrocarbonate water
DMEM	Dulbecco's Modified Eagle medium
DMSO	Dimethyl Sulphoxide
dNTPs	Deoxynucleotide triphosphate
DPBS	Dulbecco's Phosphate Buffered Saline
DSB	Double-Stranded DNA Breaks
E	Molar Extinction Coefficient
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-Mesenchymal Transition
EPCAM	Epithelial cell adhesion molecule

ER	Estrogen Receptor
ErbB2	Avian erythroblastic leukaemia viral oncogene homolog 2
FA	Fanconi Anemia
FACS	Fluorescence-activated cell sorting
FAM	Fluorescein amidites
FANCJ	Fanconi anemia complementation group J
FBC	Familial breast cancer
FBS	Fetal Bovine Serum
FGFR2	fibroblast growth factor receptor 2
FISH	Fluorescence in situ Hybridization
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCC	Gulf Cooperation Council
GST	Glutathione S-Transferase
HBOC	Breast and Ovarian Cancer syndrome
HDI	Human Development Index
HER2	Human Epidermal Growth Factor Receptor 2
HR	Homologous Recombination
HRP	Horseradish Peroxidase
HuMEC	human mammary epithelial cells
ICLs	Inter-strand Cross-links repair
IHC	Immunohistochemistry

LPA	urokinase-type plasminogen activator
LSP51	Lipoprotein Signal Peptidase
MAP3K1	Mitogen-Activated Protein Kinase Kinase Kinase 1
MCAM	Melanoma Cell Adhesion Molecule
MECs	Mammary Epithelial Cells
METABRIC	Molecular Taxonomy of BC International Consortium
MG2+	Magnesium ion
MGAT5	Alpha-1,6-Mannosylglycoprotein 6-Beta-N Acetylglucosaminyltransferase
MLH1	Mismatch Repair Protein
MMP	Matrix Metalloproteinase
mRNA	messenger RNA
MYC	Avian myelocutomatosis viral oncogene homolog
ND	NanoDrop
NF- κ B	Nuclear factor kappa B
NLS	Nuclear Localization Sequence
OD	Optical Density
PALB2	partner and localizer of BRCA2
PBS	Phosphae Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase

PI3KCA	phosphatidylinositol-4,5-bisphosphate 3-kinase
PR	Progesterone Receptor
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase
RAD51L1	DNA repair protein RAD51 homolog
RAS	Retrovirus-Associated DNA Sequences
RB	Retinoblastoma
RhoA GTPase	Ras homolog gene family, member A
RHOC	Ras Homolog Family Member C
RIPA buffer	Radioimmunoprecipitation assay buffer
RNAi	RNA interference
RNA	Ribonucleic acid
RPA	Replication Protein A
RPM	Revolution Per Minute
RPMI medium	Roswell Park Memorial Institute medium
RQ	Relative Quantity
RT	Reverse Transcriptase
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
SCD	SQ/TQ Cluster Domain
SCP1	synaptonemal complex protein 1
SD	Standard Deviation

SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SES	Splicing Enhancer Site
SFM	Serum Free Medium
si-Ctrl	si-Control
siRNAs	siRNA oligonucleotides
SMAD 4	SMAD family member 4
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
STK11	Serine/Threonine Kinase 11
TBS-T	Tris-buffered saline with Tween-20
TCGA	Cancer Genome Atlas
TEMED	Tetramethylethylenediamine
TF	Transcription factor
TGF-β	Tumor Growth Factor beta
TNBCs	Triple Negative Breast Cancers
TOPBP1	Topoisomerase-II Binding Protein 1
TOX3/TNRC9	TOX/ Trinucleotide repeat-containing gene 9 protein
TP53	Tumor protein 53
TSG	Tumor Suppressor Gene
UTR	Un Translated Region

VIC	2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein
W/V	Weight/Volume
Wnt	Wingless-related integration site
WR	Working Reagent
WT1	Wilms' tumor 1

CHAPTER 1 : INTRODUCTION

Breast cancer (BC), a worldwide health problem, is the most common cancer in women worldwide, including the State of Qatar (Bener, Ayub, Kakil, & Ibrahim, 2008; Bray et al., 2018; Jones, 2008; Maughan, Lutterbie, & Ham, 2010). In 2018, BC was also considered the most frequent female cancer in both developed and developing countries with ~2.1 million new cases diagnosed globally (11.6 % of all female cancers) (Bray et al., 2018). In addition, 268 600 (30.1%) new cases of BC and 41,760 (14.6%) deaths among women were predicted by The American Cancer Society in 2019 (Siegel, Miller, & Jemal, 2019). An estimated number of 19.3 million new cases will be diagnosed each year by 2025 (Bray & Soerjomataram, 2015). In the Gulf Cooperation Council (GCC) region, including Qatar, the rate of BC was 39.41 % of all female cancer cases in 2015, and the risk for women developing BC in the population was 56 per 100,000 (Qatar Cancer Registry, 2015). In 2018, BC was ranked the most frequent cancer (with a percentage of 38.7 % new cases among women compared to other cancers (Cancer & Organization, 2018), with a particular tendency to affect younger ages at advanced stages of the disease (MS Al-Moundhri, Al-Ansari, Al-Mawali, & Al-Bahrani, 2013; Phelan et al., 1998).

The etiology of BC in the GCC region including Qatar, encompasses numerous risk factors, such as late menopause, prolonged hormone replacement therapy, older age at first live childbirth, family history of BC at a young age, and the genetic mutations of the *BRCA1/2* genes (MS Al-Moundhri et al., 2013; Phelan et al., 1998). Curiously, in Oman, a recent

study showed that the majority of BC cases present to the clinic at late stages of the disease (I. Gupta et al., 2018). BC is a heterogeneous disease with variable biological and clinical distinguished traits, including ethnic and racial factors and their influence on ***invasiveness*** or ***metastasis***, which is the worst aspect of cancer. Therefore, new prognostic biomarkers need to be developed to guide the design of better-targeted therapies against invasive stages of BC in order to enhance the chance for long-term survival and patient's quality of life. To achieve this goal, it is imperative to understand the exact signalling pathways associated with the multistage process of metastasis. In fact, the process of BC metastasis involves several highly selective, sequential, and interrelated steps that begin with dissociation of cells from the primary tumor and their invasion; this process is followed by intravasation, extravasation, and establishment of cell growth at secondary site (Martin, Ye, Sanders, Lane, & Jiang, 2013). ***Invasion***, the hallmark of malignancy, is the recurring and defining event in the metastatic process, and elucidation of its mechanisms is critical for developing effective anti-metastatic therapies.

In an attempt to identify a unique set of genes associated with the transition from normal epithelial breast cells to malignant invasive cells, we used microarray gene expression profiling and compared RNA samples isolated from 40 malignant breast tumor tissues and 40 normal/benign breast tissues (I. Gupta et al., 2018). Among several differentially expressed genes, the *BRCA1* interacting C-terminal helicase 1 (*BRIP1*), showing 5-fold induction, was identified as a potential gene that might promote BC progression. *BRIP1*, also known as *FANCJ* or *BACH1*, was first identified using tandem mass spectrometry by

its physical interaction with *BRCA1*, and also belongs to the Fanconi anemia (FA) genes family. *BRIP1* is located on chromosome 17q22, spanning a region greater than 180kb starting from 61,679,185 to 61,863,558 base pair with 20 exons and 19 introns (Rutter et al., 2003). Interestingly, *BRCA1* is also located on chromosome 17q21 region, hence in close proximity with *BRIP1*.

BRIP1 plays major roles in DNA repair, development of breast and ovarian cancers as well as type J Fanconi anemia, and increasing the risk for development of leukemia and several other solid tumors, including head, neck and skin cancers (Rutter et al., 2003). BRIP1, a DNA-dependent ATPase and a 5' to 3' helicase that belongs to the DNA dependent RecQ DEAH helicase family, interacts with BRCA1 and is involved in double-stranded DNA breaks (DSB) repair during the G2-M phase of the cell cycle as well as in tumor suppression. *BRIP1* is expressed in both normal and malignant cells, controls genome integrity *via* regulation of replication and homologous recombination (HR), DNA damage responses and checkpoints, which are crucial for genomic stability (S. B. Cantor & Guillemette, 2011; London et al., 2008). While *BRCA1* and *BRIP1* work as tumor suppressor genes (TSGs) (Godwin et al., 1994), when BRIP1 fails to bind BRCA1 in certain conditions, cells will be sensitive to different genotoxic stress with aberrant homologous DNA repair function (Litman et al., 2005). The clinical finding that implicates *BRIP1* in the onset of BC was the identification of *BRIP1* germline mutations in a patient that showed an early onset of BC with wild type *BRCA1* and *BRCA2* genotypes, suggesting a major link between moderate penetrance of BC and *BRIP1* mutation (De

Nicolo et al., 2008; L.P.Ren, 2013; Rafnar et al., 2011; Seal et al., 2006).

The rationale of the present study is based on the following observations: **1)** in GCC countries, a significant number of younger patients, with advanced BC are admitted to the oncology clinic; **2)** In the GCC countries, including the state of Qatar, the rate of consanguinity is significantly high (~50%); **3)** A previous study conducted among Omani females with BC showed no significant pathogenic *BRCA1* gene missense mutations, suggesting the involvement of other genes in BC development; and **4)** microarray analysis of Omani patient breast tumors identified *BRIP1* as a potential candidate gene for BC progression, showing a 5-fold induction (I. Gupta et al., 2018).

Hypothesis:

We generated the following hypothesis based on the observations described above. We hypothesized that beyond its function as a DNA repair gene, *BRIP1* plays a novel role in malignant progression and tumor cell invasion/metastasis. In addition, to a better understanding of the BRIP1-mediated breast tumor cell invasion mechanisms, this study has the potential to identify BRIP1 as a target gene that can be used to design efficient therapeutic strategies against BC.

To address this hypothesis, we proposed the following specific aims:

1) To structurally validate the differential expression of *BRIP1* in different BC cell lines.

2) To elucidate the effect of *BRIP1* suppression, using RNAi technology on cell proliferation and motility *in vitro*.

3) To identify *BRIP1* pro-invasive target genes using the TaqMan Array analysis and predict the mechanisms that underpin *BRIP1*-promoted BC progression.

Innovation:

The innovative aspect of this investigation is to provide evidence of the novel role of *BRIP1* to promote breast tumor progression, and further, identify the pro-metastatic gene targets that underpin its novel function in promoting BC metastasis. In addition to a better understanding of the novel role of BRIP1 in promoting BC malignancy, this study has the potential to identify *BRIP1*-inducing pro-invasive genes that could serve as biomarkers and/or targets to guide the design of appropriate BC targeted therapies.

CHAPTER 2 : LITERATURE REVIEW

Although most BC cases are sporadic, some have a clear familial inheritance caused by germline mutations. Germline mutations passed through generations may contribute to increased cancer risk in these families. Although hereditary BC follows an autosomal dominant pattern, germline mutations in one allele of a tumor suppressor gene are inherited in a recessive manner. Consequently, it has been difficult to explain inherited cancer development until the "two-hit hypothesis" was formulated by Knudson in 1971 (Knudson, 1971). Indeed, in order to develop cancer, both germline mutation (first hit) and somatic mutation (second hit) have to occur to initiate tumor formation (Berger, Knudson, & Pandolfi, 2011).

2.1 Breast cancer

As with other hormone-responsive tissues undergoing many rounds of proliferation, mammary epithelium has a high risk of developing malignancies. Mammary carcinoma, or BC as it is called in humans, is the aberrant growth of mammary epithelial cells (MECs) that make up either the ducts or the lobules at the terminal end of the ducts. If confined by the boundaries of the basement membrane, the growth is termed ductal carcinoma *in situ*, but when it breaches the basement membrane it is called invasive BC. If the cancer reappears in distant sites, most commonly the bone, liver, brain, lymph node and lung (Ursaru, Jari, Naum, Scripcariu, & Negru, 2015), the BC has metastasized and is called metastatic BC (Weigelt, Peterse, & Van't Veer, 2005). BC, the most common cancer in

women, is the second most common malignancy after lung cancer worldwide (**Figure 2.1**) (Bray et al., 2018; Jones, 2008; Maughan et al., 2010). In 2018, BC is also the most frequent female cancer in both developed and developing countries with ~2.1 million new cases diagnosed globally (11.6 % of all female cancers) (Figure 2.1) (Bray et al., 2018), however, there are large geographical variations in the incidence of this disease (Figure 2.2) (Bray et al., 2018; Cancer & Organization, 2018; J Ferlay et al., 2019).

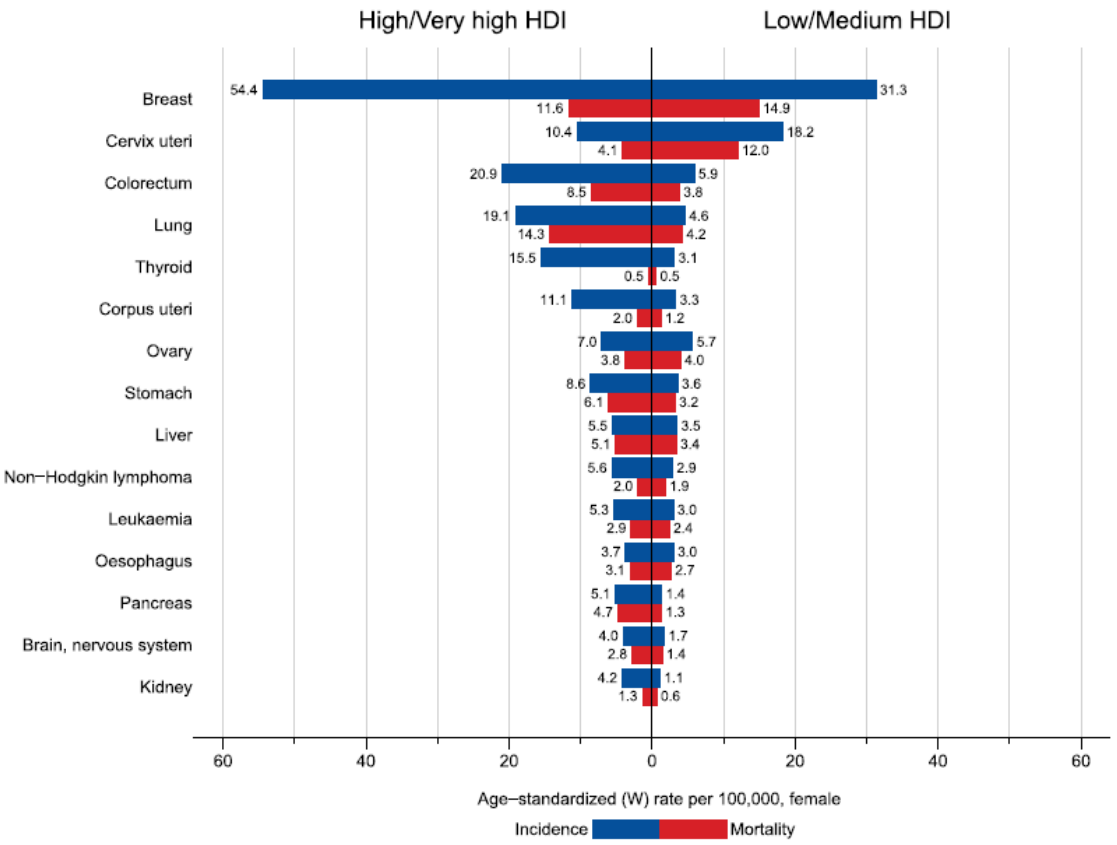

Figure 2.1: Bar Chart of incidence and mortality of most common cancers among women in Low/Medium Human Development Index (HDI) regions compared to High/Very-High HDI regions in 2018 (Bray et al., 2018).

Furthermore, the mortality rate from malignant diseases was estimated to be 8 million cases in 2008, and is estimated to reach 11 million by 2050 (Momenimovahed & Salehiniya, 2019). BC is the major cause of death among women in developing countries (14.3% of total, 324,000 deaths), estimated to reach even higher by 2020. However, it is considered the second cause of death after lung cancer in developed countries (15.4% of total, 198,000 deaths) (Jacques Ferlay et al., 2015; Momenimovahed & Salehiniya, 2019; Organization,

2014). Actually, the mortality rate is less than that among different regions, because of the higher survival rate of BC patients in developed countries (high incidence regions), ranging between 6 per 100,000 and 20 per 100,000 in Eastern Asia and Western Africa, respectively (Organization, 2014). Overall, BC is considered the second most common cancer worldwide (1.7 million cases, 11.9%). However, because of the relatively favorable prognosis, it is ranked as the 5th cause of death globally (522,000 of total deaths, 6.4%). Despite all the advances made in early detection and treatment over the last 20 years, BC is still a major burden in today's society, largely because of complications from metastatic disease and lack of effective treatments for metastasis.

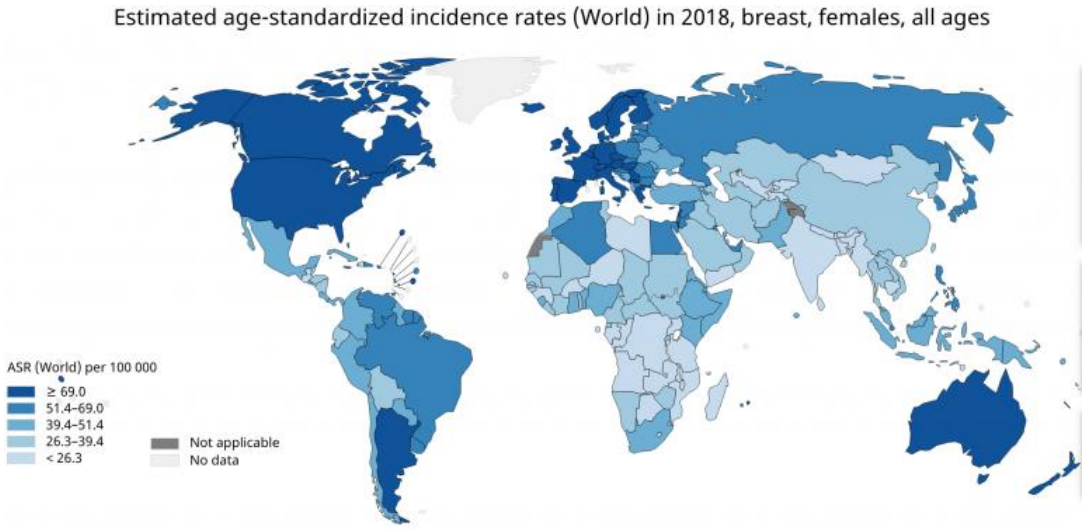

Figure 2.2: Global breast cancer incidence in 2018. Colored bars indicate age-standardized incidence rates per 100,000 (Cancer & Organization, 2018).

2.2 Predisposition to breast cancer

Both environmental and genetic factors have been identified as possible inducers of BC. Obviously, gender is important although both women and men can develop BC, and the incidence of this disease is much higher in women. It has been estimated that out of every 150 cases, only one BC case will occur in a male (Hill, Khamis, & Tyczynski, 2005). Moreover, age is also an important factor as the risk for developing the disease increases with age. Generally, the risk of developing BC increases with age. It is rare for BC to occur before the age of 25 without predisposing genetic factors. The incidence then increases gradually from ages 30 to 49, and continues to rise after age 50, while the incidence of BC in women ages 70 years and over is significantly lower. More than 75% of BC are diagnosed in women aged 50 years and above (P. S. Carroll, Utshudiema, & Rodrigues, 2017; van den Broek et al., 2015). In the west, approximately 5% of women diagnosed with BC are aged 40 and under. However, in Eastern countries, including GCC, a significant proportion of BC cases tend to have 25-40 years of age, and present to the clinic with BC advanced stages (M Al-Moundhri et al., 2004; Brenner et al., 2016). Other risk factors include ethnicity, alcohol consumption, low physical activity, obesity and exposure to sex hormones, both endogenous and exogenous (Oldenburg, Meijers-Heijboer, Cornelisse, & Devilee, 2007). However, the major risk factor, besides age, is history of the disease in the family (Hálfdánarson; Liaw et al., 2019; Oldenburg et al., 2007). The risk increases, although to a lesser extent, if BC is diagnosed in a second-degree relative or any relative at all. BC risk is age-specific, and the risk is higher in women under 50 years of age, who have a relative with early-onset of BC (Pharoah, Day, Duffy, Easton, & Ponder,

1997). Moreover, family history of ovarian cancer increases the risk of BC given that both cancers are part of Hereditary Breast and Ovarian cancers (HBOC) syndrome caused by defects in *BRCA1* and *BRCA2* (Lynch et al., 2009). More interestingly, exposure to radiation from the recent wars in the Arab world might also explain the increase in the incidence of BC in the GCC countries in particular (Fathi, Matti, Al-Salih, & Godbold, 2013).

2.2.1 Genetic Factors

Cancer development can be attributed to genes being altered. The two types of genes related to cancer genetics are oncogenes and tumor suppressor genes. The first evidence that showed that genetic alteration could result in cancer, was obtained in the study of Burkitt's lymphoma, where it was shown that c-*Myc* proto-oncogene mutations can initiate cancer development (Conacci-Sorrell, McFerrin, & Eisenman, 2014)**.**

2.2.1.1 Tumor Suppressor Genes

They are considered as genes that encode proteins that prevent cells from becoming cancerous. It is believed that, in some cases, they can act by negatively regulating cell proliferation (Lai, Visser-Grieve, & Yang, 2012). For tumor cells to thrive, TSGs need to be inactivated, which can occur via loss of function mutations, loss of heterozygosity, gene inactivation by epigenetic mechanisms (i.e histone modifications and DNA methylation), somatic mutations (spontaneous tumors), inherited syndrome mutations, and acquisition of the overall ability to lead malignant cells to overgrow and escape apoptotic control (Fouad

& Aanei, 2017; Hanahan & Weinberg, 2011). In BC, *BRCA1* and *BRCA2* are the two most commonly classic TSGs. These proteins take part in two fundamental cellular processes, transcriptional regulation and DNA damage repair (Scully & Livingston, 2000).

2.2.1.2 Oncogenes

Proto-oncogenes are genes that have essential roles in normal tissues. As a result of gain of function or inappropriate increase of their activity due to (mutations, gene duplication, or altered DNA transcription), these genes have the potential to become oncogenes that can contribute to cancer (Bishop, 1991). Oncogenes have the potential to transform normal cells into tumor cells by inducing a high proliferative status or by evading programmed cell death (Labi & Erlacher, 2015). In BC, studies have implicated alteration in a defined group of oncogenes, such as *ErbB2, PI3KCA, c-MYC, RAS and CCND1* (encodes cyclin D1) (Botezatu et al., 2016). Moreover, amplification of the *ERBB2/HER2* oncogene in BC is a well-known biological marker with therapeutic value. Amplification of this gene is seen in approximately 20-30% of BCs, and is associated with aggressive BC disease (Uscanga-Perales, Santuario-Facio, & Ortiz-López, 2016).

Altogether, a mutation in TSGs along with the activation of oncogenes can promote the insurgence and progression of cancer, by promoting cell proliferation, cell cycle progression and evasion from apoptosis (J. Zhang, Chen, & Lu, 2010). The hallmarks of cancer have recently been revisited to include genomic instability, re-programming of energy metabolism, tumor-induced inflammation, and escape from immune destruction.

Interactions with the tumor stroma also contribute to the acquirement of hallmark traits.

2.3 Breast cancer progression

Metastasis is the major cause of cancer-related mortality, and understanding its underlying mechanisms might ultimately lead to the establishment of novel anti-metastatic therapies. In the initial stages of tumorigenesis, some luminal cells acquire the ability to avoid anoikis (apoptosis induced by lack of contact with ECM) and become able to sustain a proliferative cell cycle phase (Guadamillas, Cerezo, & del Pozo, 2011). These transformed cells result from activation of oncogenic stimuli and/or loss of tumour-suppressing regulators (Shortt & Johnstone, 2012). The rapid proliferation of the cells results in the lumen of ducts or alveoli to be filled with transformed cells, which initially remain encapsulated by the surrounding myoepithelial cells and the basement membrane. This pre-malignant condition is called carcinoma *in situ* (Pandey, Saidou, & Watabe, 2010). Once transformed cells manage to force themselves through myoepithelial cells and the basement membrane, escaping into the surrounding matrix, the disease has become an invasive carcinoma with poor prognosis (Pandey et al., 2010).

BC tumor cells disseminating from the primary site enter the circulation and travel with the blood (hematopoietic route) or lymphatic system to distant organs typically brain, lungs, liver and bone, where they form secondary tumors, known as metastases (Leong & Tseng, 2014). Throughout the process of tumor progression, an epithelial-mesenchymal transition (EMT) process takes place to initiate the process of motility (Kalluri & Weinberg, 2010). A combination of intrinsic programs in tumor cells themselves and the

involvement of the microenvironment "both in the primary tumor and the metastatic tissue" are essential for metastatic success. First, a cancerous cell must breakdown the extracellular matrix and break contacts with adjacent cells to migrate from the original tumor through blood or lymphatic vessel wall. During this process, the epithelial features of the cells are replaced by mesenchymal properties, characterized by loss of polarity and cell adhesion as well as increased motility (Kalluri & Weinberg, 2010). The cancerous cell then circulates through the bloodstream, adheres to the vessel wall at a distant location, and migrates through the blood vessel again. Ultimately, the metastatic cell establishes a new site for growth, forming a secondary tumor. Tumor metastases are found in the lymph nodes first (near the primary tumor), and only later at other distant locations. Metastatic tumors often prove difficult to treat because they may continually metastasize to multiple locations (Fidler, 2003; Mlecnik et al., 2016). Metastasis is not a random process, although cancer cells might be widely dispersed, the formation of metastases like the formation of primary tumors, require the hallmarks of cancer but also additional changes, including adaptation to foreign microenvironments and activation of protein degradation (Mlecnik et al., 2016). New metastatic mechanisms are continuously discovered, as recent studies have identified common markers on circulating tumor cells, which may allow for their characterization and provide further insights into the mechanisms of tumor metastasis.

2.4 Prognosis of breast cancer

Rather than being a disease of a single specific origin, BC is a collection of tumors of different nature with varying prognosis, hence not all BC tumors can be considered similar

(Colombo, Milanezi, Weigelt, & Reis-Filho, 2011). BC is pathologically complicated, and the majority of these tumors are classified as *in situ* non-invasive (absence of invasion to surrounding tissues), or invasive (infiltration into surrounding tissues) (Lakhani, 2012; Stephens et al., 2009) **(**Figure 2.3).

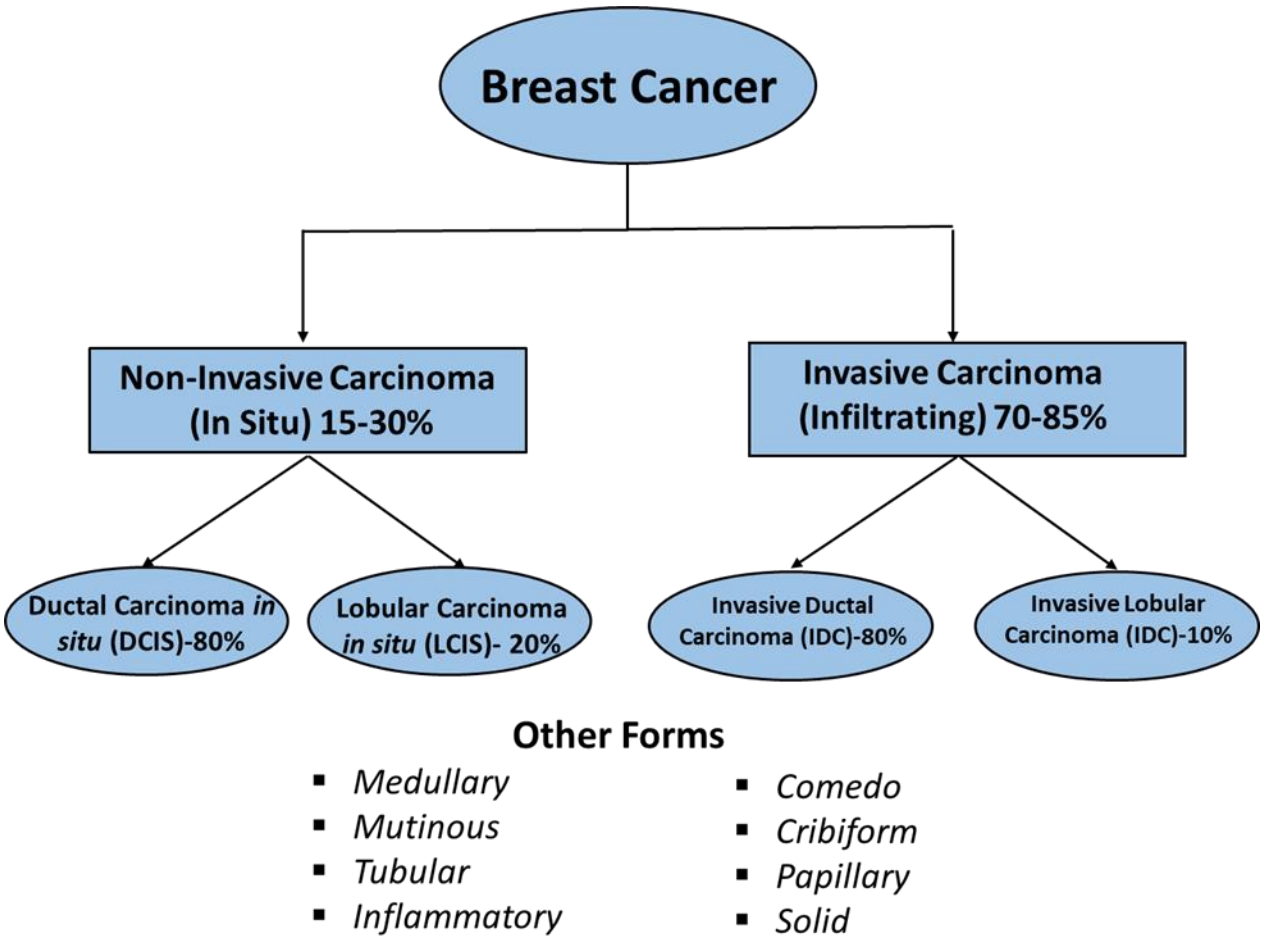

Figure 2.3: Histological types of breast cancer (Adapted from (Lakhani, 2012))

2.4.1 Morphological classification

The classification of breast tumors can be based on a number of factors such as the tumor origin within the organ, i.e. whether it is lobular or ductal, the size of the tumor, and the histological grade (Colombo et al., 2011). In the clinic, breast tumors are classified based on their stage, grade, and receptor status. The grade designates how abnormal the cells look like histologically, where the score can range from 1 (low grade, well-differentiated) up to 3 (high grade, poorly differentiated). As measured by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH), the stages of tumor can be classified as (Stage 0); non-malignant, (Stages I-III); malignant, or (Stage IV); metastatic (Edge & Compton, 2010).

2.4.2 Immunohistochemical molecular classification

Classically, breast tumors are classified based on the expression profiles of certain of their hormonal receptors including, estrogen receptor (ER), progesterone receptor (PR), as well human epidermal growth factor receptor 2 (HER2) (Colombo et al., 2011). These IHC profiles are now being used as biomarkers for disease prognosis and treatment options (Jones, 2008). The patterns of gene expression in these breast tumors have also been used for another type of classification that has emerged during the last decades (Sørlie et al., 2001; Sørlie et al., 2003; Sotiriou & Pusztai, 2009). This classification divides tumors into six distinct molecular BC subtypes; the luminal A, luminal B, HER2, basal-like, normal-like, and claudin-low subtypes. Each subtype displays a different expression pattern of a

given group of genes (Tang & Tse, 2016); These different subtypes are shown in (Table 2.1)

Table 2.1: Classification of breast carcinoma subtypes (Network, 2012; Santos et al., 2015; Sørlie et al., 2001)

Subtype	Grade	Receptors	Prognosis
Normal breast-like	Similar to luminal A, Resemblance to normal tissue	PR+ and/or ER+, HER2-	Good Prognosis
Basal-like/ Triple-negative	Grade 1 Histologically	PR - , ER -, HER2 - or low expression	Worst Prognosis
Luminal A	Grow slowly, Low-grade, Grade 1 Histologically	PR+/- and/or ER+, HER2-	Best Prognosis
Luminal B	Grow slightly faster than luminal A, Grade 3 Histologically	PR+/- and/or ER+, HER2 + or -	prognosis is slightly worse than Laminal A
HER2-enriched	Grow faster, Grade 3 Histologically	PR - and/or ER - , HER2 +	Poor Prognosis
Claudin-low	Low expression of claudin genes contributed in cell-cell adhesion and tight junctions such as CLDN, CDH1	ER/PR-, HER2-	Poor Prognosis

There is an overlap, although incomplete, between tumors that are classified on the basis of gene expression profiles of different receptors (Colombo et al., 2011). For example, the HER2 subtype contains tumors that overexpress HER2 gene, while the luminal A tumors are ER and PR positive, while the luminal B tumors generally express the ER and sometimes they are also PR and HER2 positive, although this varies. The basal BC subtype contains tumors that are characterized by the expression of basal cytokeratins and are also known as triple-negative BCs (TNBCs); they are ER, PR and HER2 negative (Couch et al., 2016; Hu et al., 2006; Sotiriou & Pusztai, 2009). These carcinomas are frequently associated with *BRCA1* mutations, are typically aggressive and of high grade with poor prognosis (Pazaiti & Fentiman, 2011). Classification of breast tumors plays a pivotal role with regards to the treatment options and the overall survival of patients diagnosed with the disease. Generally, the best prognosis is for patients with tumors that belong to luminal A subtype while the worst prognosis is for patients diagnosed with triple-negative tumors. TNBCs have a lower recurrence and overall survival, regardless of the stage of disease at the time of diagnosis, and are more difficult to treat, as no specific treatment is available. The presence of hormone receptors in breast tumors is a strong predictor of response to endocrine agents such as tamoxifen, a competitive inhibitor of estradiol (an ER antagonist), but ER^+ patients continue to relapse due to an intrinsic or acquired resistance (Eiermann & Group, 2001). HER2-positive breast tumors respond well to treatment with HER2-targeting drugs such as trastuzumab (Herceptin) (Jones, 2008). The molecular pathology classification system of BC tumors is, however, a dynamic field that is constantly evolving. This is evidenced by a further classification and characterization, using microarray gene

expression profiling, that led to identification of another subgroup of breast tumors; the claudin-low subtype of triple-negative tumors that may have further therapeutic and prognostic implications (Colombo et al., 2011; Prat et al., 2010; Prat & Perou, 2011; Sabatier et al., 2014). Interestingly, existence of normal-like tumor subtype is today being questioned by some researchers who claim that it is more likely to be an artefact than a real breast tumor subtype (Prat & Perou, 2011; Sørlie et al., 2001). Further refining and classifications of these distinct subgroups are likely to be revised in future studies.

2.5 Heterogeneity of breast cancer

Although the majority of BC cases are sporadic, about 10% may harbor predisposing germline mutations. These mutations differ in their penetrance and associated BC risk (Network, 2012).

2.5.1 Familial breast cancer

Familial breast cancer (FBC) is generally an early onset and aggressive disease at age < 40 years (van den Broek et al., 2015). However, most BC cases have unknown etiologies or known as non-hereditary or sporadic BCs, and are estimated to account for approximately 75-80% of all cases, with the remaining attributed to inherited genetic anomalies, categorized as FBC (Hálfdánarson; Mitrunen & Hirvonen, 2003; Rich, Woodson, Litton, & Arun, 2015). A proportion of 20-25% of FBC cases has a disease family history, with one or more family members affected and attributed to inherited genetic aberrations (Ford,

Easton, Bishop, Narod, & Goldgar, 1994). A number of FBC cases cluster together, and the disease has a dominant appearance, mainly caused by germline mutations (Oldenburg et al., 2007). Around 25-40% of these FBC families have germline mutations in known highly penetrant cancer genes, about 25% of hereditary BC are associated with mutations in *BRCA1*, *BRCA2*, *STK11*, *PTEN*, *TP53*, and *CDK1* (Shiovitz & Korde, 2015), with *BRCA1* and *BRCA2* being the most common (Fanale et al., 2012; Lux, Fasching, & Beckmann, 2006). However, around 80% of inherited BC cases do not result from *BRCA1/BRCA2* mutations, suggesting that other unidentified genetic aberrations are present in these patients. Identification of these genes will ultimately lead to accurate diagnosis and improved or personalized treatments (MS Al-Moundhri et al., 2013; Skol, Sasaki, & Onel, 2016).

2.6 Breast cancer penetrance genes

Three distinct classes of BC susceptibility alleles have been associated with increased risk of FBC. The first class includes high penetrance genes, with rare variants that confer high BC risk. The second class harbors moderate penetrance genes, also with rare variants that confer a moderate BC risk. The third allele class is that of common but low penetrance variants conferring a low BC risk (Larsen, Thomassen, Gerdes, & Kruse, 2014; Stratton & Rahman, 2008).

2.6.1 High penetrance genes

In fact, a number of chromosomal regions within the human genome might harbor possible BC susceptibility alleles that spread across all classes of penetrance type. In 1994, BC susceptibility gene 1 (*BRCA1*) was discovered as the first BC susceptibility gene (Gene, 1994; J. Li et al., 2015). A year later, BC susceptibility gene 2 (*BRCA2*) was identified (Wooster et al., 1995). Together, these two genes are responsible for 40 % of hereditary/familial breast and ovarian cancer cases and are identified as high penetrance genes (S. B. Cantor & Guillemette, 2011; Olopade, Grushko, Nanda, & Huo, 2008). Since then, many other pathogenic mutations were identified in *BRCA* genes. Both genes frequently display a loss of heterozygosity (LOH), characterized by the loss of the wild-type allele in the tumors. Moreover, *PTEN*, *CDH1*, *STK11*, and *TP53* genes have been identified as high-risk BC susceptibility genes (Economopoulou, Dimitriadis, & Psyrri, 2015; Stratton & Rahman, 2008). The relative risk for carriers of pathogenic mutations within any of these genes, compared to non-carriers, ranges from 5 to over 20 %. Although these mutations confer high risk, they are quite rare in the general population and therefore each mutation explains only a small fraction of the increased BC risk (Easton et al., 2015). Notably, some of these genes are involved in cellular pathways that control cell growth and signalling, which differ from the pathways previously reported as high BC risk. Primarily, these genes are involved in the repair process of damaged DNA (Easton et al., 2007), suggesting a different mechanism of action of the low-risk variants, mediated through activation of oncogenes, including those that promote cell growth (Stratton & Rahman, 2008).

2.6.2 Moderate penetrance genes

A set of genes found to confer a moderate increase in BC risk, identified through mutation screening analyses, are *ATM* (Renwick et al., 2006; Seal et al., 2006), *BRIP1* (Economopoulou et al., 2015; Seal et al., 2006), *PALB2* (Rahman et al., 2007) and *CHEK2* (Meijers-Heijboer et al., 2002). The pathogenic mutations found within these moderate penetrance genes share certain characteristics with the pathogenic variants within high penetrance genes (Stratton & Rahman, 2008). In that, they have turned out to be quite rare and uncommon in the general population, and most of them are loss of function variants, i.e. they result in premature protein truncation. *ATM*, *BRIP1*, *PALB2*, and *CHEK2* proteins are all associated with DNA repair pathways (Economopoulou et al., 2015; Mavaddat, Antoniou, Easton, & Garcia-Closas, 2010). Variants within these moderate penetrance genes count for approximately 5% of the hereditary BC risk (see Figure 2.4) (Mavaddat et al., 2010; Stratton & Rahman, 2008).

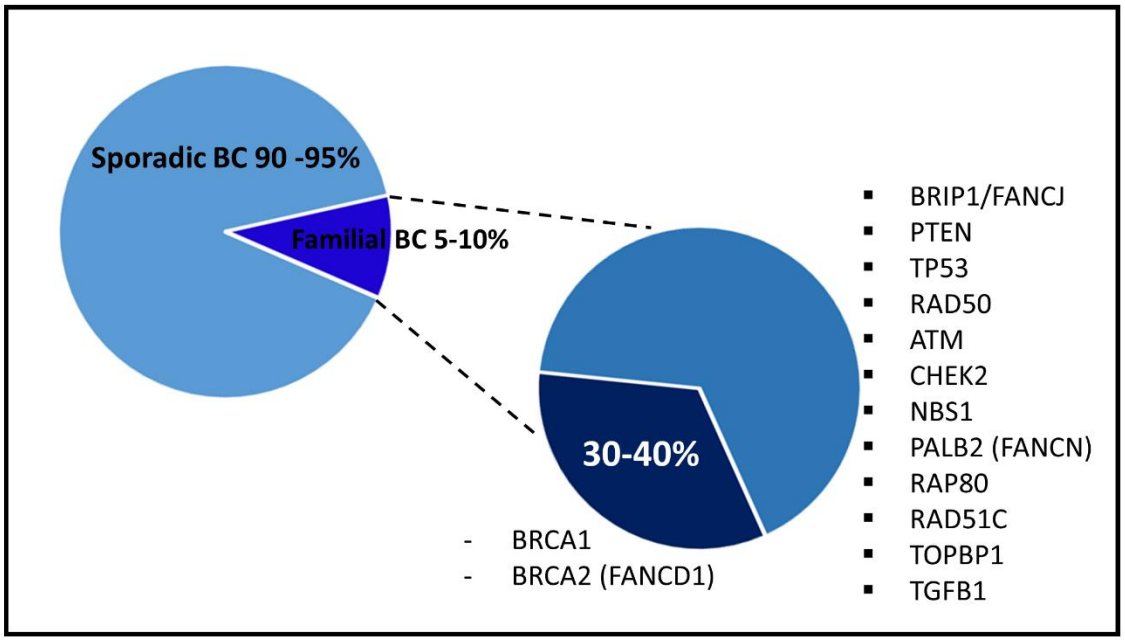

Figure 2.4: The most common DNA repair genes associated with Familial and/or sporadic BC and linked to Fanconi anemia. Germline mutations in either *BRCA1* or *BRCA2*, as well as other genes, increase the genetic susceptibility to BC, especially those genes expressing interacting proteins (BRIP1 and PALB2) that interact with *BRCA1* and *BRCA2*, respectively (S. B. Cantor & Guillemette, 2011).

2.6.3 Low penetrance genes

High and moderate genes explain less than half of BC cases; thus, the polygenic model might explain the majority of the remaining BC cases (Fanale et al., 2012). In recent years, several genomic regions that harbor another group of risk variants have been identified by genome-wide association studies. The variants of this class confer only a small BC risk, defined by an estimated risk ratio below 2, and they are predominantly common SNPs that are carried by a high proportion of the general population (Chung, Magalhaes, Gonzalez-

Bosquet, & Chanock, 2009; Skol et al., 2016). Some of these common low-risk SNPs were located in regions, either within or in close proximity to known genes such as *FGFR2*, *TOX3/TNRC9, MAP3K1, LSP51,* and *RAD51L1* (Fanale et al., 2012).

2.7 *BRCA1* domains and interacting proteins

Identification and characterization of BRCA1-associated proteins have revealed that BRCA1 binds and recruits DNA damage repair DDR proteins in response to DNA damage. Most likely, these proteins are bridged together by BRCA1 to facilitate DNA damage-induced cell cycle checkpoint activation and repair (Roy, Chun, & Powell, 2012). In fact, BRCA1 can interact directly or indirectly with nearly one hundred proteins. Many of BRCA1-interacting proteins, including BRCA1 associated RING domain 1 (BARD1) and BRCA1 associated protein 1 (BAP1), can bind to BRCA1 RING domains (Brzovic et al., 2003); These proteins function to facilitate BRCA1 E3 ubiquitin ligase activity. On the other hand, CtBP-interacting protein (CtIP), BRIP1, and Abraxas interact with BRCA1 through BRCT domains and facilitate DNA damage response and cell cycle (Figure 2.5) (S. B. Cantor & Guillemette, 2011; B. Wang et al., 2007).

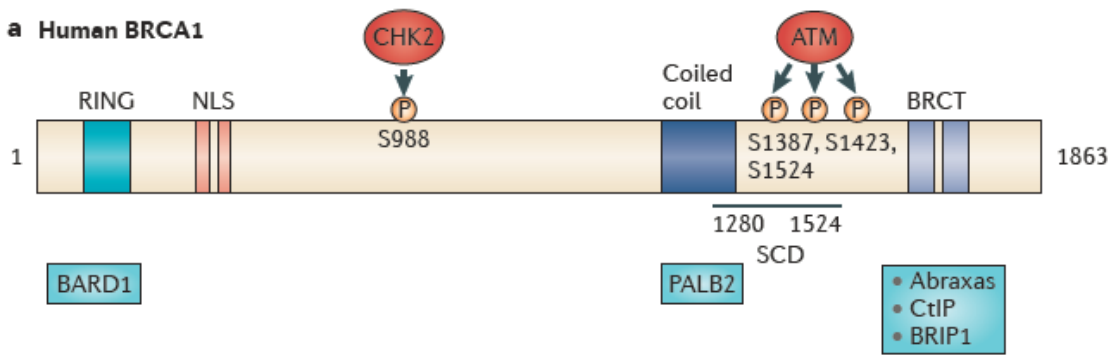

Figure 2.5: Schematic representation of *BRCA1* domains and interacting proteins. Functional domains of BRCA1 proteins. (a) The N-terminus of BRCA1 associates with (BARD1) by the RING domain as well as a nuclear localization sequence (NLS). CHK2 phosphorylates a serine residue in position 988 of the protein. C-terminus of BRCA1 associates with PALB2 protein by a coiled-coil domain, a SQ/TQ cluster domain (SCD) that harbors' a BRCT domain associated with three proteins, Abraxas, CtIP and BRIP1 and the ATM phosphorylation sites (Roy et al., 2012).

The closest protein that binds to BRCA1 is BRIP1, which is of the DNA damage complex.

The following remaining chapters will focus on BRIP1 structure and function.

2.8 *BRIP1* Gene

2.8.1 Identification of *BRIP1* gene

In an effort to understand the contribution of BRCT sequences to *BRCA1* function, Glutathione S-transferase-BRCT motifs (GST-BRCT) fusion was generated (S. B. Cantor & Guillemette, 2011). In these experiments, a protein of ~130 kDa was identified with the GST-BRCT fusion protein that was labelled with protein kinase A by *in vitro*

phosphorylation. Furthermore, GST-BRCT fusion protein containing clinically relevant point mutants, P1749R and M1775R, reduced or failed to bind to the 130 kDa protein, respectively. Subsequently, the 130 kDa protein was characterized with 1249 residues that contain seven helicase motifs that are characteristic of the DEAH helicase family. Its N-terminal 888 residues revealed strong homology to the DEAH helicase family (Schmekel et al., 1996). Unlike DEAH helicases, the -terminal region of the 130 kDa protein shares 39% homology with the synaptonemal complex protein 1 (SCP1) (Schmekel et al., 1996). Given the interacting domain with BRCA1 and its helicase domains, this 130 kDa protein was named BRCA1 interacting protein c-terminal helicase 1 or BACH1 for BRCA1 associated C-terminal helicase 1 (S. B. Cantor et al., 2001). In addition to the interacting domain of BRIP1 with BRCA1, the phosphorylation of Serine 990 (S990) of BRIP1 is regulated by cyclin-dependent kinases, and appears critical for its interaction with BRCA1 (Yu, Chini, He, Mer, & Chen, 2003). Although *BRIP1* expression is stable throughout the cell cycle, S990 is phosphorylated only from S to G2/M phase (Yu et al., 2003). Thus, the interaction of BRIP1 and BRCA1 is cell cycle regulated.

2.8.2 Structure of *BRIP1*

BRIP1, located on the long arm of chromosome (17 q22) distal from *BRCA1* locus (17 q21) in a region showing loss of heterozygosity in BC (Callahan, 1997; Phelan et al., 1998), encompasses 20 exons and 19 introns encoding a protein of 1249 amino acids (Figure 2.6) (Levitus et al., 2005).

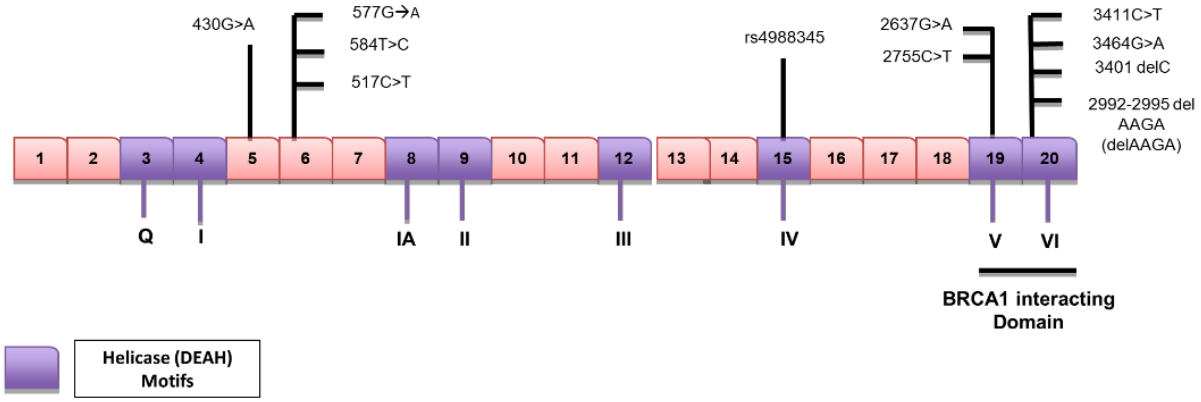

Figure 2.6: Structure of *BRIP1* gene and its common mutations. BRIP1 consists of 20 exons and seven conserved motifs involved in helicase ATP-core binding domain (I, IA, II, III, IV, V, VI). Exons 19 and 20 contain BRCA1 interacting domain (A. Ouhtit, Gupta, & Shaikh, 2015). It is normally restricted within the nuclear envelope and the cytoplasm, but BRIP1/RPA1 complex translocate into the nucleus after DNA damage (S. B. Cantor et al., 2001; L.P.Ren, 2013). BRIP1 belongs to the Rec Q Helicase comprised of seven conserved DEAH helicase motifs (mentioned above). These domains are involved in the helicase ATP-core binding domain responsible for DNA strand separation (Tanner, Cordin, Banroques, Doère, & Linder, 2003; Y. Wu et al., 2010). The primary iron-sulfur cluster motif (Fe-S), encompasses four conserved cysteine residues, which are fundamentally required for ATP helicase activity (S. Cantor et al., 2004; Rudolf, Makrantoni, Ingledew, Stark, & White, 2006). Furthermore, BRIP1 C-terminal interacts with BRCA1 through BRCT repeats, thereby contributing to the onset of FA and BC (Moldovan & D'Andrea, 2009; Shiozaki, Gu, Yan, & Shi, 2004).

2.8.3 Function of *BRIP1*

2.8.3.1 Function of BRIP1 in normal cells

BRIP1 is physiologically expressed in both normal and malignant cells. DNA damage response in normal cells triggers BRIP1 acetylation at lysine 1249, which subsequently

enhances checkpoint signalling and the repair of DNA damage (Jenny Xie et al., 2012). Regulation of DNA repair is mediated by BRIP1 phosphorylation at S990, that promotes BRIP1-BRCA1 interaction through BRCT, a phosphorylation-protein binding domain important in HR process (J Xie et al., 2010; Yu et al., 2003). Interestingly, the same site also influences the catalytic activity of BRIP1 by binding to BLM protein associated with Bloom syndrome, an autosomal recessive disease with a predisposition to genomic instability and cancer development (Suhasini et al., 2011). In addition, BRIP1 impedes RAD51 protein single-strand exchange by HR process in a process regulating DNA repair, through interaction with Topoisomerase-II Binding Protein 1 (TOPBP1) at site Thr133, as a consequence of stalled replication fork associated with ATP-dependent phosphorylation process (Gong, Kim, Leung, Glover, & Chen, 2010). The mechanistic function of BRIP1 in DSB repair remains elusive, but due to its ability to unwind Rad51 nucleoprotein filaments, BRIP1 may either function to exit the repair process or monitor homologous strand exchange.

The fundamental function of BRIP1 is to establish genome integrity by controlling replication stress response, DSB repair by HRs, cross-link repair, and Inter-strand Cross-links (ICLs) repair **(**Figure 2.7) (London et al., 2008). Prior to BRIP1 binding to replication protein A (RPA), it removes other DNA binding proteins in order to stall the replication fork and unwind damaged DNA that hampers genomic integrity (R. Gupta et al., 2007; Sommers et al., 2009).

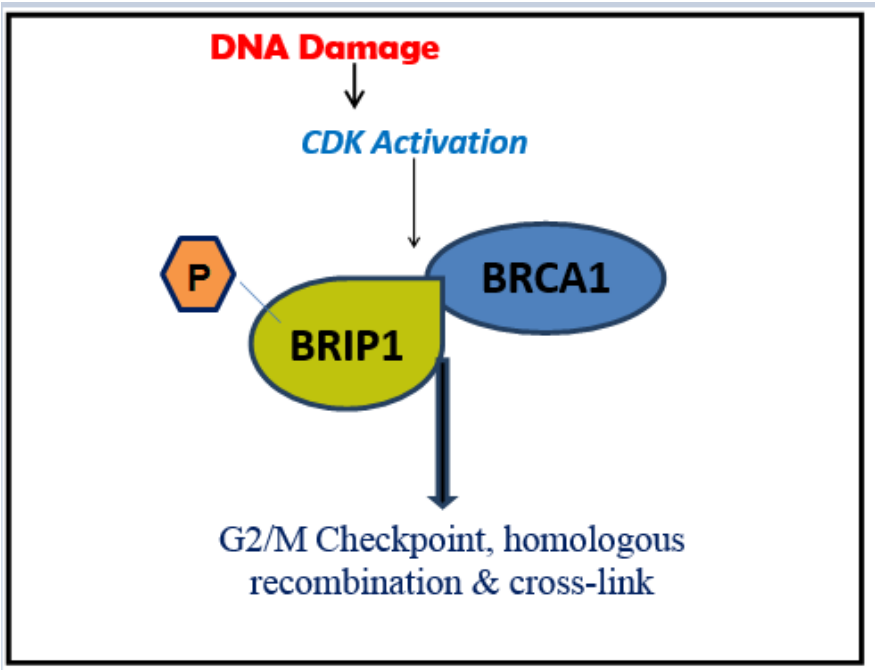

Figure 2.7: Schematic representation of BRIP1 function. Cyclin dependent kinases are activated upon DNA damage in G2 phase of the cell cycle; this phosphorylates BRIP1 protein and promotes its interaction with BRCA1 to repair the damage.

Interestingly, BRIP1 function in DSB repair is potentially not conserved between species. In mammalian cells, BRIP1 is critical for DSB repair, while in chicken DT40 cells, BRIP1 functions independently of BRCA1 and HR (Bridge, Vandenberg, Franklin, & Hiom, 2005). BRIP1 in chicken DT40 cells lacks the binding domain required for BRCA1 interaction; thus BRIP1 may still function in mammalian HR, but requires the interaction of BRCA1. Whether BRIP1 functions independent of BRCA1 remains to be determined in mammalian cells.

When DNA damage accumulates within a cell, it causes uncontrolled cell division, BRIP1 and BRCA1 proteins are then recruited to repair DSB, by HR (Gong et al., 2010). In the nucleus, BRIP1 protein acts as a helicase and unwinds the two strands of DNA double helix. This permits BRIP1 protein and other proteins like BRCA1 to repair DNA damages during G2-M phase and promotes chromosome stability, thus both *BRCA1* and *BRIP1* act as TSGs (S. B. Cantor & Guillemette, 2011; Jiang & Greenberg, 2015).

In addition to DSB repair, BRIP1 also functions in ICLs repair. ICLs is introduced into DNA either endogenously during cellular metabolism, through the acidification of nitrites, or exogenously by agents such as melphalan, cisplatin, and mitomycin C. ICLs are extremely toxic due to their ability to inhibit DNA replication, transcription, and segregation resulting from the impediment of DNA strand separation. The first indication that BRIP1 functions in ICL repair originated from the finding that BRIP1 deficient cells were extremely sensitive to these agents (Litman et al., 2005). The function of BRIP1 in ICL repair required its helicase and a mismatch repair protein (MLH1) binding activities, but not BRCA1 binding (Peng et al., 2007). Thus, the contribution of BRCA1 binding to the function of BRIP1 in ICL repair remains elusive. These data put together implicate BRIP1 function in DDR. However, the mechanism affecting DDR following BRIP1 binding to BRCA1 or MLH1 remains to be elucidated.

2.8.3.2 BRIP1 in Fanconi Anemia (FA) pathway

In addition to its interaction with individual DDR proteins, BRCA1 is involved in the FA pathway. The FA pathway encompasses 16 complementation groups, the upstream proteins that include FA- (A, B, C, E, F, G, L, and M). In response to DNA damage, these eight core complex proteins become activated through phosphorylation by ATR. Once activated, this complex translocates to the chromatin and is recruited to sites of DNA breaks. The E3 ligase (FANCL) of the core complex works in concert with the E2 subunit UBE2T and facilitates ubiquitination of FANCD2 (Longerich, San Filippo, Liu, & Sung, 2009; Machida et al., 2006; Smogorzewska et al., 2007). Following their mono-ubiquitination, FANCD2 and FANCI form a heterodimer and translocate to chromatin, where they are recruited to nuclear foci containing BRCA1 and downstream FA proteins (Taniguchi & D'Andrea, 2006). Since BRCA1 mutations have not been linked to FA, as it is not an official FA protein, however, BRCA1 is indirectly linked to the FA pathway on multiple levels. First, BRCA1 not only co-localizes with FANCD2 and FANCI in nuclear foci, but also regulates their ability to form damage-induced foci (Garcia-Higuera et al., 2001). Second, BRCA1 facilitates the re-localization of FANCD2 to sites of stalled replication forks (Vandenberg et al., 2003). Third, BRCA1 interacting partners were identified as downstream FA proteins (Litman et al., 2005). Downstream FA proteins include FANCD1, FANCN, and FANCJ, and all the three groups were later identified as BRCA2, PALB2, and BRIP1, respectively (Figure 2.8). The intimate connection between FA and BRCA pathways demonstrated the overlapping nature and complexity of the DDR network.

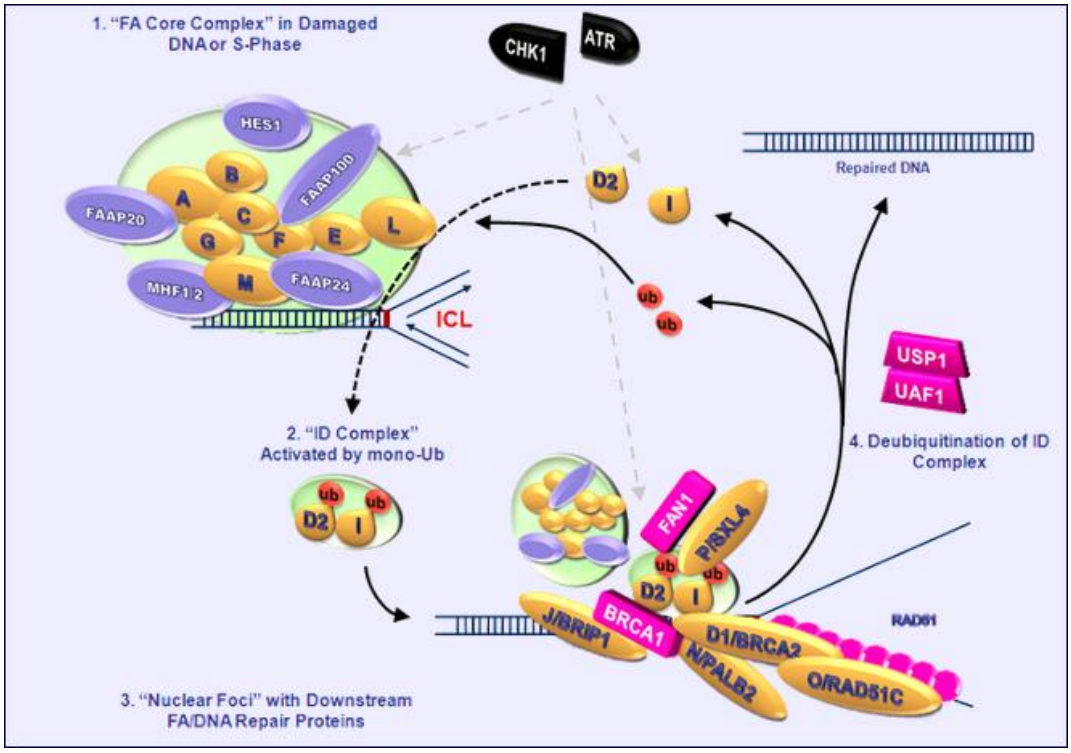

Figure 2.8: Overview of the Fanconi Anemia and BRCA DNA repair pathways: FANCI and FANCD2 are mono-ubiquitinated in response to DNA damage by FA core complex proteins, and form a heterodimer. This heterodimer translocates to DNA repair foci where it colocalizes with BRCA1 and the downstream proteins FANCD1/BRCA2, PALB2, and BRIP1. This complex recruits RAD51 and RAD51C along with other proteins. The FA pathway is critical for ICLs (Particles, 2013) and maintaining genomic stability (Valeri, Martinez, Casado, & Bueren, 2011).

2.8.3.3 Physiological function of BRIP1 in cancer

As mentioned earlier, BRIP1 directly interacts with BRCA1 through its BRCT domain and both function as tumor suppressors. *BRIP1* plays a significant role in DNA repair, and mutations in *BRIP1* gene mediate the development of breast and ovarian cancers as well as

FA (Kobayashi, Ohno, Sasaki, & Matsuura, 2013; Levitus et al., 2005). The upcoming section will highlight various mutations linking *BRIP1* to BC development.

2.7.3.1 Association of BRIP1 with breast cancer

The relevance of *BRIP1* in BC was clinically evident upon the identification of *BRIP1* germ-line mutations in early BC patients displaying wild type *BRCA1* and *BRCA2*; thus highlighting the role of *BRIP1* as low/moderate predisposing penetrance gene for BC (De Nicolo et al., 2008; Haley, 2016; L.P.Ren, 2013; Rafnar et al., 2011; Seal et al., 2006). Rahman and colleagues were the first to link *BRIP1* gene to the increased risk of BC in a case-control study of British FBC case (Rahman et al., 2007; Seal et al., 2006).

The interaction of BRIP1 and BRCA1 suggests that *BRIP1* could be linked to FBC (Guénard et al., 2008). Interestingly, the disease-associated mutations in *BRIP1* altered its helicase activity *in vitro*, providing a direct link between the helicase activity of *BRIP1* and disease development (S. Cantor et al., 2004). Moreover, loss of heterozygosity produced a short deficient BRIP1 protein that failed to bind BRCA1, thus resulting in abnormal cell growth as well as mis-regulation of DNA damage repair mechanisms leading to increased risk of BC in non-*BRCA1/2* BC patients (Moran et al., 2016; Scalia-Wilbur, Colins, Penson, & Dizon, 2016; Seal et al., 2006).

In fact, *BRIP1* mutations that affected domain activity or mRNA expression were observed during the early onset of BC, thus indicating a role of *BRIP1* in BC susceptibility (S. B. Cantor & Guillemette, 2011). Recently, whole exome sequencing identified rs552752779

BRIP1 mutation in TNBC cases confirming the association of BRCA1 interacting mechanisms towards TNBC (Buys et al., 2017; Kumar et al., 2018). Also, a rare missense mutation in *BRIP1* was detected with low frequency in BC family (rs201047375; c.550G>T) in Spain. This mutation disrupted the Splicing Enhancer Site (SES) in exon 6, causing exon 5 skipping. This resulted in an impairment of BRIP1 helicase function that affected DNA repair efficiency and as a result modulating BC risk (Velázquez et al., 2019). Moreover, the mutation c.2992–2995 del AAGA, is a germline mutation that led to the loss of wild-type allele, thereby impairing the binding of BRIP1 to BRCA1 (A. Ouhtit et al., 2015). Other germline mutations, two independent missense M299I and P47A, were identified in other cases of early-onset FBC targeting the helicase domain of *BRIP1* (Antoinette Hollestelle, 2010). Surprisingly, the heterozygous phenotype of these two mutations did not exhibit any loss of the wild-type function as the classical TSG paradigm (S. B. Cantor & Guillemette, 2011). Thus, these patients showed tumors due to the negative dominant like effect (S. B. Cantor & Xie, 2010; Y. Wu et al., 2010). However, the familial significance of these mutations could not be established due to the absence of co-segregation analysis (Fostira, 2013). In fact, P47A was found also in controls and was unlikely to be associated with high risk of BC compared to *BRIP1* truncating mutations (Seal et al., 2006).

BRIP1 mutation spectrum of FBC Japanese patients with no *BRCA1/2* mutations detected a splice donor site variant c.918+2T>C, and three novel missense mutations, c.2131A>G, c.736A>G, and c.89A>C (Sato et al., 2017). In addition, in a study of high-risk Jewish

patients with no *BRCA1/2* mutations, seven germline *BRIP1* mutations were identified, including three missense (p.Ala745Thr, p.Ser919Pro, and p.Val193Iso), two intronic (c.508-31C>G and c.346+21A>G) mutations, and two silent (Tyr1137Tyr and Glu879Glu) (Catucci, 2012). In another study, 10 amino acid substitution mutations (728A>G, 653G>T, 571G>A, 2971C>G, 2564G>C, 3418C>G, 3736G>A, 3715G>C , 3829G>C, and 3798C>A) were detected with a heterozygous novel missense mutation 2971C>G (exon 19) also linked to BC development. Other variants, including S919P and 4049 C>T mutations were found in both controls and BC patients, most likely not associated with BC (Cao et al., 2009). Mutational analysis of tumors from Korean BC patients reported eight missense mutations in 15 individuals and one novel 1018 C>T truncating mutation. Among these eight missense mutations, five were novel (1442G > A, 1421T > C, 2543G > A, 2854A > G and 787C > T) and three were previously reported (2830C >, 587A > G, and 430G > A) (Haeyoung Kim et al., 2016; H Kim, Cho, Choi, Park, & Huh, 2014). However, large systematic study in European region identified the truncating *BRIP1* variant rs137852986 (p.Arg798Ter); 2392C>T to show no substantial increase in BC (Easton et al., 2016).

Genotyping of *BRIP1* identified two alternative human isoforms of *BRIP1*, with different SNPs; both mediate different molecular functions such as DSB repair, helicase activity, DNA duplex unwinding and DNA damage checkpoints, ATP- dependent DNA binding, and protein binding (Seal et al., 2006). On the other hand, complete analysis of different *BRIP1* polymorphisms by mass array analysis identified different SNPs in Chinese BC

patients, including rs4988344, rs2048718, rs8077088, rs4986764, rs6504074, rs7213430, rs4988345, rs34289250, rs11079454, rs12937080, and rs4986763. The rs7213430 polymorphism was significantly associated with BC (Ren et al., 2013). In addition, the rs4986764 (exon 18) that was previously linked to high BC risk also showed a high correlation to BC although an association between the rs4986764 SNP and BC was not observed (Pabalan, Jarjanazi, & Ozcelik, 2013; Sigurdson et al., 2004). Further investigation is still required because this study did not cover some of the crucial SNPs in order to include a link between these SNPs (rs4986764, rs2048718, rs11079454, rs7213430, and rs4986763) and the onset of BC risk (Pabalan, Jarjanazi et al. 2013).

Several studies proclaimed that *BRIP1* mutations are associated with a higher predisposition to BC as well as other cancers including ovarian (Rafnar et al., 2011; Weber-Lassalle et al., 2018), prostate (Kote-Jarai et al., 2009), cervical cancers (Ma et al., 2013a, 2013b), and recently colorectal cancer (Ali, Delozier, & Chaudhary, 2019; Ren et al., 2013). The relationship between *BRIP1* polymorphisms and Cancer susceptibility were inconsistent in numerous molecular epidemiology studies due to genomics variations in different ethnic groups. To evaluate the relationship between four common SNPs of *BRIP1* (rs2048718, rs4988344, rs4986764, and rs6504074) and common cancer risk, meta-analysis of different 18 studies was performed. They showed that both rs2048718, rs4988344, and rs4986764 SNPs associated with decreased risk of cervical cancer instead of BC (D. Liu et al., 2018; Ren et al., 2013). However, ethnicity subgroup analysis showed a significant association of rs4988344 and cervical cancer among Chinese population (D. Liu et al., 2018).

A Swedish BC patients study, showed three known polymorphisms (2755C-T, 2637G-A and 3411C-T) and one novel 517C-T, which resulted in Arg173Cys substitution, leading to protein localization into the nucleus (I, 2003), hence suggesting its role in BC susceptibility (Luo et al., 2002). A novel heterozygous Pro1034Leu mutation was also identified in normal and BC patients, and rarely related to BC (Pabalan et al., 2013; Sigurdson et al., 2004).

Interestingly, among Italian men patients, *in-silico* mutation analysis of *BRCA1/2*, *PALB2*, *CHEK2*, and *BRIP1* reported a pathogenic mutation R245W in *BRIP1* gene, while haplo-insufficiency analysis showed no correlation between this mutation and BC (Meijers-Heijboer et al., 2002; Silvestri et al., 2011). Furthermore, three previously reported mutations 3'UTR 4049C>T, IVS4-28G>A, and P919S (S. Cantor et al., 2004; Seal et al., 2006) and a synonymous variant E879E were also identified in females. This study clearly highlighted no correlation between theses variants and BC onset in males (Silvestri et al., 2011). Additionally, in an attempt to analyze *BRIP1* mutations associated with male BC, a case of male BC patient with significantly high FBC history and normal BRCA was studied in India. Two variants; 2755C-T (silent mutation) and 2637G-A (missense mutation) variants were identified (Venkateshwari et al., 2017).

Based on the results from these studies, and with the identification of several mutations (truncated, germline and missense), *BRIP1* plays a critical role in the onset of BC (Lindor, Hopper, & Dowty, 2016). Although, the pathogenicity of *BRIP1* mutations has not been

convincingly proven so far, it can possibly help understand better the non-*BRCA1/2* BC cases (S. B. Cantor & Guillemette, 2011; Moran et al., 2016). Several European studies resulted in the identification of few hotspot regions within the *BRIP1* gene related to BC. However, the association of *BRIP1* with BC development has not been studied in the Arab world, where *BRCA* genes are particularly rarely mutated (MS Al-Moundhri et al., 2013). Surprisingly, a recent study was conducted in the Omani population, specifically on BC patients with no *BRCA1/2* mutations, showed that *BRIP1* was overexpressed in BC tissues compared to control/benign breast tissues using IHC analysis. Overexpression of *BRIP1* in the Omani cohort was confirmed to be associated with poor outcome (I. Gupta et al., 2018). Nevertheless, although many studies have implicated *BRIP1* in genetic stability and DNA repair mechanisms associated with the onset of BC, the role of these mutations in BC cell invasion and metastasis remains nascent (A. Ouhtit et al., 2015).

2.8.4 *BRIP1* transduction signalling pathways

The ErbB2/HER2 signalling pathway has been associated with BC poor prognosis (Johnston & Leary, 2006), while the Amphiregulin (AR)/EGF receptor pathway is essential in pubertal ductal epithelial tree outgrowth (Sternlicht et al., 2005). Interestingly, however, loss of BRIP1 protein caused abnormal mammary morphogenesis modification *via* different signalling pathways, including Myc, Wnt, PTEN, PI3K, LPA receptor and DNA damage response (Daino et al., 2013). Other signalling pathways including, integrin, NF-kappa B (NF- κ B), Notch, STAT, and TGF-β are altered in BC (Bon, Folgiero, Di Carlo, Sacchi, & Falcioni, 2007; Stylianou, Clarke, & Brennan, 2006). A recent study showed a

significant role of *BRIP1* in controlling cell proliferation and acinar formation by regulating major cell function such as cell adhesion and DNA repair mechanisms, which are important in mammary gland normal development (S. B. Cantor & Guillemette, 2011).

CHAPTER 3 : MATERIALS AND METHODS

3.1 Ethical Compliance

This work was done under Qatar University/ Institutional Biosafety Committee (IBC) approval QU-IBC-2018/025.

3.2 Cell culture

The human BC cell lines HCC-2218, T47D, BT474, MCF-7, CAMA-1, and the immortalized human breast cell line MCF 10A were obtained from the American Type Culture Collection (ATCC, USA). Immortalized HuMEC cells, MDA-MB-468, and MDA-MB-231 were provided by Dr Ala-Eddin Al Moustafa from the college of Medicine at Qatar University, while HCC-1500 cell line was obtained from Weill Cornell Medical College-Qatar. HCC-2218, T47D, BT474, MCF-7, CAMA-1 cell lines were grown in DMEM medium (Gibco, USA), while MDA-MB-468, MDA-MB-231, and HCC-1500 cells were cultured in RPMI 1640 medium. All culture media were supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Thermo Scientific, USA) and 1% of an antibiotic suspension (Penicillin and streptomycin, Gibco, USA). MCF 10A nonpathogenic breast cell line was cultured in HuMEC Basal serum-free medium (Gibco, 12753018) supplemented with HuMEC Supplement Kit (Gibco, 12755013), while HuMEC cells were cultivated in keratinocyte SFM supplemented with bovine pituitary extract (BPE) (Gibco,

17005042). All cells were maintained in a humidified incubator adjusted to 37°C with 5% CO_2.

3.3 RNA Extraction and Quantification

For all cell cultures, media was discarded, and the cells were washed with 1X DPBS twice prior to RNA purification. Total RNA was extracted using Thermo Scientific GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. For elution, the column was placed in 1.5 ml collection tube and eluted using nuclease-free water. RNA concentration was determined using NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, USA), and the sample was stored at - 80°C for further experiments. Nucleic acids absorb UV light at 260 nm, while proteins absorb at 280 nm. Nucleic acid, including RNA concentration, was determined at OD_{260}. OD_{260}/OD_{280} ratio was used to estimate the purity of RNA samples. Ratios of 2.0 - 2.1 indicated pure RNA samples, while lower ratios indicated protein and/or phenol contamination.

3.4 cDNA Reverse Transcription

Total RNA was reversed transcribed into single-stranded cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK) and ProFlex™ 3x32-well PCR thermocycler (Applied Biosystem, UK). Briefly, 1 µg total RNA was added to the master mix following the manufactured protocol in a final volume of 20 µl per sample. The cDNA was synthesized from RNA using the Reverse Transcriptase (RT) enzyme that extends the RT random primers, bound to RNA templates, with dNTPs, as described in

(Table 3.1). The reactions were incubated according to the kit at 25°C for 10 min, 37°C for 2 h, and at 85°C for 5 min for inactivation. Subsequently, the cDNA samples were collected and diluted 1 in 4 with nuclease-free water and stored at -20°C until use. A mock reverse transcription reaction served as a negative control, without the presence of reverse transcriptase.

Table 3.1: Reagents and volumes used for one cDNA reaction

Reagent	**Volume (µL)**
RT Buffer (10X)	2.0 µl
RT Random Primers (10X)	2.0 µl
MultiScribe™ Reverse Transcriptase	1.0 µl
dNTP Mix (25X)	0.8 µl
RNA Sample	1.0 µg
Nuclease-Free water	Up to 20 µl
Total Final Volume	20 µl

3.5 TaqMan quantitative Real-Time PCR

Real-time quantitative PCR (RT-qPCR) was performed on cDNA samples to measure and compare mRNA expression of target genes. In RT-qPCR, each cDNA sample was

exponentially amplified through a series of PCR cycles, while a fluorescent signal was measured after each cycle and displayed in an amplification plot. The signal was proportional to the amount of target cDNA as well as the target gene mRNA expression in each sample. An endogenous control was used to correct the variations in cDNA initial concentration. In our study, Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as endogenous control because it is unaffected upon up- or down-regulation of *BRIP1* expression.

The RT-qPCR assay was carried out to determine the expression of *BRIP1* using TaqMan® Advanced Master Mix (Applied Biosystems, UK). In Brief, 1 µl of diluted cDNA was added to 7 µl of nuclease-free water, 10 µl TaqMan Master Mix, 1 µl of *BRIP1* Gene Expression TaqMan® Assays FAM-MGB (4351372, Applied Biosystems, UK), and 1 µl of *GAPDH* Gene Expression TaqMan® Assays VIC-MGB (4448489, Applied Biosystems, UK). A negative control containing nuclease-free water instead of cDNA was included. All the reactions were performed in triplicate in a Fast Optical 96-well reaction plate (Applied Biosystems, Inc., USA). The plate was sealed and then centrifuged at 1,600xg for 2 min at 4°C, prior to the incubation of the samples into the QuantStudio™ 6 Flex RT-qPCR System (Applied Biosystems, Inc., USA). The RT-qPCR reaction included the following steps: 10 min at 95°C (stage 1); 20 sec at 95°C and 20 sec at 60°C for 40 cycles (stage 2). Finally, the results were analyzed accordingly using the QuantStudio™ 6 analysis software. Relative quantity (RQ) of the target gene mRNA in each sample compared to the calibrator (normal cells or si-Ctrl) was calculated using the formulas below.

RQ values > 1 indicated overexpression of the target gene while values < 1 indicated downregulation, when compared to the calibrator.

ΔCt of testes sample = Ct of the target gene – Ct of the endogenous control

ΔCt calibrator sample = Ct of the target gene – Ct of the endogenous control

ΔΔCt = ΔCt (tested sample) – ΔCt (calibrator sample)

$RQ = 2^{-\Delta\Delta Ct}$

3.6 Immunoblotting Assay

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an *electrophoretic* system used to separate proteins based on differences in their molecular mass. SDS is a detergent that binds to hydrophobic regions leading to their unfolding and dissociation from other molecules.

3.6.1 Protein extraction and Quantification

RIPA buffer was used to prepare the whole cell lysates. Cells were washed in ice-cold PBS three times prior to harvesting them in RIPA Extraction Buffer (89901, Thermo Scientific, USA), supplemented with 1x Halt protease inhibitor cocktail (78437, Pierce, USA). The cells were removed by a sterile plastic cell scraper, transferred to 1.5 ml microfuge tubes, and placed on ice for 20 min. The whole lysate (supernatant) was centrifuged at 15,000x g for 20 min at 4°C, and stored at - 80°C until use.

3.6.1.1 Protein Quantification

Quantification of extracted Protein was carried out using Pierce™ BCA Protein Assay Kit (Thermo Scientific™, USA) according to their detailed protocol. Pierce Bicinchoninic acid (BCA) protein assay is one of the commonly used methods to quantify total protein concentration. It relies on protein forming complexes with Cu^{2+} under alkaline conditions, followed by the release of Cu^{+} from the reduction of copper-protein complexes; This leads to a colourimetric change. The amount of protein present in the sample represents the amount of reduction and the resulting quantifiable color modification. This method measures the amount of tyrosine, tryptophan, cysteine/cystine, and peptide bonds, all of which are capable of producing the reduced Cu^{+}.

The diluted Bovine Serum Albumin (BSA) standards were made starting from 2,000 µg/ml BSA Ampules (Thermo Scientific™, USA), and diluted in RIPA buffer/Protease inhibitor, according to the manufacturer's procedure. This buffer was used for dilution since all protein lysates were eluted in this buffer during the extraction procedure. A blank was prepared containing only the dilution buffer. BCA Working Reagent (WR) was prepared by mixing the two reagents A and B (Thermo Scientific™, USA) at a ratio (50:1, reagent A:B).

Measurement of the samples and the standards as follows; Briefly, 25 µl of each standard and each protein sample were added to 96-well plate in triplicates with 200 µl of WR. The plate was incubated at 37°C/30 min avoiding light, and the absorbance of each well was measured at (562 nm) using the BioTek $Epoch_2$ (Synergy Multi-Mode Reader,

Inc., USA). The blank measurement was subtracted from all standards and samples' measurements in order to calculate the average-absorbance values. By plotting the average-absorbance values for each BSA standard *versus* their relative concentrations in μg/ml, a standard curve was prepared. This curve was used for protein concentration determination of all unknown samples.

3.6.2 Electrophoresis of protein lysates (SDS-PAGE)

SDS-PAGE consists of two parts: a stacking gel (upper part) and a resolving/separating gel (lower part). The percentages of resolving gel were adjusted depending on the size of the protein of interest. In our study, 7.5% resolving gel and 4% stacking gel were prepared. The polymerization of the gel was catalyzed by ammonium persulfate (APS) (17874, Thermo Scientific™, USA) and tetramethylethylenediamine (TEMED) (15524010, Invitrogen™, USA). Approximately, 20 μg of each cell lysate was denatured, using Laemmli SDS Sample Buffer supplemented with β-mercaptoethanol (Novex, CA), and then heated at 100°C for 5 min prior loading. Electrophoresis was performed at 70 V for 30 min and then increased to 100 V for 1-2 h in 1X electrophoresis running buffer (25mM Tris, 190mM glycine, 0.1% (w/v) SDS) using a thermos 200 CL vertical electrophoresis device (Thermo Scientific™, USA). PageRuler™ Plus Prestained 10-250 kDa Protein Ladder (26619, Thermo Scientific™, USA) served as a marker for estimating the molecular sizes.

3.6.3 Immunoblotting (Western Blotting)

Separated proteins were then transferred and immobilized onto a nitrocellulose membrane (88014, Thermo Scientific, USA) using an electrical current. Blotting was performed using the omniPAGE Mini Vertical Protein Electrophoresis System (Cleaver Scientific, UK). Initially, the blotting sandwich was prepared in the following order: 2 blotting pads + filter paper (originally surrounding membrane) + gel (protein side towards membrane) + membrane + filter paper + 3 blotting pads. The blotting procedure was performed using the electrophoresis tank filled with Towbin blotting buffer (25 mM Tris, 190 mM glycine, and 20% methanol) at 100 V for 180 min and surrounded with ice for cooling. After removing nitrocellulose membranes from the transfer tank, protein transfer was confirmed by Ponceau S stain (Sigma-Aldrich, UK). The red stain from Ponceau S was washed out by Tris-buffered saline with Tween-20 (TBS-T) (20 mM Tris pH 7.6, 136 mM NaCl, and 0.01% (v/v) Tween-20). After removing the stain, the membranes were blocked with 5% (w/v) of non-fat dry milk in TBS-T for 1 h. The membranes were then probed with the primary antibodies (Table 3.2) overnight at 4°C using a shaker. The next day, the membranes were washed with TBS-T (three times/ 5 min each). The membranes were then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h (Table 3.2). Following three times 5 min washing, the SuperSignal™ West Pico PLUS Chemiluminescent Substrate developing reagent (Thermo Scientific, USA) was added to enable visualization. Luminescence signal was detected using the Chemiluminescent GeneGnome system (Syngene. Rome).

Relative quantitative analysis was performed using ImageJ software (NIH Image Software) to determine the bands' intensities.

Table 3.2: Antibodies used in western blotting

	Type	Dilution	Target	Source
Primary antibodies	Rabbit Polyclonal antibody	1:2500	BRIP1	B1310, Sigma
	Mouse Monoclonal antibody	1:2500	β-ACTIN	A2228, Sigma
Secondary antibodies	IgG anti-rabbit-HRP	1:40000	Rabbit antibodies	A0545, Sigma
	IgG anti-mouse-HRP	1:2500	Mouse antibodies	PAB0096, Abnova

3.6.4 Quantification of western blot protein bands

The bands shown on blot images were quantified to determine the amount of protein contained in each band. Collectively, the densitometry quantification of the western blotting protein bands was performed using ImageJ analysis software. After saving images as TIFF, ImageJ software was used for densitometry analysis.

3.7 RNAi transfection experiments

3.7.1 Screening of siRNA oligonucleotides transfection efficiency

The efficiency of siRNA oligonucleotide (siRNAs) transfection was tested using BLOCK-iT™ Alexa Fluor® Red Fluorescent control (14750-100, Invitrogen, USA); This labelled modified stable siRNA mimicked the standard siRNA and was used as a transfection efficiency control through the assessment of fluorescent signal in mammalian cells using Cy®3-filter in the Olympus IX73 inverted microscope. The cells were transiently transfected with labelled siRNAs using RNAi/Max Lipofectamine® reagent (ThermoFisher Scientific, USA), according to their detailed protocol. Lipofectamine reagent consists of positively charged lipids that form circular lipid bilayers, called liposomes, which enclose the siRNAs. The positively charged liposomes fuse with the negatively charged plasma membrane, thus transferring the siRNAs across the membrane and into the cytoplasm (Zhao et al., 2008).

3.7.2 Optimization of BRIP1 downregulation with siRNA oligonucleotides in BC cells

In order to optimize *BRIP1* inhibition, small interfering smart pool BRIP1 siRNA (M-010587-00-0010, Dharmacon Products, USA) and Non-Targeting siRNA Pool (si-Ctrl) (D-001206-14-05, Dharmacon Products, USA) were transfected into cells, using RNAi/Max Lipofectamine® reagent (ThermoFisher Scientific, USA). The smart pool BRIP1 siRNA used in our experiments was a pool of four different siRNAs targeting *BRIP1* to provide

both specificity, potency, and knockdown efficiency. The use of a mixture of four siRNAs with different sequences targeting BRIP1 was significantly effective in inhibiting BRIP1 expression; this can significantly reduce the probability that the observed gene expression changes are caused by the off-target effects.

Initially, it is essential to evaluate the silencing capability before moving forward with large-scale experiments. On the first day, cells were cultured in a 12-wells plate in a medium with no antibiotics at a density of 60% confluency at 37°C and 5% CO_2 overnight. The next day, the cells were washed with sterile PBS for 2 h prior to transfection. RNAi/Max Lipofectamine® mixture was prepared according to the manufacturer's protocol as follows; solution A: (RNAi/Max Lipofectamine in Opti-MEM (Invitrogen, USA)) and solution B: different concentrations of *BRIP1*-siRNA (10, 20, 30, 50, and 100 nM) and si-Ctrl each were mixed separately in Opti-MEM media. Then solution B was added to solution A, gently mixed, and incubated for 20 min at RT. The mixed solution was added to the cells and then incubated in 5% CO_2 incubator at 37ºC. RNA and Protein samples were collected at 48 and 72 h post-treatment, and BRIP1 gene expression was examined using RT-PCR and Western blot analyses, respectively. Extraction procedures of both RNA and protein are described in (sections 3.2 and 3.5.1) above.

Unfortunately, the first attempt for inhibition of BRIP1 expression, especially at protein level was not efficient when a standard knockdown method was applied. Therefore, in order to generate an effective gene silencing method, the transfection protocol was

standardized by including an additional transfection step, performed 24 h following the first one, which showed a better result. Furthermore, different siRNA concentrations ranging from 30 to 100 nM at different time points were tested. The best siRNA concentration that showed the most efficient BRIP1 silencing was **50 nM** at **72 h** in all tested BC cell lines; these criteria were applied in all functional assays' experiments, including gene expression, cell proliferation, and cell motility.

3.8 Cell proliferation assay

Alamar Blue (Resazurin) cell proliferation assay is a simple and popular assay characterized by its non-toxic property, and thus can be used for a longer period of time in the experiments. Furthermore, this test is suitable for most cell types. It is also highly sensitive, as it can detect as few as 50 cells, leading to reliable results (Mikus & Steverding, 2000). In Alamar Blue assay, Resazurin is reduced to resorufin using the natural reducing power of living cells with strong fluorescence proportional to the metabolic activity of living cells and number of cells. After incubating the Alamar-blue with living cells, the color of the solution changes from blue to red, and can be detected using fluorescence or light absorbance.

3.8.1 Optimization of Alamar Blue Assay

In Alamar Blue reduction assay, the optimal cell density and incubation time were initially optimized. For this purpose, cell densities of 1×10^3, 5×10^3, and 1×10^4 cells/well were

seeded in 96 wells tissue culture plates (flat bottom Corning® Costar® cell culture plates) in 200 µl of CGM/well and incubated for 1-3 days. To perform the assay, the cells were washed twice with PBS and 10% µl of Alamar Blue reagent, diluted in corresponding CGM was added. The cells were incubated for various periods of time from 1 - 4 h at 37°C avoiding light. The absorbance of the reduced Alamar Blue form (570 nm) and the oxidized form (600 nm) were measured using the BioTek Epoch$_2$ plate reader (Synergy Multi-Mode Reader, Inc., USA). The percentage of reduction of Alamar Blue was calculated using the following equation:

$$\% \text{ Reduction of Alamar Blue} = \left(\frac{(E_{oxi}600 \times A_{570}) - (E_{oxi}570 \times A_{600})}{(E_{red}570 \times C_{600}) - (E_{red}600 \times C_{570})}\right) \times 100$$

E_{oxi} 600 = molar extinction coefficient (E) of oxidized Alamar Blue at 600 nm = 117216.

E_{oxi} 570 = (E) of oxidized Alamar Blue at 570 nm = 80586.

A_{600} = absorbance of test wells at 600 nm.

A_{570} = absorbance of test wells at 570 nm.

E_{red} 600 = (E) of reduced Alamar Blue at 600 nm = 14652.

E_{red} 570 = (E) of reduced Alamar Blue at 570 nm = 155677.

C_{600}= absorbance of negative control well at 600 nm (medium, Alamar Blue, no cells).

C_{570}= absorbance of negative control well at 570 nm (medium, Alamar Blue, no cells).

% Difference between control and tested cells =

$$\left(\frac{(E_{oxi}600 \times A_{570}) - (E_{oxi}570 \times A_{600})}{(E_{oxi}600 \times P_{570}) - (E_{oxi}570 \times P_{600})}\right) \times 100$$

P_{570} = absorbance of positive control well at 570 nm (medium, Alamar Blue, cells).

P_{600} = absorbance of positive control well at 600 nm (medium, Alamar Blue, cells).

3.8.2 Alamar Blue cell proliferation Assay

To explore the effect of *BRIP1* knockdown on the proliferation rate of the BC cell lines, Alamar-Blue assay was performed to determine whether cells are still metabolically active according to the manufacturer recommendations (Invitrogen, USA). Briefly, on the first day, 1×10^4 cells (optimal cell density) of BRIP1-siRNA and si-Ctrl transfected BC cells were seeded in triplicates in 96-wells tissue culture plates. On the next day, cells were washed twice with PBS and 10% µl of Alamar Blue reagent, diluted in corresponding CGM, was added. The cells were incubated at 37^{o}C for 4 h (optimal incubation time) in dark chamber. Oxidation-reduction measurements of absorbance at 570 and 600 nm wavelengths were measured. For each biological replicate, the average and the standard error of the mean values were determined, and the results were calculated as a percent difference in reduction between BRIP1-siRNA and si-Ctrl transfected cells, according to the manufacturer recommendations, using media alone and Alamar Blue with no cells as controls.

3.9 Flow Cytometry cell cycle assay

Cell cycle profiles were analyzed using fluorescence-activated cell sorting (FACS) analysis. In both siRNA-BRIP1 and si-Ctrl transfected BC cells and the si-Ctrl, $1x10^6$ cells were trypsinized using TrypLE reagent (Gibco, USA) and washed twice with ice-cold DPBS. The cells were fixed using 70% ice-cold ethanol and stored overnight at 4°C. The next day, cells were centrifuged, washed, and incubated with 100 µg/ml RNAse and 40 µg/ml propidium iodide (PI) (Sigma, Germany) for 30 min at room temperature in the dark. Stained cells were analyzed using BD Accuri™ C6 Flow Cytometer (BDbiosciences, USA). The non-stained and PI-stained cells were used as controls. Cell cycle distribution analysis was obtained using the FlowJo™ v10.6.1, LLC software.

3.10 Cell Migration and Invasion Assay using Transwell assay

Cell migration and invasion were studied using Transwell assay (Boyden Chamber method). Boyden chamber is a system composed of an upper part (an insert) that had different size porous membrane (either coated or non-coated with matrigel) where the cells were added. The other component is the lower part of the insert (wells of the plate) contained the chemoattractant in order to attract cells to migrate or invade through the porous membrane to the lower side (Figure 3.1).

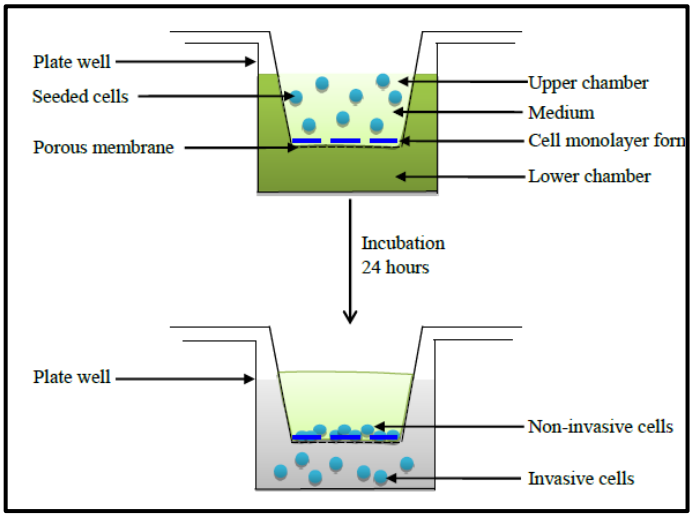

Figure 3.1: Schematic presentation of Boyden Chamber assay.

3.10.1 Cell Migration Assay

Boyden chamber assay was used to examine cell migration and invasion following siRNA BRIP1 inhibition as we have previously described (Abdraboh et al., 2011). Briefly, for migration assay, 3×10^5 of MDA-MB-231, or 5×10^5 of MCF-7 and CAMA-1 BRIP1-siRNA and si-Ctrl BC transfected cells were washed twice in serum-free media and the cells were added directly to the 8 mm PEC Transwell (without matrigel) chambers (Corning, USA). The cells were applied to the upper chamber in serum-free media. A volume of 650 µl media, containing 10% FBS was added to the lower chamber as a chemoattractant. Cultures were maintained for 24 h for MDA-MB-231 and 48 h for MCF-7 and CAMA-1 treated cells. Non-migrated cells were removed gently from the upper well using a cotton swab moistened with PBS. Subsequently, the cells were fixed with 4%

formaldehyde for 10 min and then stained using 5% crystal violet in ethanol in order to stain the cells that have migrated across the membrane to the lower side. Excessive stain was washed out with PBS. After staining, migrated cells were photographed at five different microscopic fields, using Cell Imaging System (Olympus IX73 inverted microscope, USA). Pictures of fields were quantified and analyzed using ImageJ software (NIH Image Software).

3.10.2 Cell Invasion Assay

Similarly to migration assay, cell invasion was assessed using transwell inserts coated with Matrigel (BD Biosciences, MA). Briefly, matrigel coated plates were incubated at 37°C for 2 h. During the incubation, transfected cells were washed with serum-free cell culture media and counted. 5×10^5 of MDA-231 or 1×10^6 of MCF-7 and CAMA-1 of BRIP1-siRNA and si-Ctrl BC-transfected cells were seeded onto the upper chambers of the pre-coated Transwell chambers (Corning, USA). Complete CGM was added to the lower chamber and incubated at 37ºC for 24 h for MDA-MB-231 and 48 h for MCF-7 or CAMA-1 treated BC cells (Germano et al., 2012; Mehner et al., 2014). Similarly to migration assay, invaded cells were washed, fixed, stained, and imaged as described above.

3.11 Wound-Healing Scratch Assay

MCF-7, CAMA-1, and MDA-MB-231 cells were transfected with BRIP1-siRNA or si-Ctrl. Then, 48 h post-transfection, the cells were counted and seeded in a 24-well plate with

serum-free media to resynchronize the cells overnight. Upon reaching confluence on the following day 72 h post-transfection, a straight scratch was made in each well using a sterile 10 µl white tip. Cells were gently washed in sterile PBS in order to remove debris, and then cultured in their corresponding media. The initial width of the scratch was measured and considered as the starting measurement point (0 h). The plates were further incubated and imaged at 24 h and 48 h, in reference to a marker line for accurate imaging, using an inverted microscope (Olympus IX73 inverted microscope, USA). Results were analyzed using the ImageJ software (NIH Image Software, USA) (C.-C. Liang, Park, & Guan, 2007).

3.12 Metastasis associated genes profiling

The main purpose of this investigation was to understand the process of invasion of BC cells through the identification of novel signaling pathways. Therefore, tumor Metastasis Fast 96-well plates (4414098, Life Technologies, USA) containing lyophilized TaqMan® Gene Expression assay was used to determine the expression levels of pro-metastatic genes in BRIP1-siRNA transfected cells. Briefly, total RNA was converted into cDNA as described above. The reaction was performed using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Inc., USA), according to the manufacturer instructions. The results were first normalized to GAPDH and further analyzed to obtain the Relative mRNA expression levels, using the formula $2^{-\Delta\Delta Ct}$. All the reactions were carried out in triplicates and repeated twice.

3.13 Statistical Analysis

Data were represented as means ± SD from at least three independent repeated experiments unless otherwise stated. Analysis of variance (ANOVA) was used to identify significant differences in multiple comparisons. For a direct comparison between two groups with normally distributed values, Student's *t*-test was applied using GraphPad Prism 8 (GraphPad Software, USA) and Microsoft Excel 2013. Any value of $P < 0.05$ was considered as statistically significant value for all experiments. All *P* value between 0.01 and 0.05 were shown with one (*) asterisk (significant), *P* value between 0.01 and 0.001 were shown with two (**) asterisks (very significant), *P* value between 0.001 and 0.0001 were designated with three (***) asterisks (extremely significant).

CHAPTER 4 : RESULTS

4.1 BRIP1 is highly expressed in different breast cancer cell lines

4.1.1 Expression of BRIP1 protein in different breast cancer cell lines

In order to explore the role of *BRIP1* in breast tumor progression, we examined BRIP1 protein expression in various BC cell lines CAMA-1, MCF-7, T47D, BT474, HCC-1500, HCC-2218, MDA-MB-468, and MDA-MB-231 in comparison to normal HuMEC and immortalized non-pathogenic breast cell line MCF-10A using Western blotting. Figure 4.1 showed that protein levels were differentially overexpressed in the BC cell lines compared to HuMEC and MCF 10A control cells. Densitometric quantification was performed by ImageJ software and the graph was plotted accordingly (Figure 4.2).

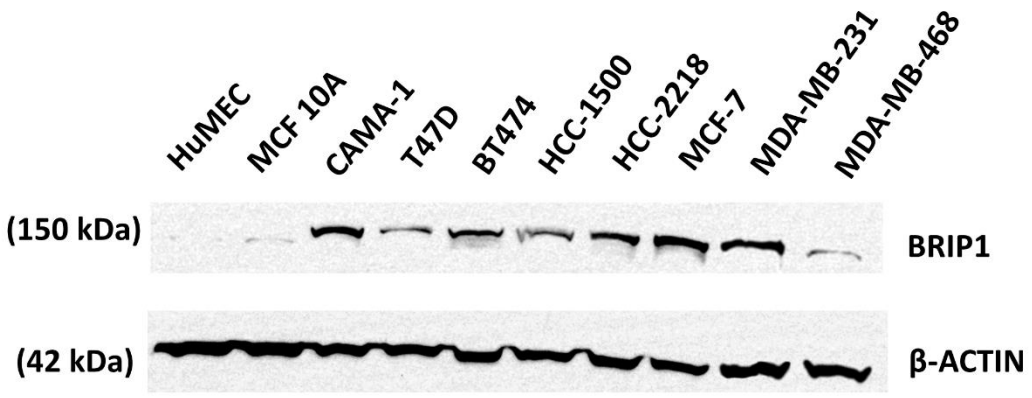

Figure 4.1: BRIP1 protein expression levels in different BC cell lines. (A) Western Blot analysis showing BRIP1 protein expression levels in tested BC cell lines. β–ACTIN was used as the loading control. All the gels from three separate experiments were performed under the same experimental conditions. Shown are the cropped immunoblot images representing indicated proteins.

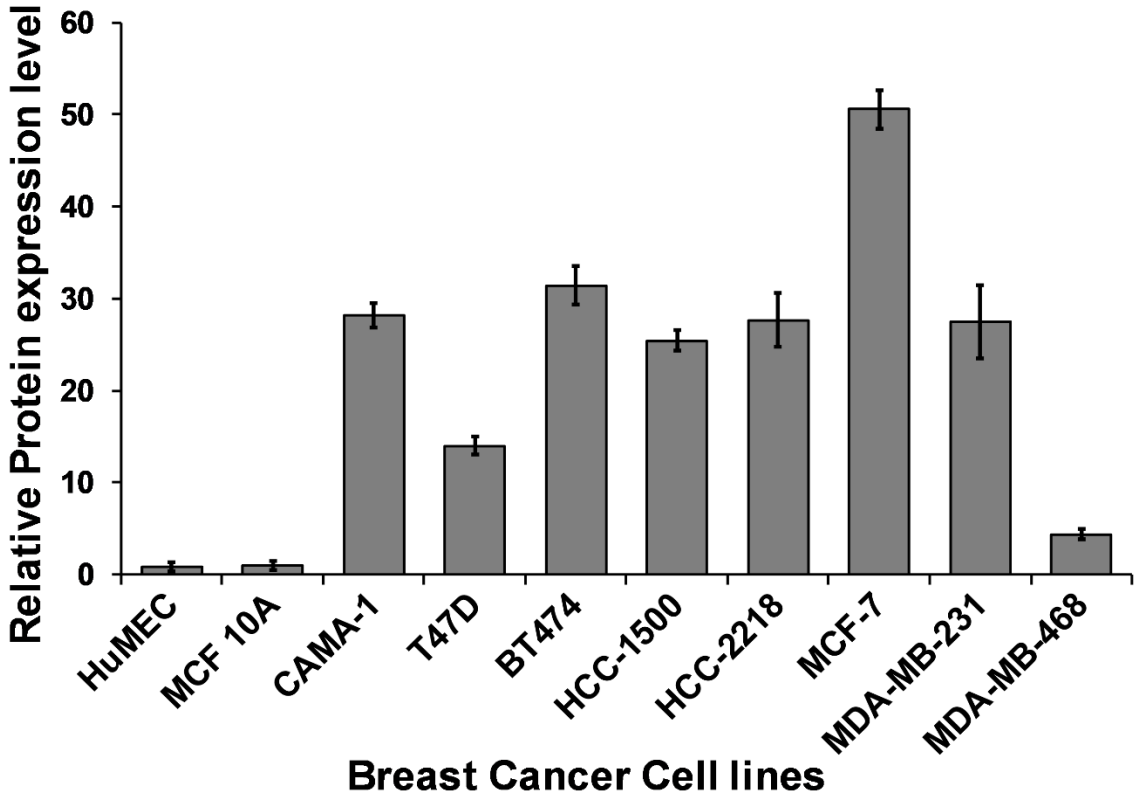

Figure 4.2: Relative quantification of BRIP1 protein expression levels in various BC cells. Quantification of BRIP1 was done by densitometry analysis and normalized to β-ACTIN as a loading control followed by relative quantitation to normal control. Mean values (n=3) ±SD of three experiments are shown.

Immunoblotting analysis of the protein lysates showed that BRIP1 was differentially overexpressed when compared to normal control HuMEC and MCF 10A cells in all tested BC cell lines (Figure 4.1). In fact, relative quantification showed that MCF-7 displayed a 50-fold increase, followed by BT474 (31-fold), (27.5-fold), [HCC-2218, CAMA-1, and

MDA-MB-231 showed (28-fold)], HCC-1500 (25-fold), T47D (14-fold), and MDA-MB-468 displayed (4-fold) in BRIP1 expression as compared to control (Figure 4.2).

4.1.2 Expression of *BRIP1* mRNA in different breast cancer cell lines

To validate western blot results described above, TaqMan quantitation PCR analysis was used to examine the cells for mRNA gene expression (Figure 4.3). *BRIP1* mRNA expression was examined in the BC cell lines CAMA-1, MCF-7, T47D, BT474, HCC-1500, HCC-2218, MDA-MB-231, and MDA-MB-468 in comparison to normal HuMEC and immortalized non-pathogenic BC line MCF-10A controls using RT-qPCR.

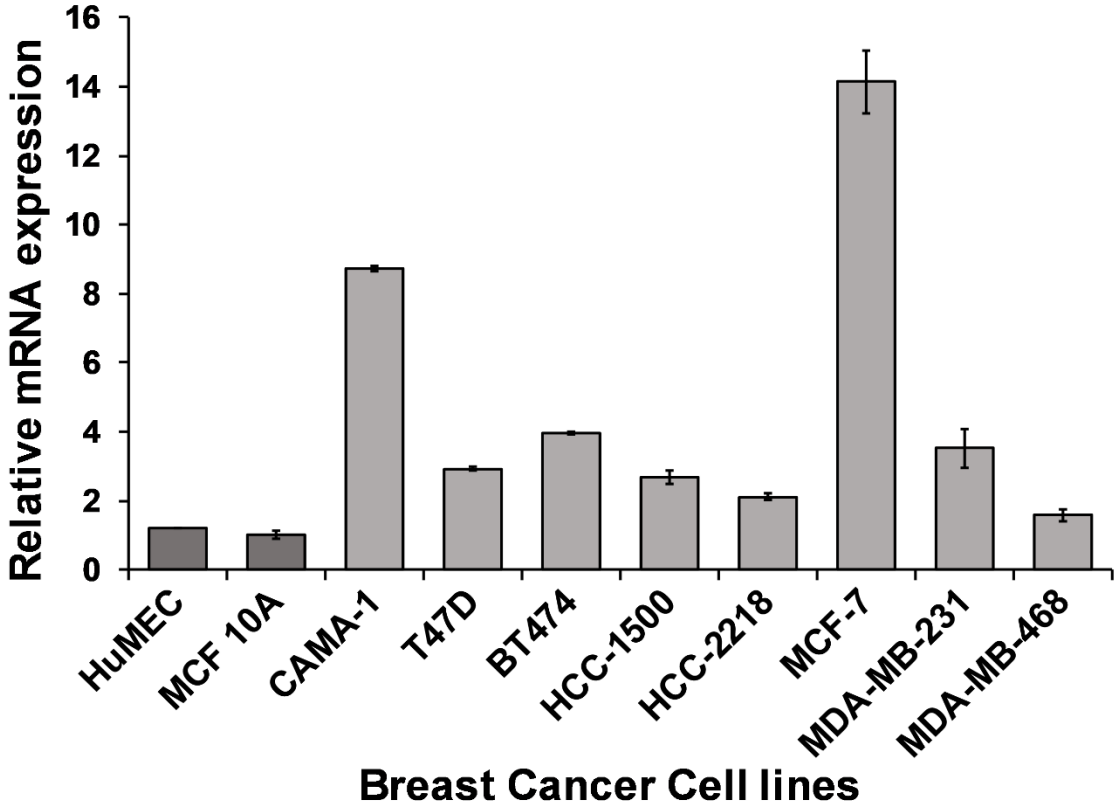

Figure 4.3: Relative *BRIP1* mRNA expression levels in various BC cell lines by using TaqMan RT-qPCR. The relative expression levels of *BRIP1* were normalized to *GAPDH* and relatively compared to normal controls. Mean values (n=3) ±SD of three experiments are shown.

MCF-7 had maximum mRNA BRIP1 expression with (14-fold), followed by CAMA-1 (8.7-fold), BT474 (4-fold), MDA-MB-231 (3.5-fold), T47D and HCC-1500 (3-fold), HCC-2218 (2.1-fold), and MDA-MB-468 displayed (1.5-fold) (Figure 4.3). In conclusion, using Western blotting and RT-qPCR analyses, data showed that both protein and mRNA were overexpressed in the BC cell lines compared to HuMEC and MCF 10A control cells.

4.2 Downregulation of *BRIP1* using siRNA interference in Breast Cancer cell lines

Next, to test our hypothesis, four BC cell lines were selected based on their BRIP1 expression levels and their tumor characteristics (MCF-7, CAMA-1, MDA-MB-231, and HCC1-500) as experimental models. Because BRIP1 is overexpressed in BC cells, RNAi suppression of *BRIP1* was favored in this study to functionally validate the role of *BRIP1* in promoting BC progression; various specific siRNA oligonucleotides were tested in this study to successfully downregulate the expression of *BRIP*, and assess its functional impact on cell growth and cell invasion. Several experimental optimization conditions were applied to set up siRNA transfection. First, to optimize the transfection of siRNA into BC cells, different **siRNA concentrations** and **time points** were assessed. The siRNA concentration that will result in the most efficient silencing of *BRIP1* with the least cell toxicity will be selected for all experiments. Therefore Smart pool BRIP1 siRNAs against *BRIP1* was used in this study. Using four siRNAs against *BRIP1* lowers the likelihood of identifying off- or non-specific targets in subsequent analyses (since the same off-targets are not normally observed with two or more different *BRIP1*-siRNAs). In addition, a non-Targeting siRNA pool was used as a negative control (si-Ctrl) for all the siRNA experiments performed in this study. The expression level of *BRIP1* in each siRNA inhibition condition was normalized against the expression level of *BRIP1* in the si-Ctrl condition; thus ensuring that the changes in mRNA levels were due to the siRNAs targeting our gene of interest *BRIP1* and are not due to any generalized effect from siRNA transfection.

4.2.1 Assessment of siRNA transfection efficiency

To insure a successful siRNA transfection, Block-iT AlexaFluor Red Fluorescent control was used. Block-iT AlexaFluor is a fluorescent dye-labelled siRNA used as a positive control to visualize transfection efficiency by lipid-based transfection reagent. This experiment was used as a model to provide evidence that the transfection efficiency was not a limiting factor in obtaining better siRNA inhibition of *BRIP1* in all tested BC cell lines.

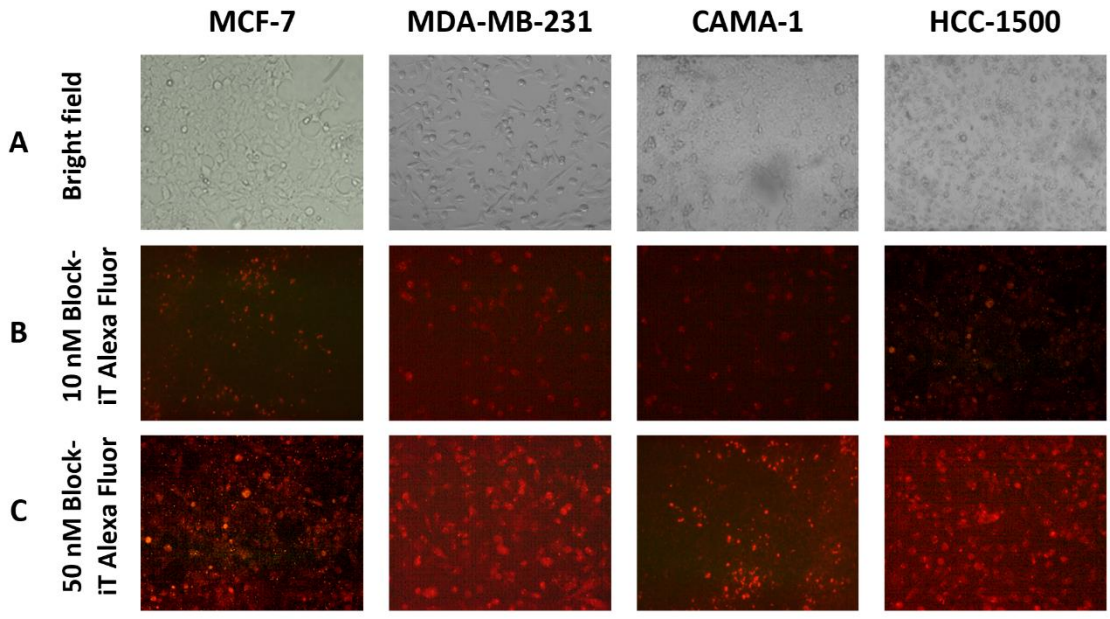

Figure 4.4: Block-iT AlexaFluor uptake by MCF-7, MDA-MB-231, CAMA-1, and HCC-1500 BC cell lines. (A) Cells before incubating with the dye. (B) Cells were transfected with 10nM Block-iT AlexaFluor. (C) Cells were transfected with 50nM Block-iT AlexaFluor. Higher uptake was achieved with 50nM of Block-iT Alexa.

4.2.2 Screening of siRNA oligonucleotides for BRIP1 downregulation in MCF-7 cells

Downregulation efficiency and specificity of the siRNA pool against *BRIP1* were tested. Initially, an optimization of transfection conditions was performed in order to achieve the best silencing results. MCF-7 BC cell line was selected as a starting control for the transfection optimization experiments as it showed the highest BRIP1 expression levels as shown in (Figures 4.1 - 4.3). *BRIP1*-inhibition experiments were evaluated at RNA level 48 h post-transfection by RT-qPCR (Figure 4.5) and at the protein level 72 h after siRNA-BRIP1 transfection by western blotting (Figure 4.6).

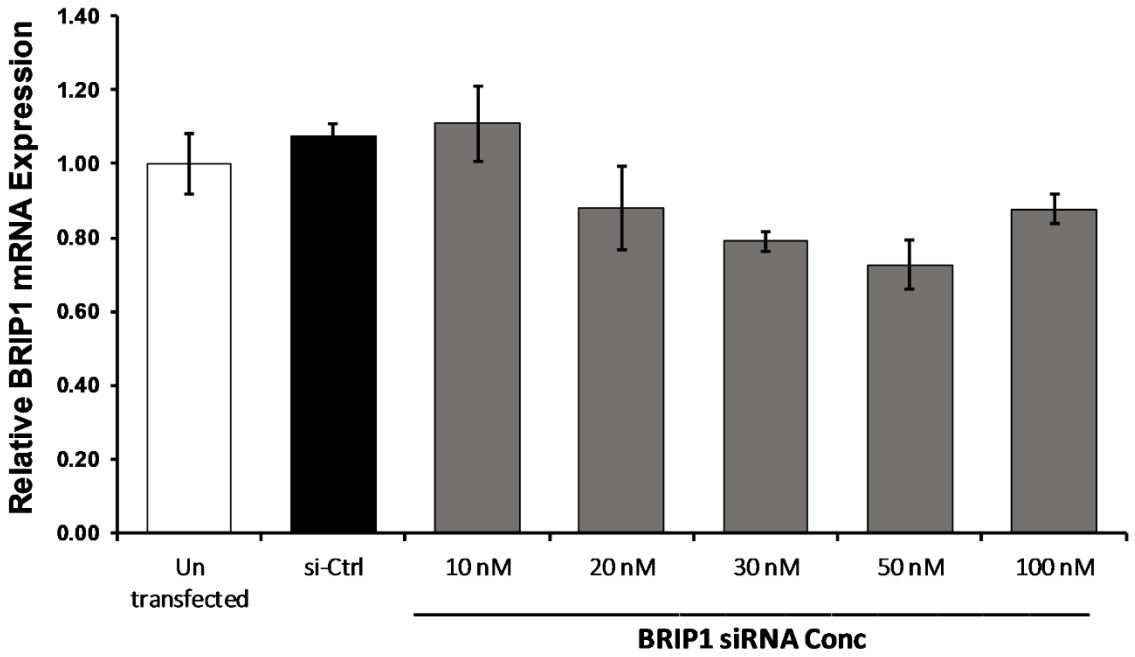

Figure 4.5: Relative *BRIP1* mRNA expression levels in BRIP1 knockdown MCF-7 cells. The *BRIP1* mRNA relative expression was measured using TaqMan RT-qPCR and normalized against *GAPDH* and the (si-Ctrl). Mean values (n≥3) ±SD of three experiments are shown.

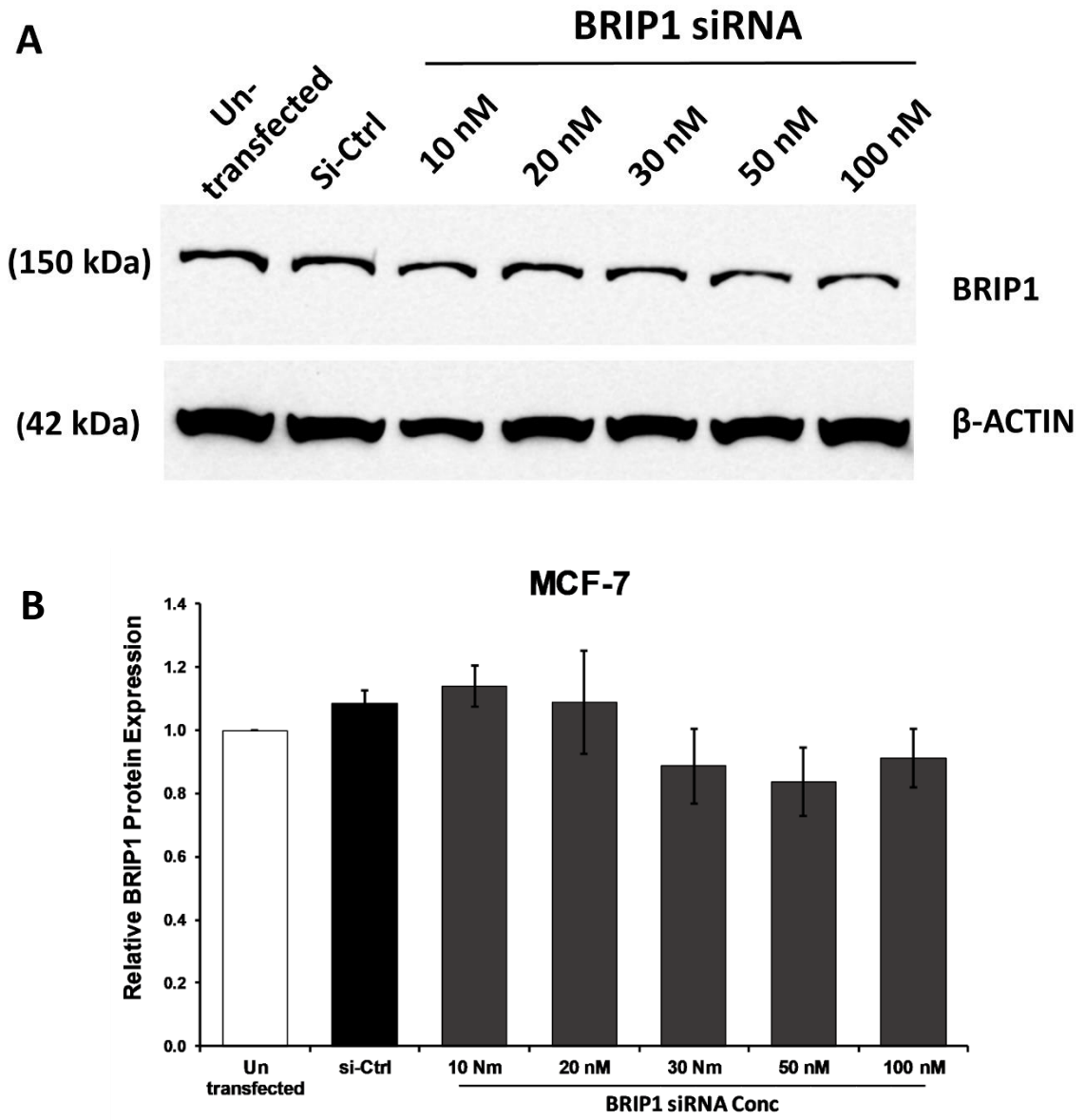

Figure 4.6: Expression levels of BRIP1 protein in MCF-7 cells transfected with different concentrations of BRIP1-specific siRNAs. (A) Representative western blot analysis of BRIP1 and β-ACTIN proteins in MCF-7 cells transfected with ascending concentrations of BRIP1 specific siRNAs (10, 20, 30, 50, and 100 nM) and (si-Ctrl) at 72 h post-transfection. (B) Representative densitometry relative protein quantification of BRIP1 normalized to its corresponding β-ACTIN control and relative to the si-Ctrl. Mean values (n=3) ±SD of three experiments are shown.

As seen in (Figures 4.5 and 4.6), transfection with different BRIP1-siRNA concentrations reduced the *BRIP1* gene expression by only 20 % at maximum compared to si-Ctrl with no significant reduction in protein expression compared to the control.

4.2.3 Successful Downregulation of BRIP1 with siRNAs in MCF-7 BC cells

After several attempts, the standard protocol for inhibition was not efficient to silence BRIP1 at both protein and mRNA levels, using different siRNA concentrations. According to this result, a second round of siRNA transfection was applied 24 h post the first transfection to increase the silencing specifically at the protein level (double knockdown). Initially, siRNAs double knockdown protocol was tested, and downregulation of *BRIP1* was evaluated in MCF-7 BC cells to determine the best dosage and time for *BRIP1* silencing. To determine the best time-point, an average dose of 50 nM was used to transfect MCF-7 and evaluate *BRIP1* inhibition at (24, 48, and 72 h) time points. Then, three siRNA dosages were tested (30 nM, 50 nM, and 100 nM) to evaluate the effective dosage for silencing gene expression. These experiments will be discussed in details below:

4.2.3.1 Relative BRIP1 mRNA expression

At first, we evaluated the effect of RNAi inhibition on *BRIP1* gene expression levels in MCF-7 BC cells by starting with an average dose of 50 nM siRNA-BRIP1 and non-targeted si-Ctrl. The mRNA expression levels of *BRIP1* were monitored 24-72 h post-transfection

using TaqMan RT-qPCR assays. Relative *BRIP1* gene expression was calculated in relation to si-Ctrl after normalization against *GAPDH* (Figure 4.7).

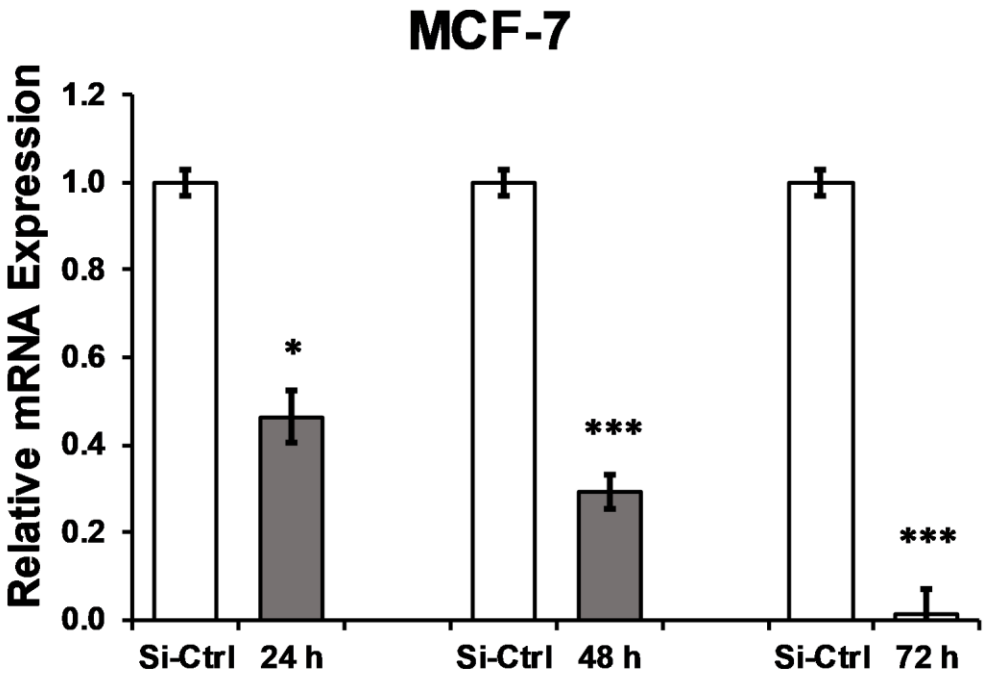

Figure 4.7: Time optimization for efficient *BRIP1* siRNAs-mediated knockdown in MCF-7 cells. Cells were transfected with 50 nM of siRNA targeting *BRIP1* and si-Ctrl. mRNA gene expression levels were determined by TaqMan RT-qPCR assay at 24, 48 and 72 h post-transfection. All relative expression levels of *BRIP1* mRNA was normalized to *GAPDH* and relative to the si-Ctrl. Mean values ±SD (n=3) of three experiments are shown; * $P< 0.05$, *** $P< 0.001$.

As shown in (Figure 4.7), the relative mRNA gene expression levels of *BRIP1* were reduced by 50%, 70%, and 90% at 24, 48, and 72 h, respectively. At this time point (72 h), different siRNA-BRIP1 concentrations (30 nM, 50 nM, and 100 nM) were assessed at mRNA level to evaluate the effective siRNA dosage as shown in (Figure 4.8).

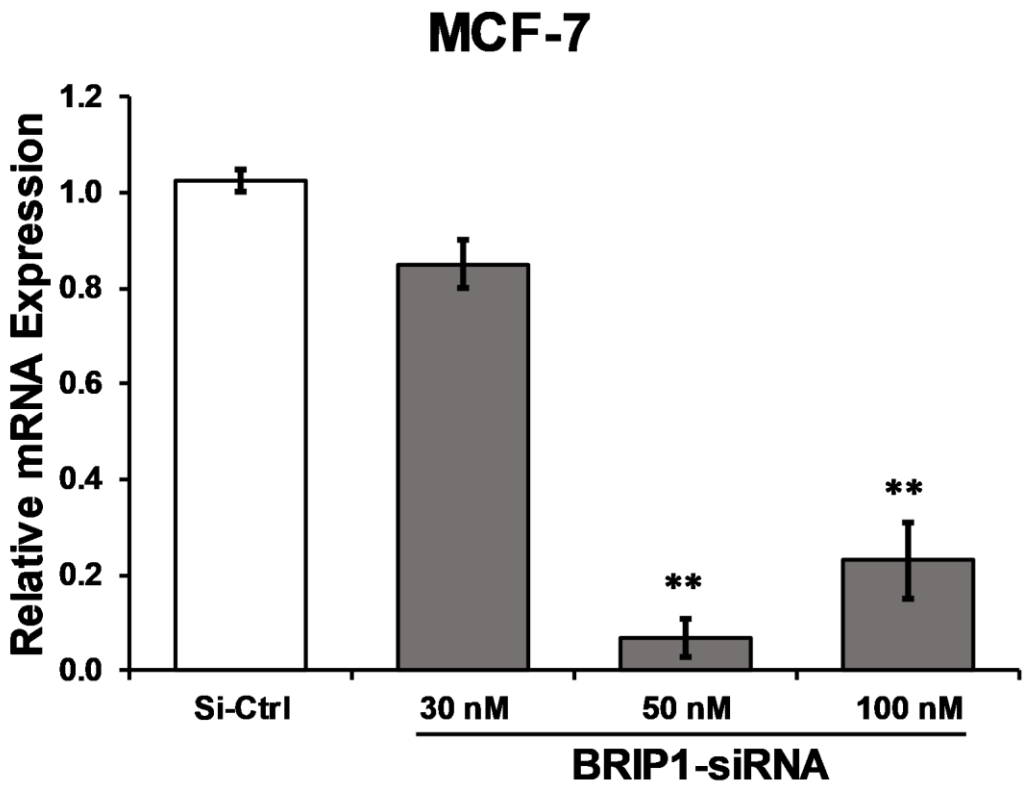

Figure 4.8: Optimization of *BRIP1*-specific siRNAs concentration for efficient *BRIP1* knockdown in MCF-7 cells. Cells were transfected with 30, 50, and 100 nM siRNAs targeting *BRIP1* and si-Ctrl. All relative expression levels of *BRIP1* mRNA were normalized to *GAPDH* and relative to si-Ctrl at 72 h post-transfection using TaqMan RT-qPCR. Mean values (n=3) ±SD of three experiments are shown; * $P< 0.05$, ** $P< 0.01$.

Relative mRNA gene expressions of different concentrations of siRNA against *BRIP1* showed a low 20 % reduction of relative mRNA expression at 30 nM. However, a significant *BRIP1* inhibition of 90 % and 75 % were obtained with 50 nM and 100 nM, respectively ($P<0.01$; Figure 4.8).

4.2.3.2 Total BRIP1 protein expression

In general, the reduction of a mRNAs using specific siRNA does not preclude that the effect would be similar at protein level. Thus, for the purpose of functional experiments, the best time point and concentration were selected based on when BRIP1 protein was reduced to its lowest levels after transfection of BC cells.

To confirm *BRIP1* knockdown at protein level in MCF-7, ascending concentrations of 30 nM, 50 nM, and 100 nM of BRIP1-siRNA were tested, total protein lysates were harvested 72 h post-transfection, and examined by western blotting and densitometry analyses (Figure 4.9).

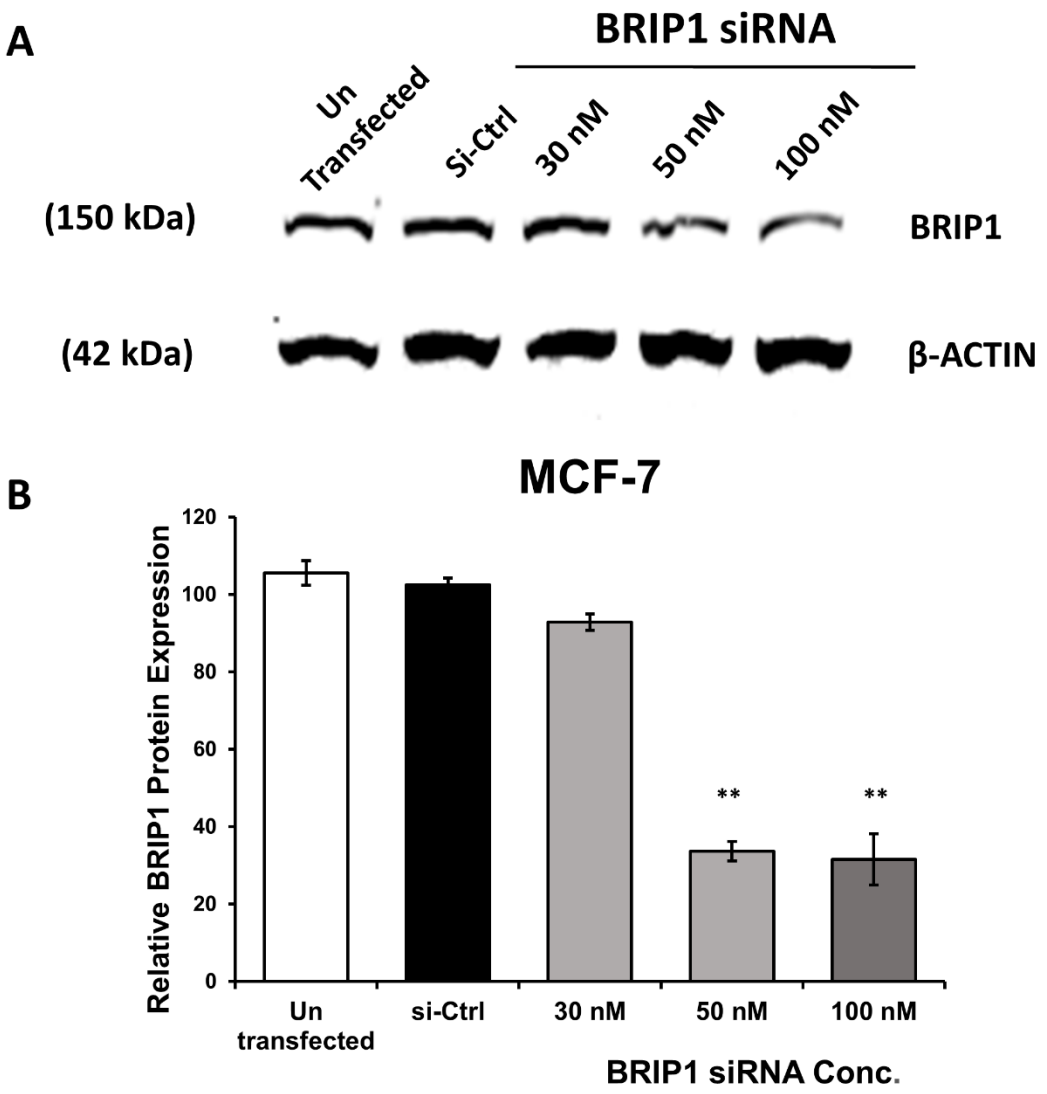

Figure 4.9: Expression levels of BRIP1 protein in MCF-7 cells transfected with BRIP1 siRNAs. (A) western blot analysis of BRIP1 and β-ACTIN proteins at 72 h after transfection with 30, 50, and 100 nM of siRNAs targeting BRIP1 and si-Ctrl in MCF-7 cells. Shown are the cropped immunoblot images representing indicated proteins. (B) Relative protein quantification of BRIP1 normalized to its corresponding β-ACTIN loading control and relative to si-Ctrl. Mean values (n≥3) ±SD of three experiments are shown; $^{**}P < 0.01$.

As shown in (Figure 4.9), transfection of MCF-7 cells with siRNA-BRIP1 showed 10%, 67%, and 70% reduction of BRIP1 protein with 30 nM, 50 nM, and 100 nM, respectively compared to si-Ctrl. Transfection with 100 nM siRNA-BRIP1 showed unhealthy pattern of the cells including changes in their shape and plate detachments, thus why it was not selected for functional analysis in our study. Altogether, the effective silencing conditions of BRIP1 expression was 50 nM concentration at 72 h time point, that showed 90% of *BRIP1* mRNA expression and 67% of protein expression levels relative to si-Ctrl ($P<0.01$; Figures 4.8 and 4.9). These conditions were used further in functional assays involving MCF-7 BC cell line. Similar experimental conditions were applied to the remaining BC cell lines, CAMA-1, MDA-MB-231, and HCC-1500.

4.2.4 Successful siRNA downregulation of BRIP1 in CAMA-1, MDA-MB-231, and HCC-1500 cells

The silencing effect of the standarized siRNA transfection conditions above were tested in on CAMA-1, MDA-MB-231, and HCC-1500 BC cells. In addition to the above standarized conditions, a second round of transfection was repeated 24 h after the first one using 50 nM BRIP-siRNAs and si-Ctrl. Proteins and RNAs were collected 72 h post-transfection and examined by western blotting and RT-qPCR, respectively,

4.2.4.1 Relative BRIP1 mRNA expression

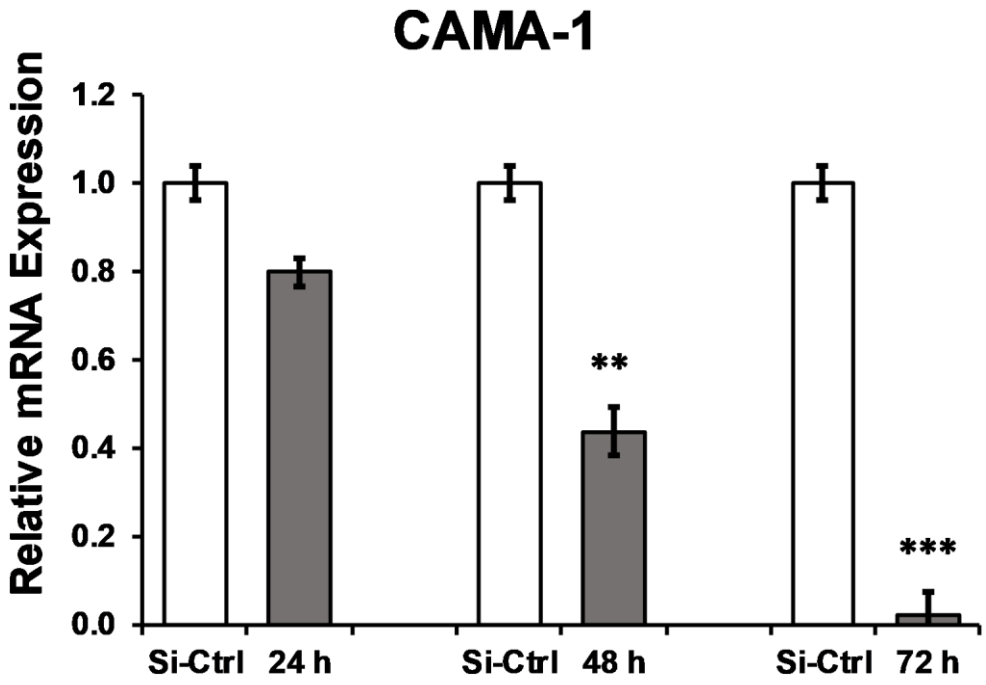

Figure 4.10: Relative *BRIP1* mRNA expression in CAMA-1 cells transfected with BRIP1 siRNAs. The cells were transfected with (50 nM) BRIP1-siRNAs and (si-Ctrl), the *BRIP1* mRNA expression was measured at (24-72 h) post transfection with RT-qPCR, and normalized against *GAPDH* relative to the si-Ctrl. Mean values (n=3) ± SD is shown. ***p < 0.01, ****p < 0.001*.

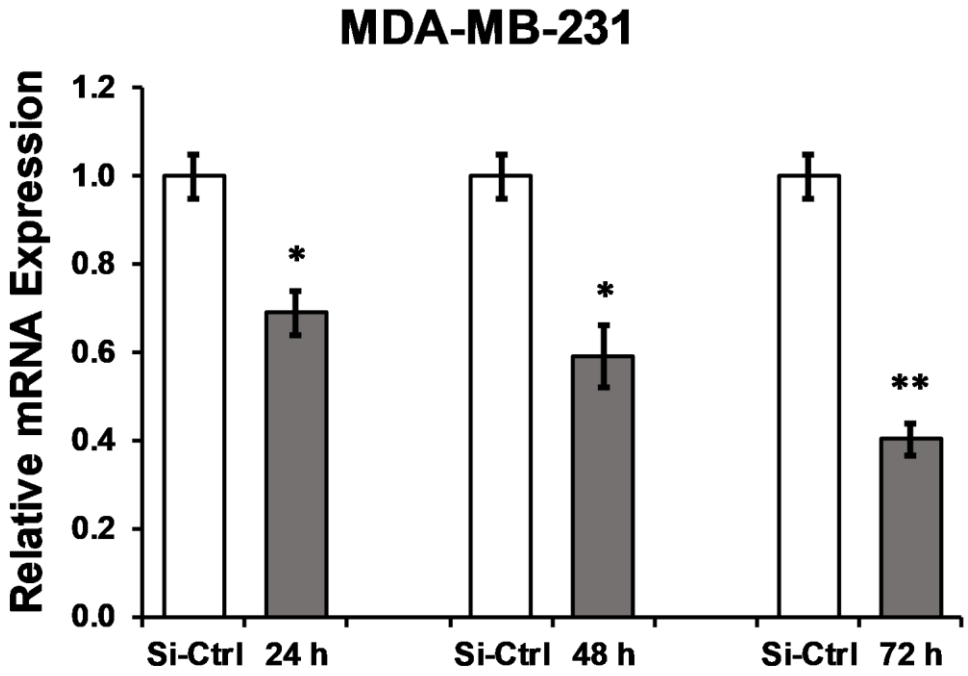

Figure 4.11: Relative *BRIP1* mRNA expression in MDA-MB-231 cells transfected with BRIP1 siRNAs. The cells were transfected with (50 nM) BRIP1-siRNAs and (si-Ctrl), the *BRIP1* mRNA expression was measured at (24-72 h) post transfection with RT-qPCR, and normalized against *GAPDH* relative to the si-Ctrl. Mean values ± SD of (n=3) is shown. *$p < 0.05$, **$p < 0.01$.

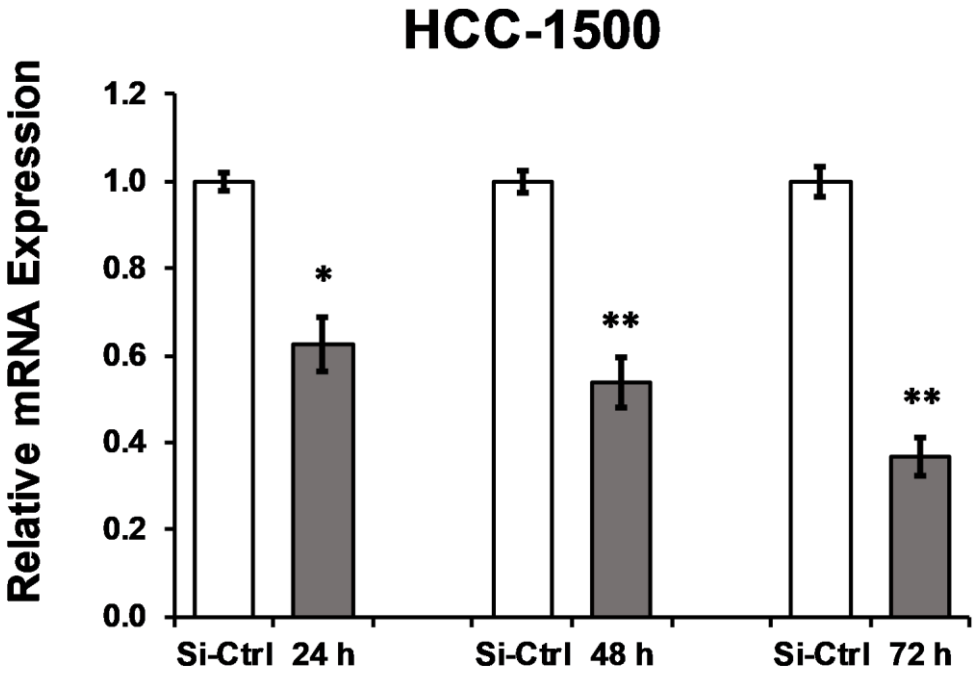

Figure 4.12: Relative *BRIP1* mRNA expression in HCC-1500 cells transfected with BRIP1 siRNAs. The cells were transfected with (50 nM) BRIP1-siRNAs and (si-Ctrl), the *BRIP1* mRNA expression was measured at (24-72 h) post transfection with RT-qPCR, and normalized against *GAPDH* relative to the si-Ctrl. Mean values (n=3) ± SD is shown. *p < 0.05, **p < 0.01.

Notably, treatment with 50 nM specific siRNAs of BRIP1 markedly suppressed *BRIP1* mRNA levels with less toxicity at 72 h time point in CAMA-1, MDA-MB-231, and HCC-1500 by 90 %, 79.1 %, 75%, respectively, (*P*<0.01; Figures 4.10 - 4.12).

4.2.4.2 Total BRIP1 protein expression

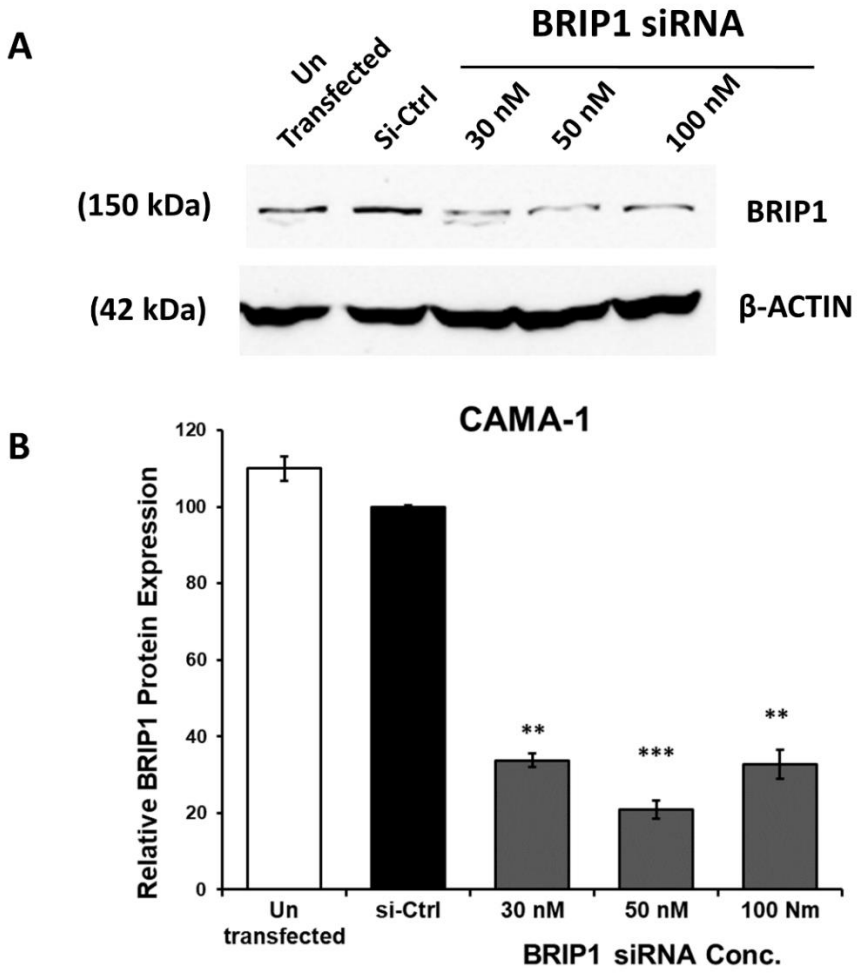

Figure 4.13: Expression levels of BRIP1 protein in CAMA-1 cells transfected with BRIP1-specific siRNAs. (A) Representative western blot analysis of BRIP1 and β-ACTIN proteins in CAMA-1 cells transfected with (30, 50, and 100 nM) siRNAs targeting BRIP1 and si-Ctrl at 72 h post-transfection. Shown are the cropped immunoblot images representing indicated proteins. (B) Representative relative protein quantification of BRIP1 normalized to its corresponding β-ACTIN control. The results were normalized to the (si-Ctrl). Mean values (n=3) ±SD of three experiments are shown; **p < 0.01, ***p < 0.001.

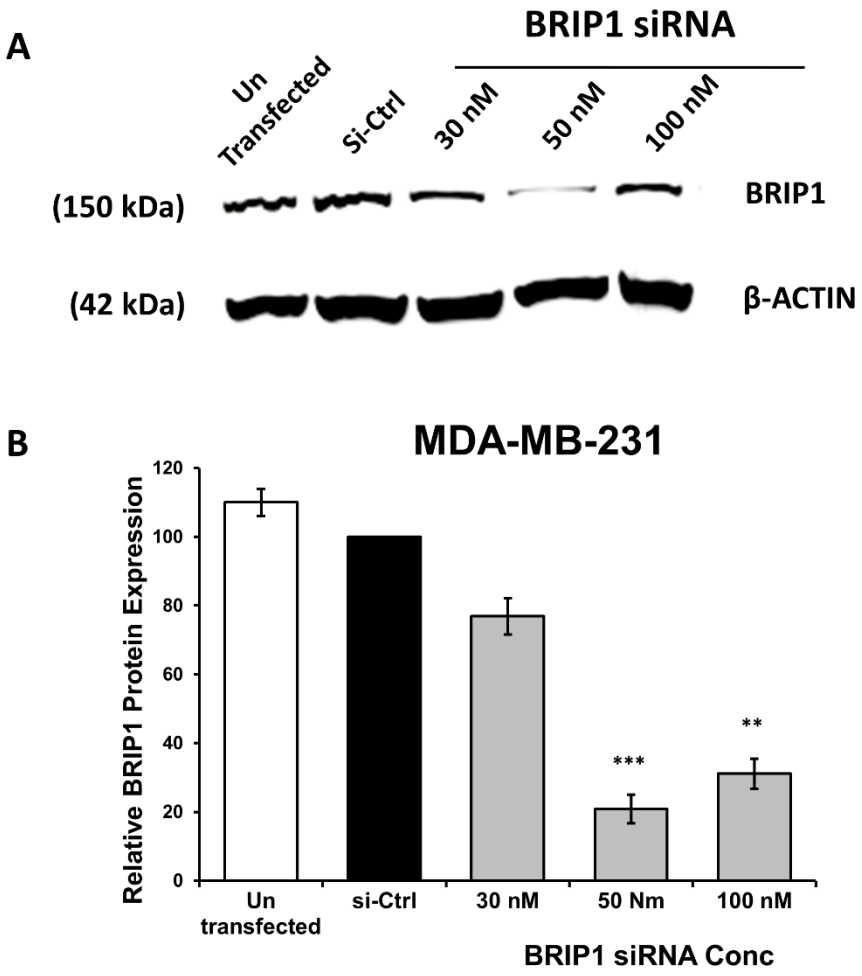

Figure 4.14: Expression levels of BRIP1 protein in MDA-MB-231 cells transfected with BRIP1-specific siRNA. (A) Representative western blot analysis of BRIP1 and β-ACTIN proteins in MDA-MB-231 cells transfected with (30, 50, and 100 nM) siRNAs targeting BRIP1 and si-Ctrl at 72 h post-transfection. Shown are the cropped immunoblot images representing indicated proteins. (B) Representative relative protein quantification of BRIP1 normalized to its corresponding β- ACTIN control. The results were normalized to the (si-Ctrl). Mean values ±SD (n=3) of three experiments are shown; **$p < 0.01$, ***$p < 0.001$.

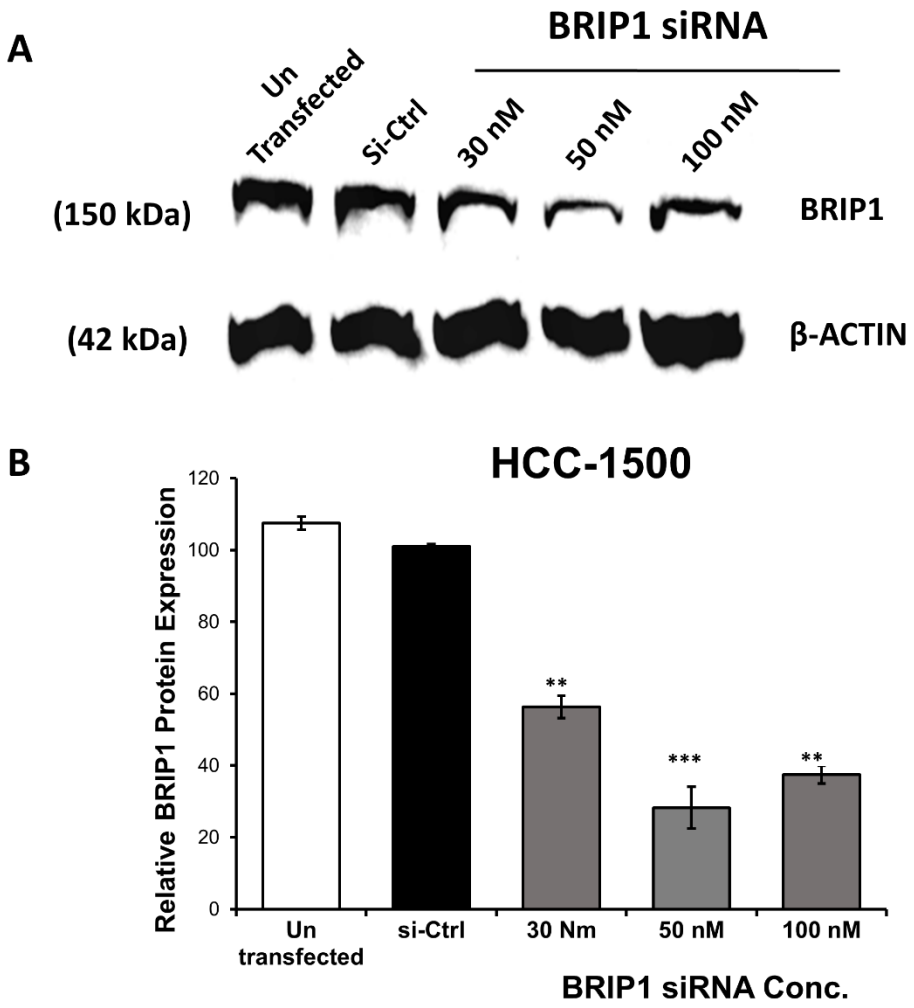

Figure 4.15: Expression levels of BRIP1 protein in HCC-1500 cells transfected with BRIP1-specific siRNAs. (A) Representative western blot analysis of BRIP1 and β- ACTIN proteins in HCC-1500 cells transfected with (30, 50, and 100 nM) siRNAs targeting BRIP1 and si-Ctrl at 72 h post-transfection. Shown are the cropped immunoblot images representing indicated proteins. (B) Representative relative protein quantification of BRIP1 normalized to its corresponding β- ACTIN control. The results were normalized to the (si-Ctrl). Mean values ±SD (n=3) of three experiments are shown; **$p < 0.01$, ***$p < 0.001$.

Transfection of **CAMA-1** BC cell line with siRNA-BRIP 50 nM 72 h post-transfection showed the highest downregulation efficiency of 90 % reduction in relative *BRIP1* mRNA expression levels and 80 % reduction in protein level in (Figure 4.13). In addition, siRNA-BRIP1 transfection of **MDA-MB-231** using same conditions showed significant silencing efficiency of 79.1 % reduction in relative *BRIP1* mRNA expression and 82% reduction at protein level (Figure 4.14). Furthermore, siRNA-BRIP1 applied to **HCC-1500** BC cell line in the same conditions showed a 75 % reduction in relative *BRIP1* mRNA expression and 71.7 % reduction of protein expression (Figure 4.15).

4.3 *BRIP1* facilitates proliferation in breast cancer cell lines

4.3.1 Optimization of the Alamar-Blue reduction assay conditions

Before assessing the effect of siRNA-BRIP1 downregulation on cell proliferation in different BC cell lines using Alamar-Blue cell proliferation assay, different cell densities of each BC cell and different incubation times with Alamar Blue were tested (*data not shown for optimization steps*). Reduction in Alamar Blue increased with increasing cell density up to a density of 1×10^4 cells/well, from this value a plateau was reached. Reduction of Alamar Blue also increased with increasing incubation time. Given all the results, Alamar-Blue assay standardized conditions were a cell density of 1×10^4 cells/well and 4 h as incubation time.

4.3.2 *BRIP1* downregulation attenuates proliferation rate of BC cell lines

To examine the effect of BRIP1 inhibition on BC cell proliferation, cells were treated with either siRNA-BRIP1 or si-Ctrl, and cellular proliferation was assessed at 24, 48, and 72 h post-treatment using Alamar-blue assay. As shown in (Figure 4.16), cellular proliferation was significantly reduced in siRNA-BRIP1 transfected cells compared to si-Ctrl groups in all studied BC cell lines.

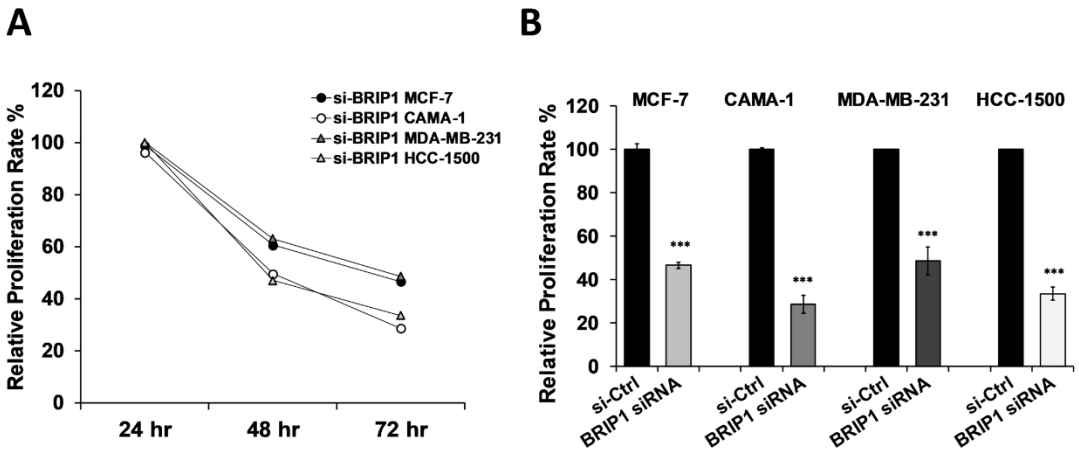

Figure 4.16: The effect of BRIP1 siRNAs suppression on cell proliferation in various BC cell lines. (A) Cell proliferation was assessed using Alamar Blue cell proliferation assay at 24, 48, and 72 h post-transfection with (50 nM) BRIP1-specific siRNAs and si-Ctrl in MCF-7, CAMA-1, MDA-MB-231, and HCC-1500 cell lines. (B) At 72 h, all tested si-BRIP1 BC cells showed the maximum proliferation inhibition rate. Mean values (n=3) ±SD of triplicate experiments are shown, ***P < 0.001.

Despite the variations between the different BC cell lines, there was a significant reduction in cell proliferation of 46.5% in MCF-7, 28.6% in CAMA-1, 48.6 % in MDA-MB-231, and 33.5% in HCC-1500 72 h post-treatment (***P < 0.001; Figure 4.16).

4.4 Cell cytometry of BC cells following siRNA mediated *BRIP1* knockdown

Knockdown of BRIP1 coincided with a decrease in BC cells' growth (Figure 4.16). Besides, Fluorescence-activated cell sorting (FACS) analysis was used to determine changes in cell cycle. BC cells transfected with siRNA pools against *BRIP1* and a non-targeting si-Ctrl were fixed 72 h after transfection and FACS analysis was carried out to determine the proportion of cells in different phases of the cell cycle.

As observed in Alamar-Blue assay, BRIP1 knockdown had similar effect on cell cycle, with a reduction in cells in G1/S phase relative to non-targeting si-Ctrls (Figures 4.17 – 4.20).

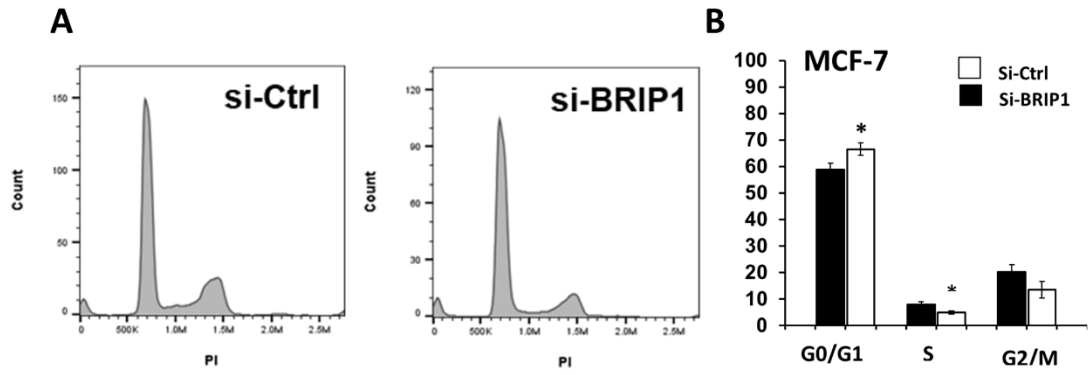

Figure 4.17: siRNA inhibition of *BRIP1* induced cell cycle arrest in MCF-7 cells. (A) Cell cycle histogram of BRIP1-siRNAs transfected MCF-7 cells vs si-Ctrl at 72 h using flow cytometry. (B) Representative quantitative analysis of cell cycle different phases population; G0/G1, S, and G2/M in BRIP1-siRNAs transfected MCF-7 cell lines compared to si-Ctrl. The quantitation was done by calculating the area under the curve using Flowjo software of three independent experiments and represented as the Mean values (n≥3) ±SD, * $P < 0.05$.

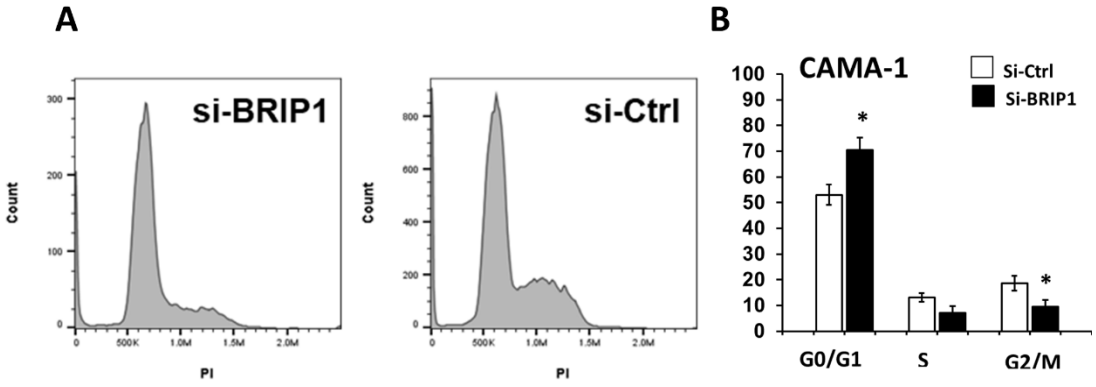

Figure 4.18: siRNA inhibition of *BRIP1* induced cell cycle arrest in tested CAMA-1 cells. (A) Cell cycle histogram of BRIP1-siRNAs transfected CAMA-1 cells vs si-Ctrl at 72 h using flow cytometry. (B) Representative quantitative analysis of cell cycle phases; G0/G1, S, and G2/M in BRIP1-siRNAs transfected CAMA-1 cell lines compared to si-Ctrl. The quantitation was done by calculating the area under the curve using Flowjo software of three experiments and represented as the Mean values (n≥3) ±SD, * $P < 0.05$.

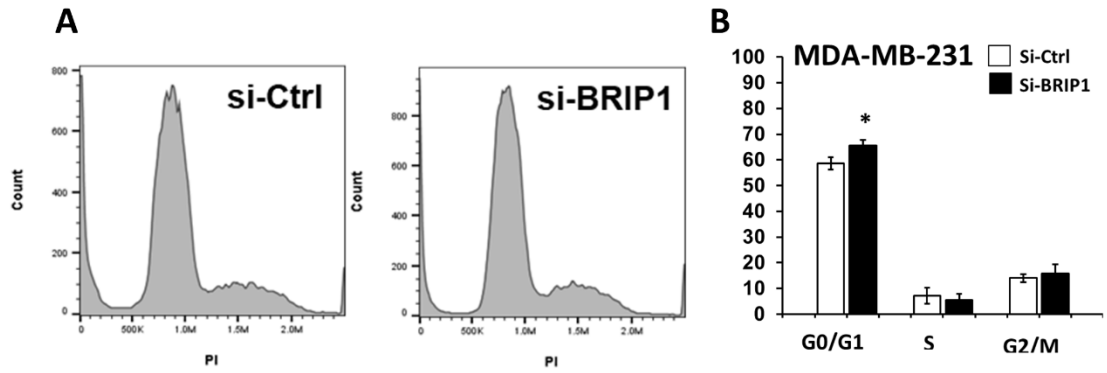

Figure 4.19: siRNA inhibition of *BRIP1* induced cell cycle arrest in tested MDA-MB-231 cells. (A) Cell cycle histogram of BRIP1-siRNAs transfected MDA-MB-231 cells vs si-Ctrl at 72 h using flow cytometry. (B) Representative quantitative analysis of cell cycle different phases population; G0/G1, S, and G2/M in BRIP1-siRNAs transfected MDA-MB-231 cell lines compared to si-Ctrl. The quantitation was done by calculating the area under the curve using Flowjo software of three experiments and represented as the Mean values (n≥3) ±SD, * $P < 0.05$.

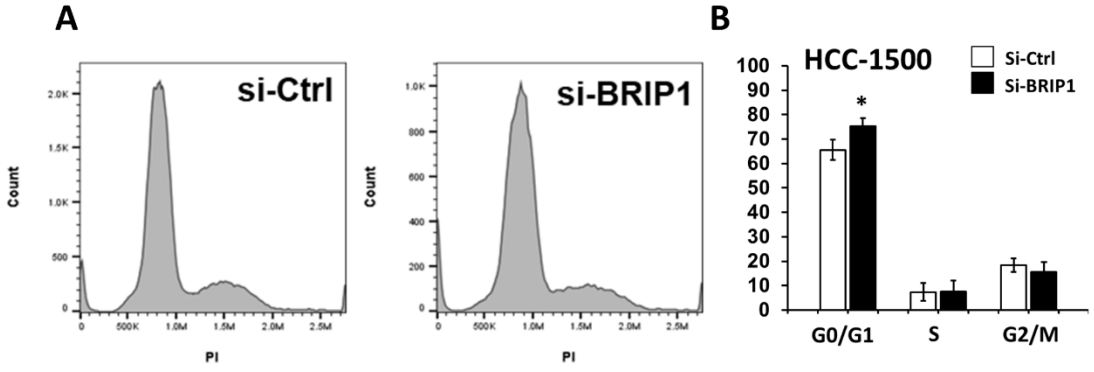

Figure 4.20: siRNA inhibition of *BRIP1* induced cell cycle arrest in tested HCC-1500 cells. (A) Cell cycle histogram of BRIP1-siRNAs transfected HCC-1500 cells vs si-Ctrl at 72 h using flow cytometry. (B) Representative quantitative analysis of cell cycle phases; G0/G1, S, and G2/M in BRIP1-siRNAs transfected HCC-1500 cell lines compared to si-Ctrl. The quantitation was done by calculating the area under the curve using Flowjo software of three independent experiments and represented as the Mean values (n≥3) ±SD, * $P < 0.05$.

Compared to si-Ctrl group, cell cycle FACS analysis showed that BRIP1 suppression induced G1/S arrest in all BC cells (Figures 4.17 - 4.20). All the data put together indicate that BRIP1 promotes cell proliferation in all the BC cells tested.

4.5 *BRIP1* promotes migration and invasion ability of breast cancer cell lines

Both scratch and Transwell assays were utilized to examine the effect of BRIP1 suppression on migration and invasion properties of siRNA-BRIP1 transfected BC cell lines compared to si-Ctrl group.

4.5.1 Scratch / Wound healing assay

Wound healing assay results revealed that BRIP1 suppression significantly attenuated the ability of the cells to close the gap. When compared to control, relative percentage of wound closures was 60% in MCF-7 and MDA-MB-231, and 50 % in CAMA-1 (Figures 4.21 – 4.23).

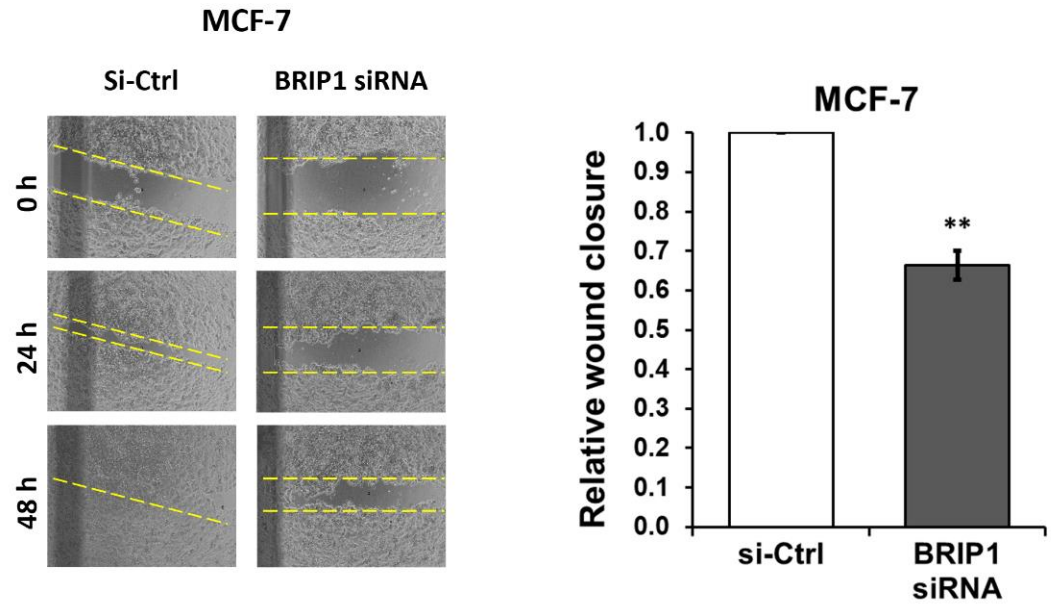

Figure 4.21: Wound-healing assay showing the effect of *BRIP1* siRNA-mediated knockdown on migration of MCF-7 cell line. Graphical representation of the relative wound closure following BRIP1- specific siRNAs treatment compared to si-Ctrl at 48 h post monolayer cell scratch in MCF-7 cells. Data shown are representatives of three independent experiments under the same conditions. The error bars represent SD. ** $P < 0.01$.

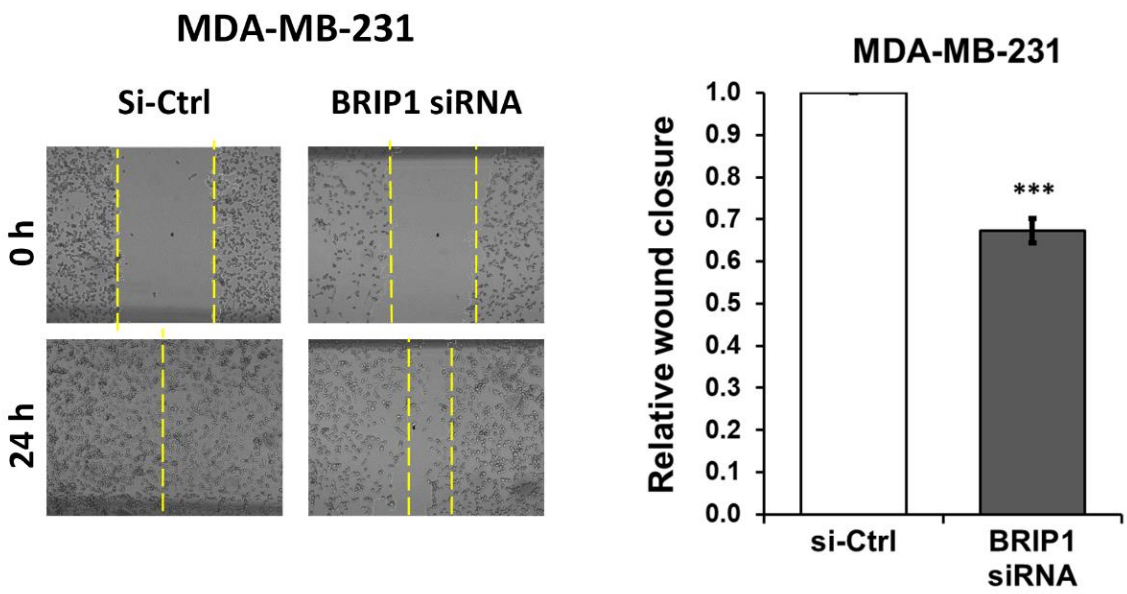

Figure 4.22: Wound-healing assay showing the effect of *BRIP1* siRNA-mediated knockdown on migration of MDA-MB-231 cell line. Graphical representation of the relative wound closure following BRIP1- specific siRNAs treatment compared to control at 24 h post monolayer cell scratch in MDA-MB-231 cells. Data shown are representatives of three independent experiments under the same conditions. The error bars represent SD. *** $P < 0.001$.

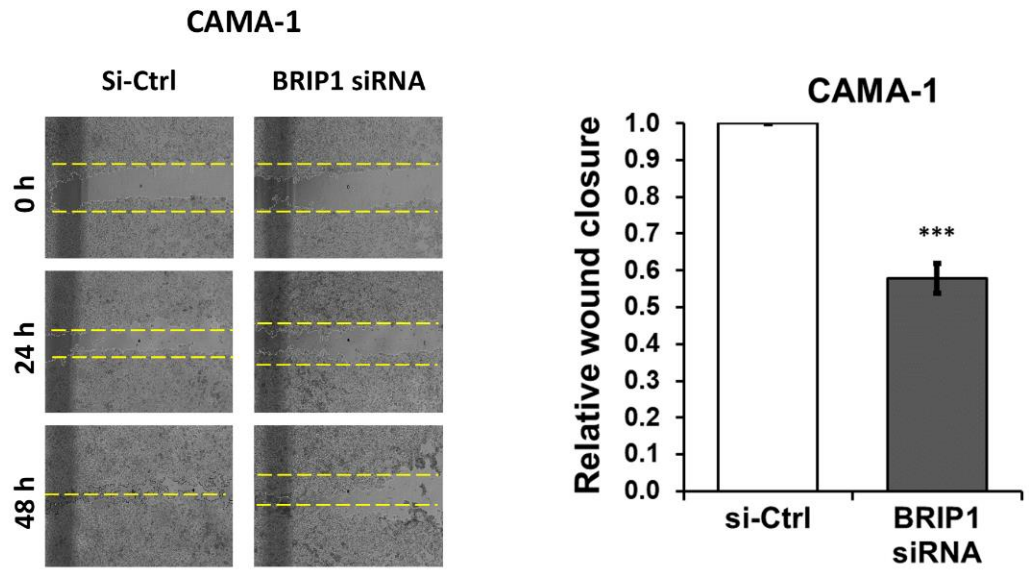

Figure 4.23: Wound-healing assay showing the effect of *BRIP1* siRNA-mediated knockdown on migration of CAMA-1 cell line. Graphical representation of the relative wound closure following BRIP1- specific siRNAs treatment compared to control at 48 h post monolayer cell scratch in CAMA-1 cells. Data shown are representatives of three independent experiments under the same conditions. The error bars represent SD. *** $P < 0.001$.

4.5.2 Transwell migration and invasion assays

BRIP1 knockdown was evaluated in reducing cell motility through trans-migration and invasion of MCF-7, CAMA-1, and MDA-MB-231 BC cells using Transwell assay. The cells entrapped within the transwell membranes' pores were fixed and counted in comparison to non-targeted si-Ctrl transfected cells.

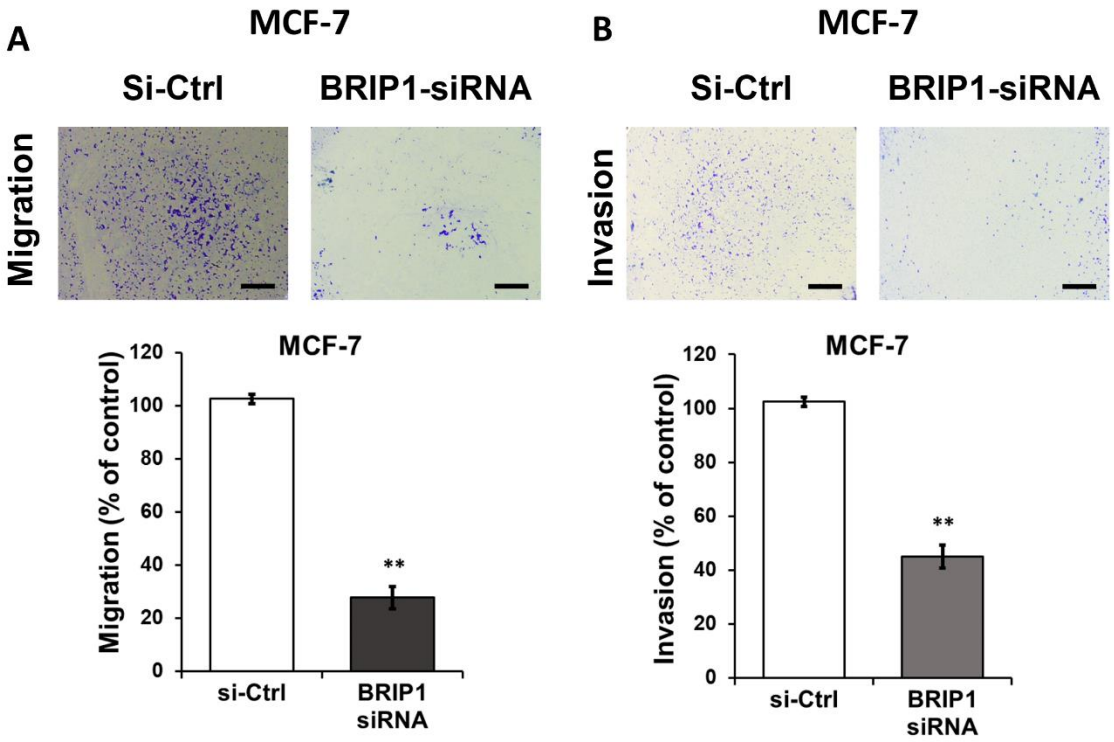

Figure 4.24: Suppression of *BRIP1* in MCF-7 cells inhibited their migration and invasive ability using Boyden chamber assay. (A Panel) Showing crystal violet stained migrated cells at the lower part of the transwell chamber membrane following BRIP1-specific siRNA transfection in MCF-7 cells. (B Panel) Showing crystal violet stained invaded cells at the lower part of the Transwell chamber membrane following BRIP1-specific siRNAs transfection in MCF-7 cells. The data represents the relative percentage of migrated (A) and invaded (B) cells after knockdown with BRIP1-siRNAs compared to si-Ctrl group. Minimum of three fields were randomly selected to count cells under an inverted microscope. (n=3, ** $P < 0.01$).

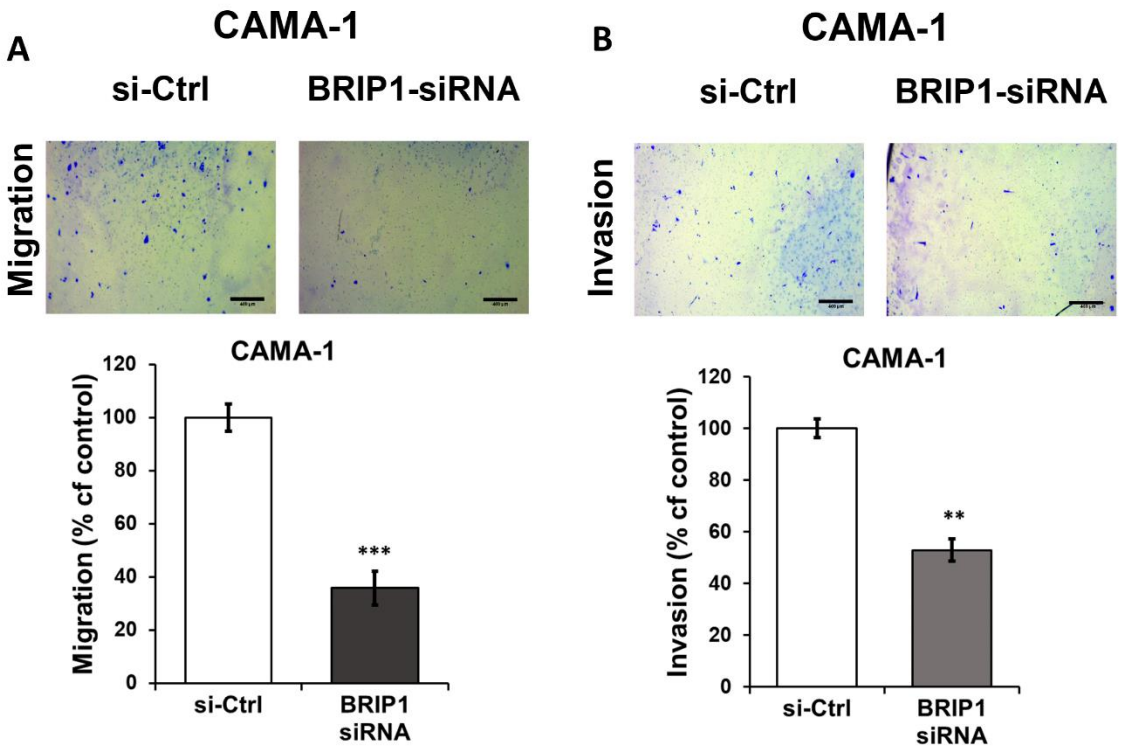

Figure 4.25: Suppression of *BRIP1* inhibited migration and invasion of CAMA-1 cells using Boyden chamber assay. (A Panel) showing crystal violet stained migrated cells at the lower part of the transwell chamber membrane following BRIP1-specific siRNAs transfected into CAMA-1 cells. (B Panel) Showing crystal violet stained invaded cells at the lower part of the Transwell chamber membrane following BRIP1-specific siRNAs transfected into CAMA-1 cells. The data represents the relative percentage of migrated (A) and invaded (B) cells after knockdown with BRIP1-siRNAs compared to si-Ctrl group. Minimum of three fields were randomly selected to count cells under an inverted microscope. (n=3, ** $P < 0.01$, *** $P < 0.001$).

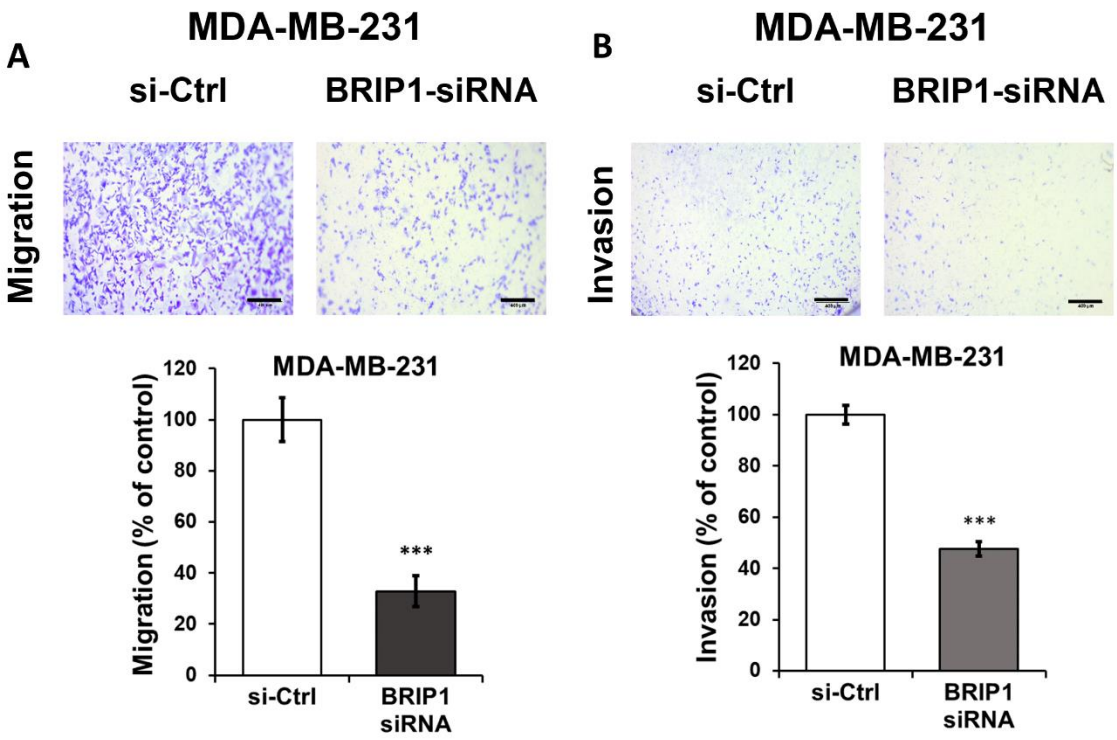

Figure 4.26: Suppression of *BRIP1* inhibited migration and invasion of MDA-MB-231 cells using Boyden chamber assay. (A Panel) Showing crystal violet stained migrated cells at the lower part of the transwell chamber membrane following BRIP1-specific siRNAs transfected into MDA-MB-231 cells. (B Panel) Showing crystal violet stained invaded cells at the lower part of the Transwell chamber membrane following BRIP1-specific siRNAs transfected into MDA-MB-231 cells. The data represents the relative percentage of migrated (A) and invaded (B) cells after knockdown with BRIP1-siRNAs compared to si-Ctrl group. Minimum of three fields were randomly selected to count cells under an inverted microscope. (n=3, ** $P < 0.01$, *** $P < 0.001$).

Compared to si-Ctrl, BRIP1 inhibition showed a significant decrease in both migration (70%, 70% and 60%) and invasion (50 %, 50%, and 50%) abilities of MCF-7, MDA-MB-

231 and CAMA-1 BC cells, respectively (Figures 4.24 - 4.26). Together, our results indicate that *BRIP1* inhibition suppressed both migration and invasion of the three examined BC cell lines.

4.6 *BRIP1* promotes cell invasion by modulating the expression of an array of related genes

In order to identify potential molecular signalling pathway(s) underpinning the novel role of BRIP1 in BC progression, TaqMan RT-qPCR of 92 different metastasis-associated genes was performed to determine differentially expressed genes in BRIP1-suppressed MCF-7 and MDA-MB-231 cells compared to controls. Analysis of our results showed a significant upregulation/downregulation of a group of genes known to regulate both cell growth and cell motility. Among an array of genes, stands out genes associated with extracellular matrix, adhesion molecules, cell proliferation, and motility (CXCL12, RB1, RHOC, H/KRAS, MYC, SMAD4, EPCAM, MCAM (CD146), MGAT5, and MMPs) were determined (Table 4.1).

Table 4.1: BRIP1 promotes cell growth and metastasis by modulating expression of an array of related genes

Symbol	Genebank accession number	Description/Main Function	Fold Change in BC cells$^{siRNA\text{-}BRIP1}$ vs BC cells $^{si\text{-}Ctrl}$
CXCL12	Hs00171022_m1	C-X-C Motif Chemokine Ligand 12/ Tumor growth and metastasis	- 5.8
RB1	Hs00153108_m1	RB Transcriptional Corepressor 1/ Cell growth and cell cycle	-4.8
RHOC	Hs00733980_m1	Ras Homolog Family Member C/ Tumor cell proliferation and metastasis.	-3.9
HRAS	Hs00610483_m1	HRas Proto-Oncogene, GTPase/ Regulation of cell proliferation	-3.8
MYC	Hs00153408_m1	MYC Proto-Oncogene, BHLH Transcription Factor/ cell cycle progression, apoptosis and cellular transformation	-3.7
SMAD4	Hs00232068_m1	SMAD Family Member 4/ Cell proliferation.	-3.5
KRAS		KRAS Proto-Oncogene, GTPase/ Regulation of cell proliferation	-3.0
EPCAM	Hs00158980_m1	Epithelial Cell Adhesion Molecule/ Proliferation, differentiation, and migration.	-2.9
MCAM (*CD146*)	Hs00174838_m1	Melanoma Cell Adhesion/ cell adhesion Molecule/ Cell adhesion	-2.9
MGAT5	Hs00159136_m1	Alpha-1,6-Mannosylglycoprotein 6-Beta-N-Acetylglucosaminyltransferase/ Proliferation, adhesion and metastasis	-2.5
MMP-1	Hs00233958_m1	matrix metallopeptidase 1/ Breakdown of extracellular matrix and metastasis	-2.0

The selected genes were analyzed using the TaqMan™ Array Human Tumor Metastasis (Applied Biosystems™, USA). Fold changes of the selected genes in siRNA-BRIP1 transfected cells was calculated by comparison Comparative Ct values to si-Ctrl transfected cells for three biological replicates. Student's t-test was performed for statistical analysis. Genes with a fold change below 2 or above -2 (Cut off point) and/or when the *p*-value > 0.05 were not considered in the table.

CHAPTER 5 : DISCUSSION

In an attempt to identify genes associated with the transition from normal epithelial breast cells to malignant invasive cells, we have previously employed microarray gene expression profiling and compared RNA samples isolated from malignant breast tumor tissues to normal/benign breast tissues. Among a number of differentially expressed genes, *BRIP1* (*BRCA1* interacting protein C-terminal helicase1), showing a 5-fold up-regulation was identified as a potential gene that might underpin breast tumor progression (I. Gupta, Ouhtit, Al-Riyami, & Al Ajmi, 2015).

Although BRIP1 interacts with BRCA1 to regulate cell cycle and DNA repair mechanisms, the role of *BRIP1* in mediating tumor growth and progression has not been examined. In the present study, we tested the hypothesis that *BRIP1* can promote both BC cell growth and metastasis. The results of the present study are summarized as follows: i) structural validation experiments were consistent with our previous findings (Ali et al., 2019), showing 5-fold overexpression of *BRIP1* in all types of BC tumor samples compared to normal/benign breast tissues; ii) functional validation approaches revealed a novel role of *BRIP1* in promoting breast tumor cell growth and progression. In fact, siRNA down-regulation of *BRIP1* attenuated cell proliferation significantly by inducing cell cycle arrest. Moreover, RNAi-inhibition of *BRIP1* significantly reduced migration and invasion of BC cell lines; iii) A unique set of differentially expressed *BRIP1*-target genes associated with both cell cycle and metastasis seem to underpin *BRIP1*-promoted BC cell proliferation and

invasion, suggesting a dual role of BRIP1 as a tumor suppressor and/or an oncogene.

BRIP1 is overexpressed in Breast Cancer

The mutational spectrum of *BRIP1* was recently determined in various BC cell lines using the *Estimate algorithm*. Comparison of RNA-Seq transcriptomes from hundreds of breast tumors showed no mutations in the selected model for functional characterization (Vincent, Findlay, & Postovit, 2015). Upregulated *BRIP1* levels in malignant breast tumors contradict its role as a tumor suppressor. As an example, the *TP53* tumor suppressor gene is overexpressed in colorectal cancer, which is not predictive with its mutational status as an early event (el-Mahdani et al., 1997), suggesting that *TP53* has an oncogenic role independent of the tumor suppression activity. This dual behavior was also reported for other genes, such as Wilms' tumor 1 (*WT1*); Mutated *WT1* led to the onset of kidney tumors, and its overexpression was detected in a subset of human cancers (Yang, Han, Saiz, & Minden, 2007).

Gene amplification is considered one of the major mechanisms that consequently upregulate and activate oncogenes of tumor cells during cancer development and progression. Although gene amplification plays a major role in tumorigenesis, especially for solid tumors, including lung, prostate, colon, gastric, ovarian, and BCs (reviewed in (Knuutila et al., 1998; Storlazzi et al., 2010)), the molecular mechanisms of how these genes are amplified are not fully understood yet. Plausible mechanisms of gene amplification may include episome excision (S. Carroll et al., 1988), re-replication and

unequal exchange (Cai et al., 2019). Initially, in episome excision model, small circular acentric molecules are formed due to extrachromosomal amplification that can cytogenetically generate minute double chromosomes (Boone & Kelloff, 1994; Papachristou et al., 2008). In the re-replication model, multiple DNA synthesis is initiated several times in one cycle. Lastly, the unequal exchange model suggests that recombination between two chromatids or two misaligned homologs or non-homologous DNA segments generate multiple gene copies (Mehta & Haber, 2014; Reams & Roth, 2015).

Datasets from the Molecular Taxonomy of BC International Consortium (METABRIC), The Cancer Genome Atlas (TCGA), and Tumorscape provided the tumor molecular patterns, including copy number variation and gene expression data in approximately 2000 BC patients. The results revealed that the 17q23 region was associated with oncogenes (Curtis et al., 2012). Comparative genomic hybridization first discovered that the chromosomal region 17q23 was amplified in BC, and thus considered as the most predominant amplified region in BC (Kallioniemi et al., 1994). Moreover, the high dose of one or more genes within the 17q23 region was correlated with BC poor prognosis and tumor progression (Pärssinen, Kuukasjärvi, Karhu, & Kallioniemi, 2007). Gain of function resulting from amplification of this region has been reported in other cancers, including liver (Marchio et al., 1997), pancreas (Solinas-Toldo et al., 1996), bladder (Voorter et al., 1995), testis (Korn et al., 1996), ovary (Arnold et al., 1996), lung (Ried et al., 1994), and brain tumors (Nicholson, Ross, Kohler, & Ellison, 1999). Interestingly, the 17q23 amplicon covers a large number of genes that can be activated by amplification, and thus

might be considered as candidate genes for the onset of BC (Sinclair, Rowley, Naderi, & Couch, 2003). Furthermore, the region 17q23, where *BRIP1* gene lies, consists of various amplified genes including *RPS6KB1*, *APPBP2*, *RAD51C*, *TBX2*, *PPM1D*, *THRAP1*, and *TRIM37* involved in BC as potential of oncogenes (Sinclair et al., 2003; G.-J. Wu et al., 2000). However, only few of these genes (*RPS6KB1*, *TBX2*, and *PPM1D*) have been validated as oncogenes both *in-vitro* and *in-vivo* by functional analyses. Thus, no study has been carried out to determine the additional role of *BRIP1* as an oncogene beside its conventional role as a tumor suppressor gene in hereditary cancers (S. B. Cantor et al., 2001; I. Gupta et al., 2015). In particular, *BRIP1* was overexpressed in higher-grade compared to lower grade breast carcinomas (Eelen et al., 2008; Sinclair et al., 2003). TCGA showed a 3.2-fold upregulation of *BRIP1* in breast tumors compared to normal breast tissues (Lee, 2012). Similarly, our microarray data revealed that *BRIP1* had an average of 5-fold overexpression as compared to normal breast tissue samples (I. Gupta et al., 2018). Mutational analysis of *BRIP1* gene 5′ flanking region showed that *BRIP1* expression is controlled by conserved E2F binding site in murine and human (Eelen et al., 2008). BRIP1 and E2F1 showed co-regulated expression in BC tissues and thus identified BRIP1 as a genuine E2F target (Eelen et al., 2008). Collectively, transcription of *BRIP1* was shown to be controlled by E2F/RB pathway to regulate cell growth and correlated with unfavourable outcome.

Furthermore, in this study, to explore the role of *BRIP1* in breast tumor progression, we examined the gene expression of BRIP1 in various BC cell lines in comparison to both normal HuMEC and immortalized non-pathogenic breast cell line (MCF 10A), using

Western blotting and RT-qPCR analyses (Figures 4.1 – 4.3). Our results showed clearly that both the protein and mRNA levels of BRIP1 were overexpressed in various BC cell lines compared to control normal/immortalized normal/control breast cells. Collectively, our results suggest that *BRIP1* might act as an oncogenic driver in BC. The clinical importance of *BRIP1* was evidenced by the identification of *BRIP1* germ-line mutations in *BRIP1* with no *BRCA1* and *BRCA2* mutations in these patients, suggesting a link between *BRIP1* mutations and BC susceptibility (Ali et al., 2019). Although *BRIP1* is considered as a tumor suppressor gene, it is amplified in sporadic cancers (Lee, 2012), thus supporting our findings in a previous study, which reported an overexpression of *BRIP1* as a major event in BC Omani cohort (I. Gupta et al., 2018). *BRIP1* amplification in sporadic cancers could be one of the plausible reasons for the elevated *BRIP1* gene expression underlining its potential role as an oncogene.

BRIP1 promotes breast cancer cell proliferation

Our siRNA experiments revealed that downregulation of *BRIP1* inhibited both BC cell growth and cell migration/invasion. In order to understand the mechanisms by which *BRIP1* mediates BC cell growth and motility, cell cycle analysis combined with metastasis TaqMan quantitative gene expression profiling were applied to various siRNA-PRIP1 inhibited BC cell lines. *BRIP1* suppression, arrested cell cycle at the G1/S phase, indicating that *BRIP1* may promote cell growth.

Analysis of the TaqMan array identified several key cell cycle regulators that were downregulated upon BRIP1 inhibition; These genes include *c-Myc* (-3.7 fold change), *Ras GTPase* (-3.8 fold change), and *Rb* (-4.8 fold change) (Matson & Cook, 2017). Of central relevance, *Ras* activation induced transcription of key genes via stimulation of transcription factors (TFs), such as serum response factor, c-Myc, and others (Blagosklonny & Pardee, 2002; Y. Zhang et al., 2016). Under the control of these TFs, D-type cyclin (cyclin D) stimulates the complex formation of cdk4/6 leading to phosphorylation of Rb, followed by the release of E2F to promote gene expression, DNA replication, and G1/S transition (Di Fiore, D'Anneo, Tesoriere, & Vento, 2013; Salazar-Roa & Malumbres, 2017). On the other hand, a meta-analysis showed clearly that a significant proportion of BC cases showed overexpression of *c-Myc* by 3-fold or greater with an average of 15% (Liao & Dickson, 2000). Normally, *c-Myc* is expressed during active cell division phase only, regulates the transition of cells from G1 to S phase, and is associated with poor prognosis (Lourenco et al., 2019) (Reviewed in (García-Gutiérrez, Delgado, & León, 2019)). RNAi-inhibited *c-Myc* reduced MCF-7 BC cells by 30% as well as tumor development in nude mice (Y.-h. Wang et al., 2005). In the present study, *Rb* gene expression was reduced by 4.8 fold, accompanied by cell cycle arrest at G1 (Flow cytometry results). Although our results showed that inhibition of BRIP1 reduced Rb expression levels, ongoing experiments aim to evaluate the expression levels of phosphorylated Rb, expected to decrease with increased BRIP1 expression to arrest the cell cycle. Nevertheless, during BC metastasis, Rb is expected to increase to promote cell growth and invasion. *Ras GTPase* pathway appears to be required in all decisions during both G1 and G2 phases (Peeper et al., 1997). In G1/S,

Ras-activation drives Myc accumulation and regulates proliferation-related genes through E2F transcriptional activity (Dong et al., 2014; Matson & Cook, 2017). Ongoing further gene selection (*Ras*, *Myc* and *Rb*), pharmacological and functional approaches aim to validate whether these genes underpin BRIP1-promoted cell growth.

BRIP1 promotes breast tumor cell invasion

To our knowledge, no study has linked *BRIP1* to cancer metastasis yet. Functional genomic approaches have led to the identification of several metastatic genes, which most likely work in concert to regulate the multistep process of BC metastasis. However, the deconvolution of the exact regulatory pathways of these genes remains elusive. Our siRNA experiments revealed that inhibition of *BRIP1* in various BC cells reduced tumor cell migration/invasion, suggesting that upregulation of *BRIP1* may promote breast tumor metastasis. A number of previous studies supported the role of Ras family proteins in cell polarity, cell proliferation, cell adhesion, and invasion (M. Liu et al., 2011; Struckhoff, Rana, & Worthylake, 2011). In fact, overexpression of *BRIP1* inhibited cervical tumorigenesis, suggesting its general role as a tumor suppressor (BRIP1 over-expression with low RhoA GTPase activity); This effect was significantly reversed by RhoA GTPase activation, suggesting that overexpression of RhoA GTPase promotes carcinogenesis (Zou et al., 2016). In the present study, RNAi-inhibited *BRIP1* downregulated *Ras GTPases* significantly by -3.9 fold and subsequently suppressed tumor cell invasion. Taken together, these data strengthen our findings that BRIP1 might promote BC tumorigenesis *via* transcriptional activation of RAS genes.

Furthermore, our Real-time qPCR experiments showed that si-BRIP1 has markedly down-regulated *MMP-1* (- 2.1 fold). MMPs are members of the endopeptidase zinc-dependent family that cleave the extracellular matrix (Massova, Kotra, Fridman, & Mobashery, 1998). *MMP-1* downregulation significantly attenuated cell proliferation, migration and invasion, and reduced the expression of c-*Myc* in MCF-7 and MDA-MB-231 cells (Q. M. Wang, Lv, Tang, Zhang, & Wang, 2019). Our Taqman array analysis showed significant downregulation of both *MMP-1* and *Myc* expression in siRNA-inhibited BRIP1 BC cells in comparison to control. In addition, our present study showed that *BRIP1* might also be regulating master regulators of BC metastasis genes; such as SMAD4, supporting the idea that *BRIP1* might contribute to BC bone metastasis by switching the SMAD pathway from the known tumor suppression role to prometastatic one (Kang et al., 2005; Y. Liang et al., 2012).

N-acetylglucosaminyltransferase V (*MGAT5*) gene is another BRIP1- transcriptional target gene that was downregulated upon siRNA inhibition of BRIP1 (- 2.5 fold). In fact, *MGAT5* overexpression has been reported in various human cancers, including hepatocarcinoma, colon cancer, and BC (Guo, Chen, Wang, & Chen, 2006; D. Li et al., 2008; Murata et al., 2000). *MGAT5* is known to promote both cell growth and cell motility in BC (Yan et al., 2019). On the other hand, MGAT5 protein suppression inhibited the expression of ECM proteins, including caspases and matrix metalloproteinases (MMPs) (Deng et al., 2015; Z. Zhang et al., 2018), leading to inhibition of tumor progression. A recent study demonstrated

that restoration of *MGAT5* expression in MCF-7 and MDA-MB-231 overcame the inhibitory effect of miR-124 on BC cell progression (Yan et al., 2019). However, the actual mechanism of action by which miR-124 regulated *MGAT5* remains unclear. Ongoing validation experiments aim to reveal the exact mechanism by which BRIP1-downstream signalling mechanism lead to MGAT5 transactivation.

It is also worth mentioning that *BRIP1* regulates *CXCR4*/*CXCL12* and *MMP-1*, two genes known to be functional drivers of metastasis, thus supporting our hypothesis that *BRIP1* overexpression might promote BC cell invasion (Y. Liang et al., 2012). Besides, our results showed that *BRIP1* downregulation reduced the expression of *CXCL12* significantly (- 5.8 fold). Structure-function studies showed that CXCL12 chemokine is the only ligand for CXCR4, and thus activation of CXC4 depends on the CXCL12 induced chemokine (Guembarovski et al., 2018). Attenuation of either one of them by using neutralizing antibodies reduced angiogenesis in human BC xenografts models. (Darash-Yahana et al., 2004). Evidence support that the CXCL12/CXCR4 axis has been implicated in BC pathogenesis including cell survival, angiogenesis, and metastasis (Luker & Luker, 2006).

Moreover, genes coding for cell adhesion molecules, known to mediate BC progression were also identified in our Taq Man experiments, including Epithelial Cell Adhesion Molecules (*EPCAM*; -2.9 fold change) and the Melanoma Cell adhesion Molecule (*MCAM* is known as CD146; - 3 fold change). *EPCAM* is overexpressed in most human carcinomas (Went et al., 2004). While the function of EPCAM in BC is not fully understood, it

abrogates E-cadherin mediated cell-cell interaction by distrusting the link between catenin and F-actin leading to loss of cell-cell adhesion (Osta et al., 2004). *EPCAM* is also overexpressed by 100 to 810 folds in primary and metastatic BC cells compared to normal breast tissues, and suppression of *EPCAM* by specific siRNA in MDA-MB-231 showed a significant decrease of cell migration and invasion (Osta et al., 2004). On the other hand, *MCAM* overexpression is associated with increased expression of epithelial-mesenchymal transition (EMT) biomarkers and cell motility (Imbert et al., 2012). Suppressed expression of these genes in BC cells in response to BRIP1 knockdown would likely result in inhibition of cell invasion and metastasis. Collectively, this study revealed potential transcriptional target genes that might underpin BRIP1-promoted BC cell invasion.

Pitfalls and Future perspectives:

Limitation of the work

In a previous work of my supervisor's team, *BRIP1* was differentially upregulated in BC tissues from Omani cases. The present investigation is an extension of this work, focusing on the impact of BRIP1 on cell proliferation, cell cycle progression, and cell migration and invasion. To achieve this objective, a panel of BC cells were used, where *BRIP* expression was downregulated by siRNA approach. Furthermore, a customized metastasis gene expression profiling was conducted to identify potential transcriptional target genes regulated by BRIP1.

Pitfalls: The main technical challenge faced during this work was the optimization of the siRNA strategy. In fact, the use of siRNA method to address the implication and function of *BRIP1* in BC progression, including cell locomotion/invasion, has limitations as revealed by the modest BRIP1 inhibition (based on mRNA and protein expression) achieved in many of the cells tested. This partial inhibition can greatly contribute to the modest effects seen on the various biological endpoints examined in cells where *BRIP1* was downregulated.

Initially, we performed lentiviral shRNA stable-transduction, using four different shRNA plasmids that target different regions on *BRIP1* mRNA (BRIP1- Human, 4 unique 29mer shRNA constructs in lentiviral GFP vector, TL306372, OriGene Technologies Inc.,USA). Transduced cells were selected with puromycin. Unfortunately, *BRIP1* was not inhibited by any one of the 4-shRNA plasmids either used individually or in combination, or even *via* re-transfection (double knockdown). The selective marker, GFP, was successfully expressed with transfection efficiency higher than 90% in all the BC cell lines tested. However, none of the cell lines showed any significant inhibition of *BRIP1*. We believe that, if the marker was expressed, the absence of knock down could not be attributed to the region of the construct used. The plausible explanations to this phenomenon could be differences in promoter activity, the regions of the integration sites (active or inactive transcription), the number of integrations in the clone, the terminator of the upstream gene may not be efficient, or by the inefficiency of the company shRNAs plasmids. These parameters might have influenced directly the level of expression and so the level of inhibition by shRNA expression.

Alternatives: Due to the time limitation and to speed up the progress of this work, we decided to use the siRNA technology to suppress BRIP1. We are totally aware that the Pros. and Cons. of siRNA methodology might be balanced due to; (1) The variation in the efficiency of an siRNA in inhibiting the intended target, and (2) the impact for an siRNA can be due to off-target effects, rather than the effect of suppressing the intended target (or a combination of both). However, it has been reported that Off-target effects usually arise from (a) the sense strand, (b) the antisense strand, or (c) competition with endogenous miRNAs (Khan et al., 2009). The last effect can be prevented by complementarity of the seeding region in the antisense strand with any 3'UTR region of any mRNA or at least with low algorithmic score number (Birmingham et al., 2007). Also, we can eliminate the sense strand from being incorporated into RISC by sequence design of chemical modifications (2-O-methyl on position 1 and 2) or (more AU in position 16-19, more GC in position 1 and 2) (Jackson, Burchard, Leake, et al., 2006).

In an attempt to overcome or reduce the limitations of the siRNA technology, we selected the smart pool BRIP1 siRNA (50 nM) (M-010587-00-0010, Dharmacon Products, USA). The use of a mixture of four siRNAs with different sequences targeting *BRIP1* was significantly effective in inhibiting *BRIP1* expression; this can significantly reduce the probability that the observed gene expression changes are caused by the off-target effects. These pools enhanced target specificity and destabilized off-target activity by modification of the antisense strand seed regions, thus Off-targets was reduced by up to 90%, compared to unmodified siRNA (Jackson, Burchard, Schelter, et al., 2006). In addition, the siRNAs'

sense strands were modified in order to favor antisense strand uptake, and also to prevent interaction with RISC (Jackson, Burchard, Leake, et al., 2006). In addition, there is also a growing list of publications showing that siPOOLs are well recognized by the research community as a method of validating loss-of-function phenotypes (Jackson & Linsley, 2010).

Obviously, the findings from *in vitro* models will require *in vivo* validation. Our future plans favor inducible systems over CRISPR technology or shRNA stable transfection combined with Xenograft models. Why? Simply because my supervisor's team have successfully established them in the past and extensively published in this area (Abdraboh et al., 2011; A. Ouhtit, Madani S, Gupta I, Shanmuganathan S, Abdraboh ME, Al-Riyami H, Al-Farsi YM, Raj MH, 2013).

Future perspectives

While these findings support our hypothesis that *BRIP1* promotes BC progression, ongoing structural and functional validation experiments in our laboratory aim to shed light on the exact mechanisms by which BRIP1-downstream signaling promotes BC cell growth and cell invasion.

- To do so, molecular and pharmacological approaches will be applied, using various well-known signal transduction pathways in order to identify each molecular player of the signaling pathway linking *BRIP1* activation to the initiation of gene targets transcription.

- BRIP1 phosphorylation deserves to be investigated in the cell models used (in particular the Serine 990 that can dictate BRIP1 function and interaction with BRCA1). BRIP1 phosphorylation is tightly regulated in contrast to total protein and phosphorylation activity can reveal interesting research avenues in relation to cancer cell signaling (e.g. most cancer cell invasion signaling pathways are regulated by complex phospho-protein networks).
- In RNAi approaches, cells will be stably transduced with (shRNAs) using lentiviruses targeting specific genes, or by using CRISPR/Cas9, or inducible system that result in heritable and stable inhibition of the corresponding gene when integrated into the genome, that will overcome the modest effect of siRNA knockdown.

CHAPTER 6 : CONCLUSION

Breast cancer (BC), a worldwide health problem, is the most common cancer in women worldwide, including the State of Qatar. BC is a heterogeneous disease with variable biological and clinical distinguished traits, including the ethnic, racial, genetic/epigenetic factors and their influence on ***invasiveness*** or ***metastasis***, which is the worst aspect of cancer. Therefore, new prognostic biomarkers are needed to develop new diagnostic gene panel and efficient targeted therapies against BC, for enhancing the chance for long-term survival and patient's quality of life. To achieve this goal, it is imperative to understand the unique disease processes associated with metastasis, the defining event in the metastatic process, and elucidation of its mechanisms for developing effective anti-metastatic therapies. Pursuant to this goal, our rcent study using microarray gene expression profiling identified BRIP1, showing 5-fold induction, as a potential gene that might promote BC progression.

Our present study first confirmed that *BRIP1* was significantly overexpressed in BC cells compared to controls. Second, functional assays further supported our hypothesis and validated *BRIP1* as a promoter of BC cell growth as well as BC cell migration and invasion, indicating its novel role in the multistep process of metastasis

Moreover, in order to identify the key signalling mechanisms that underpin *BRIP1*-promoted BC cell invasion, the present investigation identified key novel *BRIP1*-induced

pro-invasive genes. Among these genes, stands out genes associated with the extracellular matrix, adhesion, cell proliferation, and motility. While these findings support our hypothesis that *BRIP1* promotes BC progression, ongoing structural and functional validation experiments in our laboratory aim to shed light on the exact mechanisms by which *BRIP1*-downstream signalling promotes BC cell growth and cell invasion.

The task ahead is to add more knowledge to the puzzle of BC and how to effectively prevent the disease. This investigation could define *BRIP1* as a biomarker and/or target to pave the way towards the design of appropriate BC targeted therapies.

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